PHOSPHITE SENSITIVITY OF *PHYTOPHTHORA CINNAMOMI* AND METHODS FOR QUANTIFYING PHOSPHITE FROM AVOCADO ROOTS

by

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DECLARATION

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SUMMARY

Phytophthora root rot caused by *Phytophthora cinnamomi* threatens the production of avocado worldwide, but the disease can be effectively managed using phosphonates. The mode of action of phosphonates is controversial and can include a direct fungistatic action and/or an indirect action involving host defence responses. In South Africa, *in vitro* radial growth inhibition studies, which can be indicative of a direct mode of action, have only been conducted on isolates collected in one orchard in previous studies, more than a decade ago. In *in vitro* studies, phosphate in the test medium can influence the *in vitro* toxicity of phosphite ($H_2PO_3^-$), but this has not been studied in large *P. cinnamomi* populations. The *in vivo* phosphite sensitivity of *P. cinnamomi* isolates in avocado, which is indicative of host defence responses, has only been investigated in two non-peer reviewed studies in South Africa. Quantification of phosphite in roots is required for elucidating the mode of action of phosphonates, but no commercial analytical laboratory in South Africa can conduct these analyses due to the lack of a validated analytical method.

The current study optimized a liquid chromatography-mass spectrometry (LC-MS/MS) method for quantification of phosphite, the breakdown product of phosphonates in plants, using avocado roots collected in orchards. Phosphite recovery rates were good (78 - 124%) and the precision was excellent with the percentage coefficient of variation (CV%) being between 1.9 to 9.7. Although the incurred sample reanalyses (ISR) precision for the method was unacceptable for samples with phosphite concentrations lower than 27 $\mu g/g_{DW}$ (~6.75 $\mu g/g_{FW}$), it was acceptable for samples with higher phosphite concentrations (0.4 and 11 CV%; 0.6 - 21% difference [%DF]). The other investigated analytical methods, ion chromatography and an enzymatic fluorescent assay, were unreliable due to unacceptable ISR precision values.

The *in vitro* phosphite ($H_2PO_3^-$) sensitivities of 42 *P. cinnamomi* isolates from avocado in South Africa were investigated, as influenced by phosphate (HPO_4^{2-}), using radial growth inhibition assays. Based on the response of isolates to all the evaluated phosphite concentrations (30 and 100 µg/ml) and phosphate concentrations in the test medium (1, 7 and 15 mM), the isolates could be grouped into a sensitive (11.04 - 89.21% inhibition), intermediate (11.26 - 66.75% inhibition) and tolerant group (3.9 - 19.09% inhibition). The inhibition of isolates as influenced by phosphate concentration was dependent on the phosphite sensitivity of isolates. In general, inhibition by phosphite for the sensitive and intermediate groups decreased as phosphate concentration increased, whereas inhibition of the tolerant group was not influenced significantly by phosphate at a phosphite concentration of 30 µg/ml.

The *in vivo* sensitivities of one isolate from each of the sensitive and tolerant groups were investigated using an excised root bioassay. The two isolates responded similar to all

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phosphonate treatments, but the tolerant isolate tended to be more virulent, making it difficult to differentiate between phosphite- and virulence responses. The roots from seedlings that received phosphonate treatments that yielded root phosphite concentrations of 9.82 µg/g_{FW} or higher, resulted in significant control. The only exception was one treatment, in one of two experiments, which contained 1.92 µg/g_{FW} that unexpectedly caused significant control. Increasing phosphite root concentrations from 9.82 to 19.30 µg/g_{FW} did not significantly improve control. Both isolates were inhibited to a greater extent (> 40%) *in vivo* than *in vitro.* Altogether, the data supports the involvement of host defence responses in suppression of *P. cinnnamomi* by phosphite.

The study has improved our knowledge on the *in vitro* and *in vivo* response of *P. cinnamomi* isolates from avocado to phosphite, and consequently its mode of action. The data suggested that the mechanism of action is most likely host defence induction. Phosphonates thus seem to be true resistance inducing crop protection products. However, further trials are required to proof this hypothesis, since limited data and some controversial data were obtained in the current study. The *in vivo* data provided preliminary indications that in phosphonate application trials root phosphite concentrations should be above 10 µg/g_{FW} to suppress *P. cinnamomi.* However, more *in vivo* trials must be conducted to confirm this. The developed LC-MS/MS method, is the only method available for root phosphite quantifications in South Africa, and is crucial for investigating the *P. cinnamomi*-avocado-phosphonate system.

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OPSOMMING

Phytophthora wortelvrot, veroorsaak deur *Phytophthora cinnamomi*, bedreig die produksie van avokado wêreldwyd, maar die siekte kan effektief deur die gebruik van fosfonate bestuur word. Die werkingswyse van fosfonate is kontroversieël en kan ‘n direkte fungistatiese aksie en/of ‘n indirekte aksie, wat gasheer verdedigingsreaksies betrek, insluit. In Suid-Afrika, is *in vitro* radiale groei-inhibisie studies, wat aanduidend van ‘n direkte werkingswyse kan wees, slegs op isolate wat uit een boord versamel is, in vorige studies, meer as ‘n dekade terug, uitgevoer. In *in vitro* studies kan fosfaat in die toetsmedium die *in vitro* toksisiteit van fosfiet ($H_2PO_3^-$) beïnvloed, maar dit is nog nie in groot *P. cinnamomi* populasies bestudeer nie. Die *in vivo* fosfiet sensitiwiteit van *P. cinnamomi* isolate in avokado, wat aanduidend van gasheer verdedigingsreaksies is, is nog slegs in twee nie-eweknie beoordeelde studies in Suid-Afrika ondersoek. Kwantifisering van fosfiet in wortels word benodig ten einde die werkingswyse van fosfonate vas te stel, maar geen kommersiële analitiese laboratorium in Suid-Afrika kan hierdie analises doen nie weens die gebrek aan ‘n gevalideerde analitiese metode.

Die huidige studie het ‘n vloeistof kromatografie-massa spektrometrie (LC-MS/MS) metode geoptimiseer vir kwantifisering van fosfiet, die afbreekproduk van fosfonate in plante, deur gebruik te maak van avokado wortels wat in boorde versamel is. Fosfiet terugwintempo’s was goed (78 - 124%) en die akkuraatheid was uitstekend met die persentasie van koeffisiënt van variasie (CV%), tussen 1.9 tot 9.7. Hoewel die ‘incurred sample reanalyses’ (ISR) akkuraatheid vir die metode onaanvaarbaar was vir monsters met fosfiet konsentrasies laer as 27 $\mu g/g_{DW}$ (~6.75 $\mu g/g_{FW}$), was dit aanvaarbaar vir monsters met hoër konsentrasies (0.4 en 11 CV%; 0.6 - 21% verskil [%DF]). Die ander analitiese metodes wat ondersoek is, ioon kromatografie en ‘n ensiem fluoresserende toetse, was nie betroubaar nie weens onaanvaarbare ISR akkuraatheid.

Die *in vitro* fosfiet ($H_2PO_3^-$) sensitiwiteite van 42 *P. cinnamomi* isolate vanaf avokado in Suid-Afrika, is ondersoek, soos beïnvloed deur fosfaat (HPO_4^{2-}), deur die gebruik van radiale groei-inhibisie studies. Gebaseer op die reaksie van isolate op al die geëvalueerde fosfiet konsentrasies (30 en 100 µg/ml) en fosfaat konsentrasies in die toetsmedium (1, 7 en 15 mM), kon die isolate in ‘n sensitiewe (11.04 - 89.21% inhibisie), intermediêre (11.26 - 66.75% inhibisie) en bestande groep (3.9 - 19.09% inhibisie) gegroepeer word. Die inhibisie van isolate, soos beïnvloed deur fosfaat konsentrasie, was afhanklik van die fosfiet sensitiwiteit van isolate. Oor die algemeen het inhibisie deur fosfiet vir die sensitiewe en intermediêre groepe afgeneem soos wat fosfaat konsentrasie toegeneem het, terwyl inhibisie van die bestande groep nie betekenisvol deur fosfaat, by ‘n fosfiet konsentrasie van 30 µg/ml, beïnvloed is nie.

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Die *in vivo* sensitiwiteite van een isolaat van elk van die sensitief en bestande groepe, is deur die gebruik van ‘n uitgesnyde wortel bio-toets ondersoek. Die twee isolate het soortgelyk op alle fosfonaat behandelings gereageer, maar die bestande isolaat neig meer virulent te wees, wat dit moeilik gemaak het om tussen fosfiet en virulensie reaksies te onderskei. Die wortels vanaf saailinge wat fosfonaat behandelings ontvang het, het wortel fosfiet konsentrasies van 9.82 $\mu g/g_{FW}$ of hoër gelewer, wat tot betekenisvolle beheer gelei het. Die enigste uitsondering was een behandeling, in een van twee eksperimente, wat 1.92 $\mu g/g_{FW}$ bevat het, wat onverwags betekenisvolle beheer gegee het. ‘n Toename in wortel fosfiet konsentrasies van 9.82 tot 19.30 $\mu g/g_{FW}$ het nie beheer betekenisvol verbeter nie. Beide isolate is tot ‘n groter mate (> 40%) *in vivo* as *in vitro* geïnhibeer. Altesaam ondersteun die data die betrokkenheid van gasheer verdedigingsreaksies in die onderdrukking van *P. cinnnamomi* deur fosfiet.

Die studie het ons kennis oor die *in vitro* en *in vivo* reaksie van *P. cinnamomi* isolate vanaf avokado teenoor fosfiet verbeter, en gevolglik sy werkingswyse. Die data dui daarop dat die werkingswyse gasheer verdedigingsinduksie is. Fosfonate blyk dus ware weerstand induserende gewasbeskermingsprodukte te wees. Verdere proewe word egter benodig ten einde die hipotese te bewys, aangesien beperkte data en ‘n mate van kontroversiële data tydens die huidige studie, verkry is. Die *in vivo* data het voorlopige aanduidings verskaf dat in fosfonaat toedieningsproewe, wortel fosfiet konsentrasies bó 10 $\mu g/g_{FW}$ moet wees ten einde *P. cinnamomi* te onderdruk. Meer *in vivo* proewe moet egter uitgevoer word om dit te bewys. Die ontwikkelde LC-MS/MS metode is die enigste metode beskikbaar vir wortel fosfiet kwantifiserings in Suid-Afrika, en is noodsaaklik ten einde die *P. cinnamomi*-avokado-fosfonaatsisteem te ondersoek.

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CHAPTER 1

A review of *Phytophthora cinnamomi*: its pathogenicity, identification and control strategy

GENERAL INTRODUCTION

Phytophthora cinnamomi Rands is a destructive soilborne oomycete plant pathogen that causes root and collar rot in more than 4000 host plant species (Zentmyer, 1980; Shearer *et al.*, 2004; Hardham, 2005). It is one of the most invasive plant pathogenic species known, since it attacks and kills trees and shrubs of all ages, from the nursery stage to large bearing trees. Consequently, the pathogen is responsible for considerable economic and ecological damage in native ecosystems, agriculture and horticulture worldwide (Shearer and Fairman, 2007; Brasier, 2008).

Avocado is one of the horticultural crops of which the production is threatened worldwide by Phytophthora root rot, since the pathogen eventually causes the death of trees if not controlled. In California, it was estimated in the early 1990's that up to 70% of commercial orchards are affected. The annual loss attributed to the disease in this region was estimated at $30 million (Coffey, 1992). In South Africa, 20% of avocado trees were reported to be affected in 1971 (Milne and Chamberlain, 1971). Subsequently, improved management practices have resulted in decreased industry losses due to root rot, but these management practices are costly (Duvenhage, 2001).

An integrated management approach of avocado root rot includes sanitation, planting of resistant rootstocks, chemical and biological control (Coffey, 1987; Menge and Ploetz, 2003). Currently, South Africa relies on the use of resistant rootstocks such as Duke 7 and Dusa® in combination with chemical control with phosphonate fungicides (Kremer-Köhne and Köhne, 2007). Phosphonate-based fungicides are fundamental to the chemical control of root rot caused by *P. cinnamomi*. These fungicides are fully systemic, since they can be translocated in both the xylem and phloem (Cohen and Coffey, 1986; Guest and Grant, 1991).

In literature, the terminologies used for phosphonates and its derivatives are confusing since various terminologies have been used including phosphorous acid, phosphonic acid, phosphonate and phosphite. According to the latest IUPAC rules, the terminologies phosphonic acid should be used for H_2PHO_3 and phosphorous acid for phosphite (Connelly *et al.*, 2005). The salts of these compounds, that are used as fungicides are referred to as phosphonates ($[PHO_3]^{2-}$), e.g. potassium phosphonate (K_2HPO_3). In plants, phosphonate fungicides dissociate into various anions (dihydrogenphosphite $[H_2PO_3^-]$, hydrogenphosphite $[HPO_3]^{2-}$ and phosphite $[PO_3]^{3-}$) (Ouimette and Coffey, 1989b). According to Ouimette and

Coffey (1989b) the hydrogenphosphite anion mainly exists at a physiological pH and has activity against *P. cinnamomi.* For the purpose of this thesis the terminology phosphonates will be used for referring to formulated fungicides whereas phosphite will be used to refer to the anions of phosphonates in plants that occur at a physiological pH.

Although phosphonates have been used extensively worldwide to reduce the spread and impact of Phytophthora root rot, the complex mechanisms by which phosphite acts to control the pathogen remains unknown. Research has shown that a combination of a direct fungistatic activity and stimulation of host defense mechanisms are likely involved in inhibiting pathogen growth (Guest and Bompeix, 1990; Hardy *et al.*, 2001). Understanding the mechanisms involved in the suppression of *P. cinnamomi* by phosphite, and knowledge on the concentrations of phosphite required in plants to suppress the pathogen is essential for maintaining sustainable and effective management strategies.

This review presents an overview of *P. cinnamomi,* with emphasis on its importance as a pathogen of avocado and the use of phosphonates for managing the disease. The review addresses important aspects of the biology of the pathogen including the life cycle, origin, genetic variation and diagnostic approaches. Some general control measures are discussed, but the emphasis is placed on phosphonates including their possible mode of action and response of the pathogen to different phosphite concentrations in *in vitro* and *in vivo* studies. The phosphonate section is concluded with information on recent studies on host plant defense responses to phosphonate treatment, and analytical methods used for quantification of phosphite in plant tissue. The conclusions section highlights the most important aspects of the review and also indicates the scope of this thesis.

PHYTOPHTHORA ROOT ROT IN AVOCADO

Causal agent

The plant pathogen *P. cinnamomi* belongs to the class of Oomycetes, which includes several water mould algal relatives. *Phytophthora* spp. are not true fungi (Mycota), even though they produce spores and grow filamentous hyphae. Since the first isolation of *P. cinnamomi* from stripe cankers of *Cinnamomum burmanii* (Blume trees) in Sumatra in Indonesia by Rands (1922), the known plant host range of this destructive pathogen has expanded to more than 4000 woody and herbaceous plant species (Zentmyer 1980; Shearer *et al.*, 2004).

Molecular phylogenetic studies placed *P. cinnamomi* along with *P. cambivora* and *P. fragariae* into *Phytophthora* Clade 7a (Cooke *et al.,* 2000). *Phytophthora cinnamomi* var. *parvispora,* also belonging to Clade 7a, was previously described as a variant of *P. cinnamomi* but was recently shown to be a distinct species *P. parvispora. Phytophthora parvispora* is the closest relative of *P. cinnamomi* and they share a common ancestor (Scanu *et al.*, 2014). *Phytophthora cinnamomi* and *P. parvispora* is the only known *Phytophthora* Clade 7a species

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occurring in South Africa (Bezuidenhout *et al.*, 2010). *Phytophthora* clade 7b mainly contains species (*P. sinensis, P. melonis, P. cajani, P. sojae* and *P. vignae*) (Cooke *et al.,* 2000; Scanu *et al.*, 2014) that do not occur in South Africa (Crous *et al.*, 2000), with the exception of *Phytophthora niederhauserii. Phytophthora niederhauserii* was first reported in 2003 on *Thuja occidentalis* and *Hedera helix* plants in North Carolina, America and seems to have a wide host range since it has been associated with 33 different plant species (Abad *et al.,* 2014).

Life Cycle

The life cycle of *P. cinnamomi* has been reviewed extensively (Weste and Marks, 1987; Shearer and Tippett, 1989; Shearer and Smith, 2000). The vegetative mycelia of *P. cinnamomi* are able to produce four types of spores including oospores, chlamydospores and sporangia that release zoospores under specific environmental conditions (Judelson and Blanco, 2005). Of these spore types, oospores are sexual, whereas chlamydospores, sporangia and zoospores are asexual. *Phytophthora cinnamomi* is heterothallic and thus requires two opposite mating types, A1 and A2, for sexual reproduction. Oospores are formed infrequently in meiosis, and may survive for long periods of time in the soil or plant debris. Due to the limited distribution of the A1 mating type in most regions of the world, oospores probably do not play a major role in the infection cycle (Zentmyer, 1961). However, recently it was shown that oospores may be important, since selfed oospores were shown to occur in naturally infected plants and that they can germinate (Crone *et al.*, 2013).

In the presence of unfavorable environmental conditions, the pathogen can survive as dormant resting spores (oospores or chlamydospores) for several years. Under ideal growth conditions, which include moist soils and ambient temperatures (15 - 25ºC), the pathogen enters the asexual sporulation cycle. Sporangia are formed from hyphae and release motile, biflagellate zoopores. Zoopores, upon losing their flagella, form walled cysts that germinate and penetrate the root and then grow within phloem tissues. The primary infectious propagule (Zentmyer, 1961) of the pathogen is thus zoospores. Following extensive colonization of the phloem tissue, the infected tissues will be killed, and the pathogen will then start sporulating from the tissues. The newly formed sporangia will release zoospores that can swim short distances to penetrate new susceptible host plant tissues, thus continuing the cycle of infection (Hardham, 2005).

The disease has a short generation time and high reproductive capacity and therefore inoculum levels can increase from low, often undetectable levels, to high levels within days, particularly in warm, moist and well aerated soils, and if feeder roots are in abundance (Zentmyer,1980). It is estimated that the asexual life cycle can be completed within 8 hours under optimal conditions, with zoospore formation occurring within minutes (Walker and Van

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West, 2007). Both the ability of surviving under unfavorable conditions for years, and the fast life cycle makes *P. cinnamomi* a very adaptable destructive pathogen.

Origin and genetic variation in *P. cinnamomi*

The center of origin of *P. cinnamomi* is unknown, but initially there were indications of the origin possibly ranging from New Guinea through to Indonesia, Sumatra and Malaysia, including Taiwan, possibly extending into northeastern Australia (Ko *et al.*, 1978; Zentmyer, 1980; Zentmyer, 1988). However, subsequent studies based on *P. cinnamomi* isozyme studies showed that due to the low levels of genetic diversity and the absence of sexual reproduction in East Asia (Zhou *et al.*, 1992) and Australia (Old *et al.*, 1984; Old *et al.*, 1988), the pathogen was rather introduced into these regions. The southern Cape Province of South Africa (Von Broembsen, 1979; Von Broembsen and Kruger, 1985) has also been postulated as being the center of origin in the 1980's. However, subsequently Linde *et al.* (1999) conducted a study on the population structure of *P. cinnamomi* in South Africa that disproved this theory. They found that based on isozyme analysis, there are low levels of genetic diversity and that sexual reproduction thus rarely occur. Currently, indirect evidence such as the presence of resistant host species and the widespread occurrence of both mating types in native vegetation suggests that South-East Asia and in particular Hainan, South China, Taiwan and New Guinea may be the center of origin (Ko *et al.*, 1978; Chang 1996; Zeng *et al.*, 2009; Brasier *et al.* 2010; Scanu *et al.*, 2014). Outside of the aforementioned regions such as in Australia and South Africa, mainly the A2 mating type is found that consists of three distinct clonal lineages (Linde *et al.*, 1999; Dobrowolski *et al.*, 2003). On avocado in California, only the A2 mating type occurred in collections after 1993. The collection of isolates was comprised of two major groups that had low genotypic diversity, which is consistent with a mainly asexual reproduction system (Pagliaccia and Pond, 2013).

Symptoms on avocado

Phytophthora cinnamomi attacks the fine feeder roots of avocado trees at all ages, resulting in roots having a necrotic, brittle, and dark brown colored appearance, which prevent the plants from absorbing nutrients and water. This results in a general decline of trees, eventually, leading to the death of the tree (Zentmyer, 1984; Erwin and Ribeiro, 1996). The observed above-ground symptoms include wilting of leaves, defoliation and branches that rapidly die back depending on root rot severity. If new leaves form, they are always small and pale green and trees have a small fruit set. The above ground symptoms may not be apparent until the root system is severely damaged (Shearer *et al.*, 2007).

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ISOLATION AND IDENTIFICATION OF *PHYTOPHTHORA CINNAMOMI*

Isolation of *P. cinnamomi*

Traditional methods for isolating *P. cinnamomi* consists of baiting techniques (Zentmyer, 1980), where infested soil or plant parts are mixed with water to form a slurry. The soil slurry is then baited with susceptible tissue, e.g. pineapple leaves (Anderson, 1951), avocado fruit (Zentmyer *et al.*, 1960), lupine radicles (Chee and Newhook, 1965) and pear fruit (Greenhalgh, 1978). After a few days of incubation, the baits are plated onto a *Phytophthora*-selective agar medium to support the outgrowth of any infecting oomycete pathogen, which is then examined microscopically and identified based on morphology. Alternatively, to baiting, plant tissue can be plated directly onto *Phytophthora* selective media such as PARPH (Greenhalgh, 1978; Tsao, 1983; Jeffers and Martin, 1986; Eden and Galpoththage, 2000). The advantage of baiting and tissue plating are that it only detects viable pathogen propagules. However, the disadvantage is that it is very slow and false negatives can occur due to the failure of pathogen outgrowth because of the presence of fast growing organisms such as *Pythium* (Nechwatal and Obwald, 2001).

Success of baiting techniques of *P. cinnamomi* depends on the susceptibility of the bait and the incubation conditions. For example, Shew and Benson (1982) found that leaf disks of azalea would detect *P. cinnamomi* more frequently than blue lupine radicles, but were less effective than pine needles (Dance *et al.*, 1975). Ferguson and Jeffers (1999) found that using Camellia leaf-disk as bait was more sensitive than shore juniper needles to detect *P. cinnamomi*. For some *Phytophthora* species, such as *P. nicotianae, P. citricola* and *P. cinnamomi* the efficiency of baiting can be improved by drying out the soil and re-wetting (double baiting) prior to baiting (Jeffers and Aldwinkle, 1987; Ferguson and Jeffers, 1999; Hüberli *et al.,* 2000). The quality of water used also significantly affects the outcome of baiting experiments, as zoospores are very sensitive to toxic ions present in unpurified water (Tsao 1983). In a study by Gerrettson-Cornell referenced within Tsao (1983), the frequency of recovery of *P. cinnamomi* was 94, 32, and 0% respectively when glass-distilled water, deionized water, and distilled water from a metal still were used.

Identification of *P. cinnamomi*

Identification of *P. cinnamomi* isolates has traditionally been made through characterization of sporangia, antheridia, oogonia and mycelia (Waterhouse *et al.,* 1983). Additionally, the unique characteristic of the hyphae with coralloid outgrowth and clusters of hyphal swellings usually provide a good diagnostic feature. Morphological identification methods are labour-intensive and time consuming when working with large collections of isolates, and require expertise in morphological identification of *Phytophthora* species (Tsao, 1990; Dobrowolski and O'Brien, 1993). It is furthermore problematic that there is a wide variation in morphological characters

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in isolates from the same species under different growth conditions (Waterhouse *et al.*, 1983; Daniel *et al.*, 2003). Due to these variations, *P. cinnamomi* is morphologically almost indistinguishable from *P. parvispora* (Scanu *et al.*, 2014).

Antibody-based assays have been used to detect *Phytophthora* species. These serological tests are based on enzyme-linked immunosorbent assays (ELISA), membrane trapping assays and dipstick formats. Several of the antibody based assays are available as commercial kits that can be used by growers for testing their crop samples within the field (Pettitt *et al.*, 2002). However, the common problems associated with the technique are a lack of specificity and sensitivity (MacDonald *et al.*, 1990; Ali-Shtayeh *et al.*, 1991; Benson, 1991; Kratka *et al.*, 1996; Pettitt *et al.*, 2002). Of particular concern is the fact that it can fail to give a species-specific reaction, since some assays can cross-react with other *Phytophthora* spp. and with *Pythium* spp., resulting in numerous false positives. Nonetheless, Hardham *et al.* (1986) was able to develop monoclonal antibodies specific for *P. cinnamomi* spore components that can be used in traditional baiting assays where the zoospores are attracted to a dipstick membrane containing the antibodies and an attractant, which is floated on the water slurry (Hardham, 2005). This assay is unfortunately not commercially available.

To overcome problems caused by antibody-based assays and isolation assays, species-specific polymerase chain reaction (PCR) assays have also often been used for identifying and detecting *Phytophthora* spp., including *P. cinnamomi*. Coelho *et al.* (1997) developed species-specific primers targeting the elicitin cinamomin gene, whereas Schena *et al.* (2008) described species-specific primers (Ycin3F-Ycin4R) that target a region of the ras-related protein gene *Ypt1.* Kong *et al.* (2003) also developed species-specific primers (LPV2F-LPV2R and LPV3F-LPV3R) that target yet another region, the *lpv* putative storage protein genes. The LPV3 primers are more specific and have a greater sensitivity than the LPV2F primers (Kong *et al.*, 2003). Subsequently, Engelbrecht and van den Berg (2013) published modified nested LPV primers for use in quantitative real-time PCR.

Alternative to species-specific primers, restriction fragment length polymerase polymorphism (RFLP) analyses of the ITS region can be conducted. In this approach, the ITS region is amplified using universal ITS primers, where after the amplified ITS PCR products are digested with specific restriction enzymes resulting in species characteristic sized fragment patterns being produced (RFLPs) upon separation by gel agarose electrophoresis (Ristaino *et al.*, 1998; Drenth *et al.*, 2005; Hardham, 2005).

MANAGEMENT OF PHYTOPHTHORA ROOT ROT

An integrated management approach is necessary to control Phytophthora root rot. Principles of an integrated approach include hygiene and sanitation, cultural and biological control, resistant rootstocks and fungicides (Coffey, 1987). The most effective control can be achieved

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by using a combination of (1) applying clean nursery practices that include heat treatment of seed, fumigation or heat treatment of soil used to grow the avocado nursery trees in and, using clean water and good sanitation (Menge and Ploetz, 2003); (2) using fertilizers to produce vigorous and healthy trees, since some nutrients such as calcium and ammonia are particularly important in the control of avocado root rot, because they are both toxic to *P. cinnamomi,* but care should be taken since *ammonia* can be toxic to avocado feeder roots (Wolstenholme and Sheard, 2010); (3) adding organic mulches and gypsum that are naturally stimulatory towards creating suppressive soils and helps to provide an oxygen-rich root environment (Pegg and Whiley, 1987). It is recommended that mulches should be applied under the canopy at the base of the tree in a layer that is 15-30cm thick. The mulches should preferably have a C:N ratio of between 25:1 and 100:1 (Wolstenholme *et al.*,1996); (4) using systemic fungicides such as phosphanate-based fungicide and phenylamides; (5) selecting resistant rootstocks, such as Duke 7 and Dusa® (Roe *et al.,* 1995; Wolestenholme and Sheard, 2010); (6) adjusting irrigation scheduling to avoid over-irrigation and under-irrigation (stressed trees) (Pegg and Whiley, 2010); and (7) proper soil selection and preparation such as planting trees on ridges to improve soil drainage and aeration (Wolestenholme and Sheard, 2010).

Several studies have investigated biological control agents, but they are not used commercially. Biological control agents such as *Trichoderma* and *Gliocladium* exert mechanism including competition, antibiosis or parasitism to suppress *P. cinnamomi* (Erwin and Ribeiro, 1996; Downer 1998). The problems with the efficacy of these microbes are that they do not always survive when used in avocado groves, and their added presence is of limited effect if mulches containing large populations of antagonistic microoroganisms are applied by growers (Menge, 2003).

Tolerant rootstocks have great potential for successfully controlling avocado root rot in the long run. However, the use of host resistance is most successful and sustainable if sanitation measures and regulation of irrigation and soil drainage are also practiced. In general, inheritance of resistance to root rot in avocado is low, less than 1%. Consequently, seedlings produced from seed collected from resistant trees usually show limited resistance (Coffey, 1992). Consequently, when seedlings or trees with tolerance are identified, the plant material must be reproduced vegetatively in order to produce clonal rootstocks that will be tolerant.

The earliest work on resistant rootstocks was documented by Zentmyer (1957, 1980) and Zentmyer *et al.* (1963). Subsequently, many reports of general resistance in avocado have been published (Kellam and Coffey, 1985; Aveling and Rijkenberg, 1989, 1991; Gabor and Coffey, 1990). Zentmyer screened a large variety of *P. americana* types and found rootstock Duke 7 showing moderate field resistance to Phytophthora root rot. Duke 7 became the first

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commercially tolerant root stock in 1975. The rootstock is able to regenerate new roots more rapidly than susceptible rootstock cultivars, which may partly explain the mechanism of resistance in Duke 7 (Kellam and Coffey, 1985).

The early success of Duke 7 gave rise to the screening of an extensive collecting of new sources of resistance to *P. cinnamomi* in avocado and other closely related *Persea* spp. Later on, rootstocks such as Thomas, Martin Grande, D9, Barr Duke, Toro Canon and Dusa® were shown to exhibit a great degree of tolerance to Phytophthora root rot (Menge *et al.*, 1992; Menge and Marais, 2000; Bijzet and Sippel, 2001; Menge *et al.*, 2001; Kremer-Köhne and Köhne, 2007). However, none of these rootstocks are able to withstand *P. cinnamomi* infections when inoculum levels are extremely high. This emphasizes the fact that several other control methods have to be used in combination with tolerant rootstocks in order to effectively control avocado root rot (Douhan *et al.*, 2011).

In South Africa, the Mexican race seedlings (originating from Mexico) were mainly used as rootstocks in the late 1920's. However, the mother blocks were later found to be infected with the sun-blotch viroid, which could not be used as propagation material. Therefore, in the 1950's the Gautemalan cultivar Edranol became the main rootstock, which were later found to be highly susceptible to *P. cinnamomi.* More root rot tolerant Mexican Duke seedling rootstocks were later used, but root rot was still problematic. In the late 1970's South Africa imported several vegetatively propagated clonal rootstock that were more tolerant to root rot. Of these, Duke 7 became the most widely used rootstock in the industry. In the late 1980's the Westfalia rootstock selection Dusa® (Merensky 2) was found to outperform Duke 7 with regards to tree health and yield, also showing more tolerance to root rot (Kremer-Köhne and Köhne, 2007). Similar results were found in California (Menge *et al.,* 2002).

Although several countries world-wide favour the use of clonal rootstocks due to their uniformity and tolerance to root rot, some countries rather use seedling rootstocks. The seedling rootstocks have some advantages above clonal rootstocks in that they can take 12 - 18 months less to establish and that some provide higher yields than clonal rootstocks. Their disadvantage is variability in physical and physiological characteristics and disease tolerance. In Australia, the seedling Velvick rootstock is widely used, since it is well adapted to the Australian growing conditions providing high and consistent yields (Lagadec, 2011). In recent studies, Lagadec (2011) found that seedling rootstocks BW2 may be superior to Velvick, whereas Degania seedling rootstocks also show potential for good quality yields in Australia. In Israel, Degania seedling rootstocks, which are West Indian rootstocks, are used due to their good yields, and tolerance to heat and salt stress (Zilberstaine *et al.* 2003). The tolerance of the aforementioned seedling rootstocks to *P. cinnamomi* has not been reported.

Fungicides remain an integral part of the management of avocado root rot. The two main fungicides that have been used are phenylamides (acylalanines) and phosphonates that are

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both systemic fungicides active against oomycete plant pathogens. The phenylamides are xylem-translocated, single-site fungicides that provide good curative control towards soilborne oomycete pathogens when applied as a soil drench (Schwinn and Staub, 1987). However, the rapid development of fungicide resistance towards this group of fungicides soon rendered their use ineffective in the control of avocado root rot (Bruin and Edgington, 1981; Lucas *et al.*, 1990; Gisi and Cohen, 1996). Phosphonates are also highly effective against root rot but does not have resistance development problems. Although there has been an isolated report of phosphite resistance development in *P. cinnamomi* (Vegh *et al.*, 1985 reference within Fenn and Coffey, 1989) in *Chamaecyparis lawsoniana* after continued use, failure in disease control in *Phytophthora* has not become a reality in agriculture or horticulture. Phosphonates are therefore widely used in the horticultural and agriculture sectors to control Oomycete diseases, particularly *Phytophthora* related diseases. Phosphonates are effective against *Phytophthora* in numerous horticultural species, including avocado (Pegg *et al.*, 1985; van der Merwe and Kotze, 1994), cocoa (Holderness, 1990; Guest *et al.*, 1994) and pineapples (Rohrbach and Schenck, 1985). In plants, phosphonates are rapidly hydrolyzed to phosphonic acid that is then ionized at physiological pH mainly to the phosphite anion ($H_2PO_3^-$), which is the key molecule active against *Phytophthora cinnamomi* (Ouimette and Coffey 1989a).

Commercial phosphonate based fungicide products can be grouped into ethyl phosphonate and phosphonate products. The first commercial phosphonate based product was an ethyl phosphonate specifically aluminum tris-O-ethyl phosphonate, known as fosetyl-Al. In plants, fosetyl-Al breaks down to the same active phosphite anions than do potassium phosphonates, the second group of commercial fungicides (Fenn and Coffey, 1989). These fungicides therefore are in principal similar. In recent years ammonium phosphonates have also become available commercially. The effectiveness of phosphonate is dependent upon a number of factors, including host resistance, environmental conditions, the phosphonate concentration applied (dosage), application method and the time of crop phenology at application (Jackson *et al.*, 2000).

PHOSPHONATES

Application methods and timing

Phosphonates can be applied to avocado trees using foliar sprays, truck injections, trunk paints, trunk sprays or soil drenches (Hardy *et al.*, 2001). These various application methods are possible since phosphonates are first translocated in the xylem and then move to the phloem (Guest and Grant, 1991). From the phloem they are translocated along with photo assimilates to the rest of the plant. Translocation is always in source-sink relationship. (Saindrenan *et al.*, 1988; Ouimette and Coffey, 1990; Guest and Grant, 1991). Since plants cannot metabolize phosphite, it persists in tissues for extensive periods (McDonald, 2001),

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and is lost most likely due to a dilution effect that occurs when plants grow and increase in volume or due to leaf fall or fruit drop (Guest and Grant, 1991). In avocado, the most effective application method for phosphonates is trunk injections. This is a standard preventative strategy used in the avocado industry in South Africa, where trunk injections are applied twice a year at a rate of 0.4 g a.i./m^2 (Darvas *et al.,* 1984). Due to increasing labour costs associated with trunk injections, foliar sprays are becoming of interest to avocado growers. In Australia, foliar sprays are mostly used instead of trunk injections in a preventative control strategy (Thomas, 2008).

The timing of phosphonate applications are important, since the amount of phosphite that is ultimately translocated to roots is directly related to the sink strength of the shoots and fruits at the time of application. The optimum time for application in avocado consists of applications made just after summer flush has hardened off, and after the spring flush has hardened off (Whiley *et al.*, 1995; Menge *et al.*, 1999; Thomas, 2008). These application times yield the highest phosphite levels in roots and coincide with root flushing (Whiley *et al.*, 1995; Thomas, 2008).

Mode of action

Phosphonates most likely have a complex mode of action including (i) acting directly by suppressing pathogen growth as a result of accumulated phosphite in plant tissue, i.e. fungistatic effect, (ii) acting indirectly by stimulating the release of stress metabolites (elicitors) from the pathogen to elicit the plant defense responses, and (iii) indirectly by stimulating host defense responses itself (Coffey and Bower,1984; Guest,1986; Saindrenan *et al.*, 1988; Smilie *et al.*,1989; Jackson *et al.* 2000; Daniel and Guest, 2006). Since *Phytophthora* can be re-isolated from phosphonated treated plants and will grow after *in vitro* exposure to phosphonates, it is rather fungistatic than fungitoxic (Guest and Grant, 1991; Dobrowolski *et al.*, 2008). With the indirect action mentioned under point ii above, phosphite is hypothesized to trigger the release of elicitors or inhibit suppressor production by the pathogen, thus assisting plant defense responses to stop pathogen infection (Guest and Grant, 1991).

Direct inhibition may be more important in situations where high concentrations of phosphite are present in plant tissue (Afek and Sztejnberg, 1989; Jackson *et al.*, 2000), or in plants with poor defense responses exist (Guest and Grant, 1991). Indirect inhibition involving host responses may be more important at low concentration of phosphite in plant tissue where defense mechanisms against the pathogen are stimulated (Afekans and Sztejnberg, 1989; Massoud *et al.*, 2012). Another fact to consider is that since *Phytophthora* species vary greatly in their *in vitro* sensitivity towards phosphite, direct inhibition may be more important in *Phytophthora* species that are highly sensitive to phosphite *in vitro* than in species that are more tolerant to phosphite *in vitro* (Saindrenan *et al.*, 1988; Fenn and Coffey, 1989; Guest and

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Grant, 1991; Darakis *et al.*, 1997). Several studies have provided supporting evidence for an indirect action of stimulation of host defense responses in phosphite-treated plants following pathogen challenge (Grant *et al.*, 1990; Jackson *et al.,* 2000; Daniel and Guest, 2005). However, several studies have also provided evidence for a direct fungistatic mode of action (Coffey and Bower, 1984; Fenn and Coffey, 1984; Fenn and Coffey, 1985; Dolan and Coffey, 1988; Ouimette and Coffey, 1989a; Fenn and Coffey, 1989).

The mode of action of phosphonates is still being debated. This is most likely because the mechanism involved, or combinations of mechanisms involved, are influenced by several factors. Jackson *et al.* (2000) found evidence that in jarrah seedlings *P. cinnamomi* can be controlled directly or indirectly, but this depends on (a) the time interval between phosphite treatment and inoculation; (b) the concentration of phosphite applied and (c) the tolerance of the host and the pathogen to phosphite. Similar findings were made in the Arabidopsis-*Hyaloperonospora Arabidopsis* host-pathogen system (Massoud *et al.*, 2012).

In vitro studies on the effect of phosphite concentrations on *Phytophthora*

Effect on pathogen

In *P. cinnamomi* and *P. citricola*, phosphite inhibits the growth of mycelium, formation of sporangia and oospores, production of chlamydospore and release of zoospore (Coffey and Joseph, 1985), but it does not kill the pathogen (Shearer and Tippett, 1989; Marks and Smith, 1992; Smith, 1994; Pilbeam *et al.*, 2000). McCarren *et al.* (2009) suggested that phosphite might induce dormancy to the pathogen. King *et al.* (2010) were able to show that growing *P. cinnamomi* isolates in the presence of 40 µg/ml of phosphite had a direct effect on *P. cinnamomi.* Phosphite inhibited the functionality of the cytoskeleton and cell lysis in microscropic (hyphal distortions) studies and in molecular level studies (the expression of 43 phosphite-regulated transcripts were changed). Perez-Tur *et al.* (1995) showed that low phosphite levels disrupt the metabolism of the pathogen, with resulting alterations in the cell wall structure of the pathogen that causes the release of elicitors leading to an induction of plant defense mechanisms. Niere *et al.* (1994) reported the effects of phosphite on the levels of acid-soluble phosphorylated metabolites in a number of species of *Phytophthora.* It was proposed that the toxic effect of phosphite appears to perturb phosphorus metabolism by causing a massive accumulation of polyphosphate and pyrophosphate but not sugar-P or nucleotide-P pools, which inhibits key phosphorylation reactions in *Phytophthora* species.

Methods for assaying in vitro phosphite sensitivity

The *in vitro* sensitivity of *Phytophthora* to phosphite can be measured using artificial media. *In vitro* methods measure the direct toxicity of phosphite towards *Phytophthora.* For *in vitro* assays the inhibition of mycelium radial growth can be measured on solid medium or by

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comparing the dry weight of mycelia grown in liquid medium (Smillie *et al*., 1989; Wilkinson *et al*., 2001a). Measurement of radial growth is a relatively simple method of determining inhibition, but the density of mycelium is not accounted for. This method may therefore not provide a realistic measure of growth reduction. According to Davison and Tay (1986) and Guest and Grant (1991), measuring mycelium growth inhibition by using liquid medium is a more accurate method than radial growth measurements, although it is a very labor intensive method.

The *in vitro* sensitivity to phosphite has been reported for many *Phytophthora* species, including *P. citrophthora, P. citricola, P. cinnamomi, P. infestans* and *P. palmivora* (Coffey and Bower, 1984; Coffey and Joseph, 1985, Fenn and Coffey, 1985, Dolan and Coffey, 1988; Ouimette and Coffey, 1989a; Komorek and Shearer, 1997; Jackson, 1997a; Wilkinson *et al*., 2001a). The sensitivity of *Phytophthora* species to phosphite varies within species, with isolates from the same species varying in sensitivity (Coffey and Bower, 1984; Ouimette and Coffey, 1989a; Komorek and Shearer, 1997; Jackson, 1997a; Wilkinson *et al*., 2001a). There are also great differences in the sensitivities between species, for example *P. cinnamomi* is much more sensitive to phosphite than *P. infestans* (Table 1; Coffey and Bower, 1984; Fenn and Coffey, 1984; Coffey and Joseph, 1985; Ouimette and Coffey, 1989a).

The *in vitro* effect of phosphate on phosphite sensitivty

Orthophosphate (HPO_4^{2-}) or phosphate (PO_4^{3-}) has been shown to affect the *in vitro* toxicity of phosphite in a few *Phytophthora* spp. Therefore, the interaction between phosphite and phosphate is important to investigate in *in vitro* studies. The phenomenon is likely due to the fact that phosphate is a competitive inhibitor of phosphite uptake by *Phytophthora* spp., i.e. phosphate inhibits phosphite uptake and transport (Barchietto, *et al*., 1989; Griffith *et al*., 1989a; Fenn and Coffey, 1989; Darakis *et al*., 1997;). Griffith *et al.* (1989a) reported that only μM concentrations of phosphate are required to inhibit phosphite transport, whereas mM concentrations of phosphite is required to inhibit phosphate uptake. Griffith *et al.* (1989b) found in the medium containing excess phosphate, the growth of mycelium of *P. palmivora,* was not markedly inhibited until phosphite concentrations above were 10 mM. In contrast, in the phosphate-deficient medium, the mycelium growth was inhibited by 0.1 mM phosphite.

Most *in vitro* studies that have been conducted on the influence of phosphate on inhibition by phosphite have used agar based assays measuring radial growth. The agar media used in most of these studies contained very low levels (< 1 mM) of phosphate (Table 1), which is not representative of phosphate levels occurring in plants. The range of phosphate concentrations occurring in plants is typically between 0.5 - 20 mM (Bieleski, 1973). In *Phytophthora palmivora,* phosphate is growth limiting at concentrations of 10 - 100 μM, whereas above 1 mM it is in excess (Griffith *et al*., 1993), but this may vary with species.

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The effect of phosphate on phosphite inhibition, however, seems to vary considerably with species (Fenn and Coffey 1989). Results can also be influenced by the type of media, i.e. liquid versus agar media. Fenn and Coffey (1984) found that a phosphite sensitive *P. citricola* and *P. cinnamomi* isolate responded differently to the influence of increasing phosphate concentrations (0.084, 0.84 and 8.4 mM). When using liquid media for the assay, the phosphite sensitivity of the *P. citricola* isolate was not influenced by the phosphate concentration, whereas the *P. cinnamomi* isolate was inhibited significantly less (~15%) by phosphite as the phosphate concentration increased. However, in an agar medium assay using 50 µl/ml phosphite, the increasing phosphate concentrations had no significant effect on phosphite inhibition of the *P. cinnamomi* isolate based on radial growth (Fenn and Coffey, 1984). This again highlights the fact that the effect of phosphate is larger in liquid than in agar assays. Darakis *et al.* (1997) also found that the inhibitory effect of phosphate on the *in vitro* toxicity of phosphite was even more pronounced in *P. capsici* in liquid media than in agar assays.

Fenn and Coffey (1989) investigated the effect of 5, 15 or 45 mM phosphate on the phosphite sensitivity of one phosphite tolerant (generated through mutagenizes) and one sensitive isolate of *P. capsici,* and one phosphite sensitive *P. parasitica* var. *nicotianae* (syn. *P. nicotianae*) isolate. Inhibition was based on radial growth on agar media. They found that for all three isolates phosphite inhibition was higher at 5mM than at 15 and 45 mM phosphate. The inhibition of growth by phosphite at 15mM and 45mM phosphate was similar, although at some phosphite concentrations inhibition at 45mM phosphate was higher than at 15 mM phosphate (Fenn and Coffey, 1989). Therefore, although there is a trend that *Phytophthora* is inhibited less by phosphite at higher phosphate concentrations, it cannot be generalized that this is always true.

In *P. palmivora,* Griffith *et al.* (1993) investigated the effect of phosphate concentrations (0, 0.01, 0.1 and 1 mM) in three isolates that differed in phoshite sensitivity using a liquid medium. It was shown that the sensitive isolate was inhibited by phosphite at all phosphate levels, whereas the resistant isolates were only inhibited when phosphate was limiting to growth. The phosphite tolerant isolates were also able to exclude phosphite more effective than the sensitive isolate at higher concentrations of phosphate. This suggested that phosphite tolerant isolates have phosphate transport system that can discriminate between phosphite and phosphate thus resulting in less phosphite being taken up by the pathogen. This discrimination ability did, however, not provide a complete explanation of the differences in sensitivities between isolates and there must thus also be differential sensitivity to phosphite at one or more internal sites in the isolates (Griffith *et al.*, 1993).

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Range of *in vitro* sensitivities in *P. cinnamomi*

Coffey and Joseph (1985) studied the effects of phosphite on different growth stages of four *P. cinnamomi* isolates. The EC_{50} value for inhibition of sporangia formation by phosphite was 1.8 µg/ml for *P. cinnamomi*, with EC_{50} values for the inhibition of zoospore release being 6 µg/ml for *P. cinnamomi.* Oospore formation was also very sensitive to phosphite, since oospore formation was inhibited by 60 - 78% at 1 µg/ml. Chlamydospores were more tolerant and inhibition was observed at 15 - 44 µg/ml.

In South Africa, Duvenhage (1994, 1999, 2001) conducted long term *in vitro* screening studies to determine if *P. cinnamomi* isolates obtained from avocado trees with prolonged use of phosphonates (Fosetyl-Al and potassium phosphonate) were less inhibited by Fosetyl-Al and phosphite. The radial growth inhibition of the isolates, 5 to 10 isolates per treatment at each evaluation time, was screened on corn meal agar with a low phosphate level (0.38 mM) and at a phosphite concentration of 100 µg/ml phosphite. Isolates collected in October 1992 and March 1993 from potassium phosphonate treated trees were inhibited significantly less by phosphite than isolates from untreated trees. However, isolates from treated and non-treated trees collected in November 1993 did not differ significantly in phosphite sensitivity, although there was a trend for decreased sensitivity (Duvenhage, 1994). Screening of isolates collected in 1998 showed that isolates from potassium phosphonate treated trees were not significantly less sensitive to phosphite, but isolates from Aliete treated trees were significantly less sensitive than untreated trees. Although the results were not always consistent in each screening that isolates from treated trees were more phosphite tolerant, Duvenhage (1999) stated that since a 13% average decrease in phosphite sensitivity was observed for isolates collected from 1992 - 1998 from phosphonate treated trees versus untreated trees, it was concluded that there is a shift in sensitivity of *P. cinnamomi* after long term treatment of isolates with phosphonates (Duvenhage, 1999). The sensitivity screening results of isolates collected in 2000 were unexpected since isolates from un-treated trees were more tolerant to phosphite than isolates from the phosphonate treated trees. This was most likely due to the introduction of a new population of *P. cinnamomi* into the study orchard due to extreme flooding occurring in 2000 (Duvenhage, 2001). Therefore, the orchard that was used for these long term studies were deemed not useful for further studies on the long term effect of phosphonate treatment on the sensitivity of *P. cinnamomi* to phosphonates.

Coffey and Bower (1984) evaluated 12 *P. cinnamomi* isolates collected from various hosts for sensitivity towards phosphite in a low phosphate medium (0.84 mM). The radial growth of the isolates was inhibited significantly different by 5 µg/ml phosphite (0% - 44.8%). The EC_{50} values were determined for three isolates and these ranged from 9 - 11.9 µg/ml.

Wilkinson *et al.* (2001a) identified three phosphonate sensitivity groups among 71 *P. cinnamomi* isolates collected from Australian native tree species. The isolates were tested on

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Ribeiro's medium containing 7.35 mM phosphate. The sensitivity groups were comprised of sensitive (9% of isolates, EC_{50} 4 - 5 µl/ml), intermediate (82% of isolates; EC_{50} 9 - 14 µl/ml) and tolerant (9% of isolates, EC_{50} 25 - 148 µl/ml) isolates. In their studies they found a significant correlation between the growth rate of isolates on un-amended medium and phosphite amended medium. The phosphite-tolerant isolates grew faster than the phosphite sensitive isolates on the un-amended medium (Wilkinson *et al.*, 2001a).

The *in vivo* (*in planta*) effect of phosphate on phosphite

It is mostly unknown whether *in vivo*, phosphate will influence the inhibition of *Phytophthora* by phosphite. Only two studies have investigated this. Dolan and Coffey (1988) found that increased phosphate levels improved control of *P. palmivora* in tomato by phosphonates at most phosphonate dosages, which was unexpected considering the known *in vitro* effects of phosphate on phosphite sensitivity. However, not all phosphonate dosages that were applied to seedlings had this effect and results were thus not straight forward to interpret. The results from this experiment are also difficult to interpret since the zoospores of the pathogen came into contact with the phosphate and phosphite solutions prior to infection of the seedling since the zoospores were added to the phoshonate and phosphate containing water solutions in which the tomato seedlings were grown (Dolan and Coffey, 1988). Therefore the *in planta* effect was not truly measured due to the direct exposure of the zoospores to the phosphate on phosphite solutions prior to host infection. Smillie *et al.* (1989) found some data that suggest, but does not prove, that in tobacco an increase in lesion length caused by *P. nicotianae* of phosphonate treated plants was associated with a concurrent increase in phosphate concentrations in the plants at flowering. Inoculation of phosphonate treated tobacco plants at weekly intervals over a 5 week period showed that phosphite concentrations remained stable throughout this period, but that plants inoculated at the 5 week time point developed significantly longer lesions, which coincided with a significant increase in phosphate concentrations when the plants started flowering. It was hypothesized that the increased phosphate level in the plants reduced the uptake of phosphite by the pathogen thus enabling it to cause larger lesions (Smillie *et al.*, 1989).

Comparing the effect of *in vitro* and *in planta* phosphite concentrations on *Phytophthora*

The research group of Coffey conducted several studies to determine if a correlation existed between the *in vitro* and *in vivo* (*in planta*) phosphite sensitivity of *Phytophthora* isolates. They found good correlations between phosphite concentrations required for *in vitro* and *in planta* suppression of *Phytophthora.* This was taken as good evidence to support a direct fungistatic mode of action for phosphite (Fenn and Coffey, 1984; Fenn and Coffey, 1985; Dolan and

Coffey, 1988; Ouimette and Coffey, 1989a). Coffey's research group also found further evidence for a direct fungistatic mode of action for phosphite against *Phytophthora,* based on the fact that with *P. capsici* (tomato) and *P. nicotianae* (tobacco) it was found that α-aminooxyacetic acid (AOA), an inhibitor of host defense systems, could only partly reduce the efficacy of phosphite against these pathogens in plants. The interpretation of the role of AOA's in suppressing host defense was not clear-cut since AOA also inhibited phosphite uptake by the two *Phytopthohra* spp. and therefore also had a direct effect on the pathogen (Fenn and Coffey, 1985, 1989).

Fenn and Coffey (1984) found that the concentration of phosphite required for suppressing *P. citricola in vitro* in a high phosphate medium (8.4 mM) was similar than the phosphite concentration required in avocado stems for suppressing the pathogen. This was taken as good circumstantial evidence for a direct fungistatic mode of action (Fenn and Coffey, 1984). The inoculation was conducted 27 h before the phosphonate applications were made, and evaluations were made 5 days after phosphonate application. These time frames would not have allowed for the induction of host defense responses to play a role, since these take a few days to be induced.

Fenn and Coffey (1985) re-investigated the influence of phosphonates (potassium phosphonate and fosetyl-Al) on the tomato-*P. capsici* host-parasite system. A phosphite sensitive *P. capsici* isolate and a mutated phosphite tolerant isolate were used to inoculate tomato leaflets 24 h prior to floating the leaves on solutions of phosphonates. Thus, in effect the curative activity of phosphonates was evaluated, excluding the launching of host defense responses. Using this approach Fenn and Coffey (1985) found strong evidence to support a direct fungistatic mode of inhibition because potassium phosphonate was unable to control lesion development in tomato leaflets inoculated with the phosphite tolerant isolate of *P. capsici* (*in vitro* EC_{50} of 415 μg/ml), with the *in vivo* EC_{50} phosphite value being > 384 μg/ml. However, it was noted that there is a difficulty in interpreting *in vivo* versus *in vitro* data due to difference in phosphate levels in the V8 agar used (188 μl/ml) for *in vitro* sensitivity and in the tomato leaflets (634 μl/ml) since it is known that phosphate can reduce the antifungal activity of phosphonates (Fenn and Coffey, 1985). Further evidence for a direct fungi toxic mode of action was the fact that lesions caused by the sensitive *P. capsici* isolate was significantly larger when the tomato leaflets contained 31 to 56 $\mu g/g_{\text{Fresh Weight (FW)}}$ phosphite than when the leaflets contained a higher phosphite concentration (151 $\mu g/g_{FW}$) (Fenn and Coffey, 1985).

Dolan and Coffey (1988) generated mutant strains of *P. palmivora* that were tolerant to phosphite to show that similar concentrations of phosphite was required to suppress the pathogen *in vitro* and *in vivo* (tomato seedlings). The EC_{50} values for the parental and tolerant strains for *in vitro* radial growth were 36.6 to 130.3 μg/ml for the tolerant strains and 6.6 μg/ml for the parental strain. The EC_{50} values for inhibition of infection of tomato seedlings was 47

to 473 µg/g_{FW} for the tolerant strains and 38 µg/g_{FW} for the parental type. This was taken as data that supported a direct fungistatic mode of action of phosphorous acid against *P. palmivora* (Dolan and Coffey, 1988). The interpretation of the *in vitro* inhibition data is however difficult since it probably overestimated the level of suppression due to the fact that assays were conducted in a low phosphate medium (0.5% corn meal agar that would have had less than 0.38 mM phosphate). Furthermore, the authors did did not quantify the phosphite concentration in the roots of the plants. The simultaneous application of phosphonates and pathogen inoculum into the sterile water in which the tomato seedlings were grown would also have caused a direct toxic effect of phosphite to the zoospores.

Fenn and Coffey (1989) investigated the *in vitro* and *in planta* (tomato leaflets and tabaco seedlings) sensitivity of one phosphite sensitive isolate and one tolerant isolate (generated through mutagenesis) in two *Phytophthora* spp. (*P. nicotianae* and *P. capsici*). The sensitive isolates of both species could not cause symptoms on their respective hosts containing high phosphite concentrations (88 - 215 µg/g_{FW}), whereas the tolerant isolates could cause symptoms on these hosts that contained even higher phosphite concentrations (484 to 554 µg/g_{FW}). The inoculations of the hosts were conducted 24 hours after phosphonate application and phosphite quantification was done 48 h after phosphonate application (Fenn and Coffey, 1989).

Saindrenan *et al.* (1988) investigated the *in vitro* and *in planta* effect of phosphite concentrations on *P. cryptogea* in susceptible cowpea leaves. They found that the phosphite levels in leaves floated on phosphite solutions was too low to account for a direct effect of inhibition on the pathogen infecting the leaves. The phosphite concentration detected in leaves would have only caused a 23 - 26% reduction in *in vitro* growth (medium with high phosphate level), whereas the pathogen growth was completely halted within the leave tissue by phosphite (Sandrenan *et al.*, 1988). The pathogen was inoculated simultaneously with phosphonate application and pathogen colonization was assessed 24 h after inoculation and phosphonate application (Sandrenan *et al.,* 1988). It seems unlikely that this would have allowed for host defense induction to play a role in the suppression.

Smillie *et al.* (1989) investigated the correlation between *in planta* and *in vitro* phosphite concentrations in three different host-pathogen systems, which included *P. cinnamomi* in lupine, *P. nicotianae* in tobacco and *P. palmivora* in papaya. In all plants the apical meristem was inoculated and assessed for lesion length. In all three systems, the phosphite concentrations achieved in the apical meristems with phosphonate (potassium phosphonate) soil applications were comparable (approximately 150 - 200 µg/g_{FW}). These phosphite concentration in all plant species were within the range that were required for *in vitro* suppression of the respective pathogens in an agar medium with 7 mM phosphate. Furthermore, in the lupine and tobacco plants a high negative correlation (-0.95 to 0.99) was

found between lesion length and phosphite concentration in the plants. It was thus concluded that a direct mode of action is involved. However, the plants' defense system was also deemed important since although *P. cinnamomi* and *P. nicotianae* isolates had similar *in vitro* phosphite sensitivities, in lupine lesion formation could never be halted whereas lesion development in tobacco was halted 48 h after inoculation. Thus, tobacco plants treated with phosphonates are able to launch a better defense against its pathogen than lupine plants, which are known to be highly susceptible to *P. cinnamomi* (Smillie *et al.*, 1989).

In jarrah (*Eucalyptus marginata*), phosphonates (potassium phosphonate) applied as a foliar spray resulted in phosphite concentrations that varied over time. Phosphite concentrations were highest (approximately 3000 $\mu g/g_{DW}$) 14 days after application, whereas it remained at comparable levels 2 and 5 days after application (approximately 2000 $\mu g/g_{DW}$). The inhibition of lesions caused by a *P. cinnamomi* isolate that had moderate tolerance to phosphite *in vitro* (EC_{50} 9 - 14 µl/ml) was highest at 14 days after phosphonate application when phosphite concentrations were highest in plants. It should, however be noted that although there was a trend for lesion lengths to be the smallest 14 days after application, there were no statistically significant differences in lesion lengths, 2, 5, 8 and 14 days after application. There was also no significant correlation (R^2 = 0.2) between root phosphite concentrations and lesion length. It was however, concluded that at high phosphite concentrations in plants, *P. cinnamomi* is controlled via a direct fungistatic effect whereas at lower phosphite concentrations an indirect mode of action is involved (Jackson *et al.*, 2000).

In *Fagus sylvatica* (European beech trees) seedlings sprayed with phosphonates (0.5% solution), the root phosphite concentrations were high (370 to 510 $\mu g/g_{DW}$). These concentrations were ten times higher than the *in vitro* EC_{50} values determined for mycelia (34 µg/ml) and zoospores (2.9 µg/ml) of the *P. plurivora* isolate used in inoculation studies. This suggests that the pathogen is suppressed *in planta* via a direct fungistatic effect. However, since an induction in defense genes were observed only in inoculated phosphonate treated plants, it was concluded that a host response may also be involved in suppression of the pathogen (Dalio *et al.*, 2014).

Studies on phosphite concentrations in plant tissues

The significance of *in planta* concentrations of phosphite required for controlling oomycete diseases has been investigated in several studies. Several studies have also investigated only the concentrations of phosphite accumulating in plants over time, without determining the effect on pathogen inhibition. The role of *in planta* phosphite concentrations in suppressing oomycetes is difficult to determine, due to species-specific differences among plants and variation in the sensitivities of various *Phytophthora* species to phosphite (Barrett *et al.*, 2001). To maintain the effective concentrations of phosphite in plants, assuming a direct fungistatic

mode of action or induction of host defense, different plant species will require different phosphonate dosages and application frequencies. The amount of phosphite in plants required to suppress *Phytophthora* vary widely according to plant and *Phytophthora* spp. (Table 2). The only host crop where a critical phosphite concentration, minimum amount of phosphite required in plants for suppressing *Phytophthora,* has been reported is for the suppression of *P. cinnamomi* in avocado (Thomas, 2008; personal communication A.W. Whiley, Sunshine Horticultural Services Pty Ltd). The critical root phosphite concentration (25 - 30 $\mu g/g_{FW}$) in Australia has been determined based mainly on long term data sets obtained from commercial root phosphite analyses from trees that responded and did not respond to phosphonate treatment (personal communication, A.W. Whiley). However, experimental evidence is still lacking.

A few studies have been conducted on root phosphite concentrations in avocado under glasshouse conditions. van der Merwe and Kotze (1994) used an innovative method to correlate root phosphite concentrations with susceptibility of roots to *P. cinnamomi.* This was done by injecting seedlings with phosphonates, and harvesting roots at different time points for phosphite quantification and *in vitro* colonization by *P. cinnamomi.* The *in vitro* colonization and thus susceptibility of roots to *P. cinnamomi,* was investigated using an excised root assay, initially developed to investigate the tolerance of avocado rootstocks to *P. cinnamomi* (Dolan and Coffey, 1988), and subsequently optimized by Botha *et al.* (1990). This method consists of the inoculation of root tips with a *P. cinnamomi* zoospore solution, and after incubation, the percentage colonized root pieces from the tip is determined by sequential plating of the excised root. van der Merwe and Kotze (1994) were able to show that a correlation exists between root phosphite concentration and colonization by *P. cinnamomi* when phosphite levels were less than 9.5 $\mu g/g_{FW}$. At concentrations above 9.5 to 53.2 $\mu g/g_{FW}$l no significant differences were found in protection of roots against *P. cinnamomi* (van der Merwe and Kotze, 1994). The *in vitro* phosphite sensitivity of the *P. cinnamomi* isolate used in these inoculation studies was not reported. Botha *et al.* (1988) obtained high phosphite concentration in avocado seedlings in the glasshouse, with root phosphite concentrations reaching > 200 $\mu g/g_{FW}$, 14 and 21 days after application. The effect of phosphite on pathogen infection and colonization was not investigated in these glasshouse trials. In an orchard trial, Botha *et al.* (1988) detected approximately 10 $\mu g/g_{FW}$ root phosphite concentrations, 2 weeks after treatment. The electrical conductivity (EC) measurements, which has been reported as being indicative of electrolyte leakage from roots and resistance to *P cinnamomi* (Silberstein and Pinkas, 1987), showed that 2 weeks after phosphonate treatment, resistance was induced in the host roots that could potentially have inhibited the pathogen (Botha *et al.,* 1988).

Ouimette and Coffey (1989b) investigated phosphite concentrations in avocado seedlings under glasshouse conditions. Phosphonate fungicides (fosetyl-Al and potassium

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phosphonate) were applied as foliar sprays or as a soil drench to avocado seedlings in the greenhouse. Soil application of potassium phosphonate resulted in high root phosphite concentrations 1 week after application (356 µg/g_{FW}), which peaked 4 weeks after application (1399 µg/g_{FW}) and then steadily decreased to 213 µg/g_{FW} 8 weeks after application. The phosphite concentration that peaked at week 4 was significantly higher than concentrations in week 1, 2, 6 and 8, but concentrations in week 1, 2, 6 and 8 did not differ significantly from each other. Foliar applications resulted in much lower root phosphite concentrations (11 to 15 µg/g_{FW}) of which the concentrations did not vary significantly over the 8 week monitoring period (Ouimette and Coffey, 1989b).

In agricultural crops, avocado trees in orchards have been best studied with regards to the concentrations of phosphites in tissues and the translocation and accumulating of phosphite in trees. Nartvaranant *et al.* (2004) investigated the effect of different timings of phosphonate applications on phosphite concentrations in different avocado tissues in orchard trees. Foliar application of phosphonates during early anthesis can results in high phosphite levels in pollen (260 - 383 µg/g_{FW}), whereas applications made at summer flush maturity resulted in the lowest phosphite concentrations (3 - 10µg/g_{FW}) in influorescence. Applications made at floral bud break resulted in somewhat higher phosphite levels (20 - 28 µg/g_{FW}) in influorescence (Nartvaranant *et al.* 2004). Whiley *et al.* (1995) showed that the measuring of phosphite concentrations in roots is important for determining the optimal time at which trunk injections must be applied. Injections applied at the maturity of summer shoot growth yielded higher and more sustained root phosphite concentrations (approximately 20 - 30 µg/g_{FW} for 5 month period) than an injection at the maturity of spring flush. The importance of summer flush applications above spring applications was also shown (Whiley *et al.*, 1995). In orchards trees, although this depends on time of application, phosphite levels usually reach their maximum (25 to 60 µg/g_{FW}) at 2 - 6 weeks after application where after it can remain stable for several months (Botha *et al.*, 1988; Schutte *et al.*, 1988; Whiley *et al.*, 1995).

Wilkinson *et al.* (2001b) investigated the time span of pathogen protection and phosphite concentrations in stems in three native western Australian species (*Banksia grandis, B. hookeriana* and *Dryandra sessilis*) evaluated 2 weeks and 12 months after phosphonate application. Phosphonates inhibited pathogen growth for at least 12 months in these species. With one exception, phosphite concentrations in the stems declined over time. The initial amount of phosphite in stems (2393 - 1284 µg/g_{DW}) after a foliar spray of 5 g/l varied between the species. The inhibition of pathogen growth was correlated in *B. hookeriana* with phosphite stem concentrations but not in *D. sessilis* and *B. grandis.* In the latter species phosphite concentrations decreased from 1284 µg/g_{DW} at 2 weeks after phosphonate application to 209 µg/g_{DW} at 12 months after inoculation, yet pathogen growth was inhibited similarly during these time periods (Wilkinson *et al.*, 2001b). Since the same *P. cinnamomi* isolate was used for

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inoculation of all hosts, this would suggest that suppression by host defense systems are also involved, which are more effective in some plant species/genotypes than others.

Groves *et al.* (2015) investigated the control of *P. cinnamomi* in lupine relative to root phosphite concentrations. Lupine seedlings were inoculated at different time points after phosphonate application and phosphite concentration and root lesions and percentage roots colonized were monitored over a 10 day period. This showed that although root phosphite concentrations peaked at 2 days after application where after it decreased, root lesion length and percentage roots infected were reduced significantly more 10 days after application than at 2 days after application (Groves *et al.*, 2015).

Although Massoud *et al.* (2012) did not directly measure phosphite concentrations in plant tissue, their experiments where a concentration range of phosphonates were applied to plants, yields important information on the mode of action of phosphite against the obligate foliar pathogen *Hyaloperonospora arabidopsidis*. It was assumed that the concentration range (5 to 100 mM phosphite that is equivalent to 410 mg/l to 8.2 g/l) of phosphonates applied during their soil drenches resulted in different internal phosphite concentration in *Arabidopsis thaliana.* The dose response curve of these application showed a bi-phasic dose dependent response where in the first phase a linear increase in pathogen spore inhibition was observed from 7.5 to 12.5 mM (65 - 820 mg/l), followed by a plateau from 12.5 to 22.5 mM (1.02 to 1.85 g/l). In the second phase a linear increase in pathogen inhibition again occurred with dosages from 22.5 to 50 mM (4 g/l). This data was interpreted in that a host response is responsible for pathogen suppression at low dosages (< 12.5 mM) and a direct fungistatic effect at high dosages (22.5 to 50 mM). Disease suppression between 12.5 to 22.5 mM is probably a combination of host defense responses and a direct fungi toxic effect. It might be that 12.5 mM is the maximum/optimal dosage that results in expression of induced host plant resistance, or that phosphite target sites in the plant are saturated. This hypothesis was further supported by the fact that salicilyc acid (required for phosphonate induced host defense s in *Arabidopsis*) impaired mutant plants exhibited no pathogen protection at a low phosphonate dosage (10 mM) but pathogen inhibition in the mutants was only partly abolished at a higher phosphonate dosage (25 mM) (Massoud *et al.*, 2012). At the highest phosphonate dosage (50 mM) pathogen inhibition did not differ significantly between the SA impaired mutant plants and wild type plants. This assumes that only salicylic acid is involved in phosphonate host induced resistance (Massoud *et al.*, 2012).

Dalio *et al.* (2014) monitored phosphite root concentrations in *Fagus sylvatica* seedlings. Phosphite concentrations measured 6 to 10 days after application was approximately 350 $\mu g/g_{DW}$. The highest phosphite concentration (approximately 500 $\mu g/g_{DW}$) was measured 14 days after application, the last time point at which phosphite was measured in the roots. The infected seedlings showed no symptoms, and *P. plurivora* DNA measured in the roots did not

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differ significantly between 6 to 14 days after phosphonate application. Thus, no correlation was found between phosphite root concentration and suppression of *P. plurivora*. (Dalio *et al*., 2014).

Studies on the induction of host plant defense responses by phosphonate treatment

As our knowledge on the mechanisms of host resistance induction by resistance inducers has increased over the past two decades, molecular studies focusing on phosphonates in this field has also increased in recent years. Several studies have been conducted that found physiological or molecular evidence for the induction of host defense responses after phosphonate applications. Interestingly, all of these studies have found that a primed response is involved, i.e. defense gene induction only occurs upon pathogen challenge (Saindrenan *et al*., 1988; Nemestothy and Guest, 1990; Jackson *et al*., 2000, Eshraghi *et al*., 2011; Massoud *et al*., 2012)

In histopathological studies conducted in *Eucalyptus marginata* (Jackson *et al*., 2000), reduced lesion development was associated with enhanced levels of activity of host defense enzymes (cinnamyl alcohol dehydrogenase) and accumulation of soluble phenolics (an end product of the phenylpropanoid pathway) in phosphonate treated seedlings inoculated with *P. cinnamomi.* The induction of defense responses was significantly higher at 2 and 5 days after phosphonate application than at 14 days after application. Interestingly, the inductions of these compounds were highest when phosphite concentrations were lowest (1500 - 1800 $\mu g/g_{DW}$) in the plants. At the higher phosphite concentration (3200 $\mu g/g_{DW}$) 14 days after application the concentrations of these compounds were much lower. This lead to the conclusion that host defense responses are induced at low but not high phosphite concentrations in this host-pathogen system (Jackson *et al*., 2000).

A few studies have found phenolic compounds and phytoalexins to accumulate in response to phosphonate applications and *Phytophthora* infection. Daniel *et al.* (2005) showed that phosphite-treated *Xanthorrhoea australis* seedlings accumulated high levels of lignin-like and phenolic compounds, especially in cortical and vascular cell walls of infected tissue that restricted *P. cinnamomi.* Increased levels of phytoalexins were also found to accumulate in phosphonate treated seedlings of citrus inoculated with *P. citrophthora* (Afek and Sztejnberg, 1989). Saindrenan (1988) investigated the accumulation of phytoalexins (kievitone, phaseollidin and vignafuran) in cowpea leaves infected with *P. cryptogea* upon phosphonate (fosetyl-Al) treatment. The concentration of phasseollidin and vignafuran increased from 12 to 48 hours after phosphonate application and inoculation, but the concentration of kievitone peaked at 24 hours and then decreased. Since vignafuran only reached low levels, it was not thought to contribute to pathogen inhibition (Saindrenan, 1988). In tobacco, sesquiterpenoid phytoalexins and to a lesser extent lignin is induced by phosphonates (fosetyl-Al) and

inoculation with *P. nicotianae.* Most, but not all of the sequiterpenoid phytoalexins peaked 24 hours after phoshonate application, whereas the remaining phytoalexins peaked and remained stable from 24 to 48 hours after application and inoculation. The role of phytoalexin induction in phosphonate efficacy was further proven by the fact that application of compounds that inhibited phytoalexin inhibition in the inoculated leaves reduced the efficacy of phosphonates (Nemestothy and Guest, 1990).

Eshraghi *et al.* (2011) studied the effect of phosphite on the induction of defense response in *P. cinnamomi*-infected *Arabidopsis thalina* leaves. They found an enhanced production of hydrogen peroxide and callose deposition following pathogen challenge, indicating that phosphite primes the plant for a rapid and intense response to infection that is systemic. Another consequence of host defense induction by phosphonates, which results in pathogen restriction and control, is the induction of cell death, also sometimes referred to as the hypersensitive response (HR).

Robinson and Cahill (2003) used an *Arabidopsis thaliana-Phytophthora cinnamomi* pathosystem to demonstrate that *A. thaliana* can mount phosphonate induced defense responses through induced cell death with the production of hydrogen peroxide and consequently a reduction in lesion formation, pathogen growth and colonization. Daniel and Guest (2006) showed that phosphonate-treated *Arabidopsis thaliana* seedlings inoculated with zoospores of *Phytophthora palmivora* resulted in the induction of defense responses in the challenged cells that consisted of the release of superoxide and subsequently hypersensitive cell death.

A few studies have indicated that resistance induced by phosphite is salicylic acid dependent, i.e. a SA-dependent plant defense pathway is involved. Jackson (1997b) first showed that phosphite induced the accumulation of SA throughout the plant, both at the point of inoculation but also away from the site of application. Molina *et al.* (1998) used transgenic *nahG Arabidopsis* plants to provide evidence that SA is required for phosphite resistance responses since *nahG* phosphonate treated plants infected with *Peronospora parasitica* (syn. *Hyaloperonospora Arabidopsis*) showed no resistance response. Massoud *et al.* (2012), also working with *Arabidopsis* but the pathogen *Hyaloperonospora arabidopsidis,* showed that phosphite at low concentrations stimulates defense responses indirectly through SA. They also showed that SA defective plants (*sid2-1,* NahG) treated with phosphonate are not protected against *H. arabidopsidis* if low phosphite concentrations are applied (< 10 mM). At the low phosphite dosages no spore reduction was observed if inoculation and phosphonate application was conducted simultaneously, whereas pathogen inhibition was observed if inoculation occurred 24 h after phosphonate soil drenching. Groves *et al.* (2015) in lupine not only showed that phosphite application induced SA accumulation, but that exogenous application of SA can also suppress *P. cinnamomi.* Phosphite application induced the systemic

accumulation of SA in root tips as soon as 24 h after phosphonate application and peaked at 4 days (Groves *et al.*, 2015).

Several signaling pathways other than SA have also been investigated for their involvement in phosphonate host defense responses. Massoud *et al.* (2012) found that jasmonate (JA), ethylene (ET) and abscisic acid signaling were not involved in suppression of *H. arabidopsidis* in *A. thaliana* phosphonate treated plants. However, Eshraghi *et al.* (2011) found that JA/ET signaling was induced in Arabidopsis leaves treated with phosphonates in non-inoculated plants. Dalio *et al.* (2014) also found that JA/ET and SA marker genes were up-regulated in phosphonate treated *Fagus sylvatica* (European Beech) seedlings inoculated with *P. plurivora.*

Another possible host defense mechanism that is mediated through phosphonates, is that phosphonates can sometimes damage root tips that results in the reduction of pathogen infections. Groves *et al.* (2015) found that in lupine, phosphonate application results in damage of the root tips (browning and cell damage) and a reduction in root growth. This was also observed in *Ecalyptus marginata* (Jackson *et al*., 2000). It was hypothesized that this could limit *P. cinnamomi* infection since the pathogen can only infect in the zone of elongation of actively growing root tips. Phosphite would thus limit the number of available infection sites for the pathogen (Groves *et al*., 2015).

METHODS FOR QUANTIFICATION OF PHOSPHITE IN PLANT TISSUE

The increased usage of phosphite-based fungicide to control Phytophthora diseases has led to an increased interest in the development of methods for quantifying phosphite concentrations in plants. Phosphite quantification from plants is important since it can be used to provide clues as to the mechanisms of action, and it can be used to optimize management of Phytophthora diseases if a critical phosphite concentration is required in plants for disease suppression. This may vary in different host *Phytophthora* systems since a great deal of variability has been reported in phosphite concentrations accumulating in plants, and their effect on *in planta Phytophthora* inhibition and host defense responses (see sections "Studies on phosphite concentrations in plant tissues" and "Studies comparing the effect of *in vitro* and *in planta* phosphite concentrations on *Phytophthora*"). A number of analytical methods have been employed for phosphite quantification in different plant tissues (Ouimette and Coffey, 1989b; Roos *et al*., 1999; Saindrenan *et al.,* 1985; Smillie *et al.,* 1988), including radiolabelling (Fenn and Coffey,1985; d'Arcy-Lameta *et al.,* 1989; Ouimette and Coffey,1990), gas chromatography (Saindrenan *et al.,* 1985; Smillie *et al.,* 1988; Hargreaves and Ruddle,1990), Gas chromatography – mass spectrometry (GC-MS) (Smillie *et al.,* 1988) and ion chromatography (Ouimette and Coffey,1989b; Smillie *et al*., 1988; Glenn *et al*., 1990; Roos *et al*., 1999).

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The first method used to quantify phosphite in plant tissue was published by Fenn and Coffey (1985). The authors were able to detect 31 - 151 µg/g_{FW} phosphite when floating tomato leaflets on a solution containing 30 - 180 µg/ml phosphite by using radioactive labeling. Ouimette and Coffey (1988) developed an ion chromatography based method using eluent suppression and a conductivity detector to quantitative phosphite residues in plants with the limits of detection being 0.5 µg/g_{FW}, and a recovery rate of 70%. Gas chromatography – mass spectrometry (GC-MS) was first published by Smillie *et al.* (1988), this method combines the features of gas chromatography and mass spectrometry with a high recovery rate of up to 95% and a low limit of detection of 100 pg, making it very suitable for analysing small samples with low phosphite concentrations. Roos *et al.* (1999) published an ion chromatography (IC) method for quantification of phosphite from plant tissue. The first GC method was published by Saindtenan *et al.*, (1985) in combination with anion-exchange chromatography to quantify as low as 40 nmol of phosphite per gram in tomato and cowpea leaves.

Ion chromatography has been used by several authors for phosphite quantification in plants, and has the advantage that it can also quantify phosphate (Smillie *et al.*, 1988). Several publications used the method published by Roos *et al.* (1999) to quantify phosphite from various plant hosts including *Corymbia calophylla* (Fairbanks *et al.*, 2000), *Banksia grandis Willd, Banksia hookeriana, Dryandra sessilis* (Wilkinson *et al.*, 2001b) and *Fagus sylvatica* (Dalio *et al.,* 2014). Roos *et al.* (1999) used a Vydac 302IC4.6 (0.46 x 25 cm) silica-based non- suppressed ion chromatography column (supplied by The Nest Group, Inc., Southborough, MA) that is no longer manufactured. The ion chromatography method published by Ouimette and Coffey (1988) used an AS4A separator column. Their method has been used in pepper (Ouimete and Coffey, 1988) and avocado (Ouimete and Coffey, 1989b) and citrus (Orbovic *et al.*, 2008) and tomato and tobacco tissue (Fenn and Coffey, 1989) for phosphite quantification. Recently Borza *et al.* (2014) used a Metrosep A Supp 7-250 with Metrosep RP2 guard column for their ion chromatography analyses on potato tissue. The problem with published ion chromatography methods is that not all columns fit on all ion chromatography systems. For example, if a Waters (Waters Corporation, Milford, USA)) system is used, mainly columns from this manufacturer can be used on the system.

Several GC methods have been published for quantification of phosphite, which requires derivatization of plant extracts before GC analyses. GC analyses with flame photometry detection (FPD) was used in Australia for quantification of phosphite in native Australian plant species (Barrett *et al.*, 2004; Shearer and Crane, 2009; Shearer *et al.*, 2012), whereas NPSD detection was used in South Africa in avocado (Schutte *et al.*, 1988; Bezuidenhout *et al.*, 1985; van der Merwe and Kotze, 1994). GC analyses with FPD or NPSD detection, however, are problematic due to limited and variable recovery of phosphites (Smillie *et al.*, 1988). Furthermore, strictly controlled conditions, especially with the formation of derivatives, and

experience in handling the NSPD detector are required. The process is also time consuming with each sample requiring about 1 hour for preparation and analysis (Bezuidenhout *et al.*, 1985). Smillie *et al.* (1988), therefore, published a protocol for detection of phosphite in plant material using GC analyses with mass spectrometry detection (GC-MS). This method has been used by to detect phosphite concentrations in *P. palmivora* mycelia grown in liquid Ribeiro's medium (Dunstan *et al.*; 1990), quantify the phosphate concentration in rye-seed broth and 20% V8 broth (Grant *et al.*, 1992), detect the phosphite and phosphate in tobacco transgenic plants (Torres Elguera *et al.*, 2013) and quantify phosphite residue in phosphonate treated cauliflower curds (Mckay *et al.*, 1992).

The aforementioned analytical methods all have some limitations such as using toxic reagents, high cost and technical complexity (Saindtenan *et al.*, 1985; Fenn and Coffey, 1989; Roos *et al.*, 1999). The GC-MS method required extensive extraction and complex procedures prior to separation, which is concerning since these tedious protocols can increase the likely hood of chemical loss during sample preparation. The low sensitivity and selectivity of conductivity detectors used in ion chromatography do not favor the determination at residue levels in complex matrix (Hernández *et al.*, 2003). Therefore, Hernández *et al.* (2003), published a robust method namely liquid chromatography with electrospray tandem mass spectrometry (LC-MS/MS) that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS) to determine fosetyl-aluminum (Al) residues in lettuce with a high recovery rate of 98 and 106% and limit of detection as low as 0.05 mg/kg.

Berkowitz *et al.* (2011) developed and optimized a cost effective and easy to use enzymatic fluorescent assay for the quantification of phosphite from *Arabidopsis thaliana.* This assay is a high-throughput screening assay that is very sensitive with a detection limit of 0.25 nmol (0.41 µg/ml) phosphite. However, Berkowitz *et al.* (2011) reported that it was not effective on woody plants. This assay is based on the principle of oxidation of phosphite to phosphate by the enzyme phosphite dehydrogenase (PTDH) from *Pseudimonas stutzeri* with nicotinamide adenine dinucleotide (NAD^+) as a co-substrate. Resazurin is included in the reaction and is reduced to resorufin through a cycling reaction where electrons are transferred from NADH via phenazine methosulfate. The reaction product resorufin is highly fluorescent at a wavelength of 590 nm with excitation wavelength of 535 nm, and the fluorescence of the reaction can thus be monitored and used to infer phosphite concentrations in samples (Berkowitz *et al.*, 2011).

Stasikowski *et al.* (2014) described a sensitive, inexpensive, direct chemical method to estimate the concentration of phosphite in different plant species using a silver nitrate reagent. The assay is very sensitive with a detection limit of 0.5 - 1 mM (depending on the background staining) in a specific plant species and plant part (root or leaves). The silver nitrate stain

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method can detect phosphite in as little as 20 µl of aqueous extract from 100 mg of fresh plant material and was deemed reliable since 95% of the samples analyzed by this method gave similar results to those obtained by gas-liquid chromatography. This test is a quick and easy way to estimate the uptake and distribution phosphite in the plant, the extension of phosphite over time and the timing of phosphite application.

CONCLUSION

Phytophthora root rot caused by the *P. cinnamomi* has been the main limiting factor for avocado production in South Africa. Phosphonates-based fungicides have been used extensively worldwide, including in South Africa, to reduce the spread and impact of Phytophthora root rot (Duvenhage and Köhne, 1995; Whiley *et al.*, 2001; Duvenhage 2001; Thomas, 2008), but the complex mechanism by which phosphite act to control the host-pathogen interaction remains controversial (Coffey and Bower, 1984; Fenn and Coffey, 1984; Fenn and Coffey, 1985; Fenn and Coffey, 1989; Guest and Grant, 1991; McDonald *et al.*, 2001). Long term use of phosphonate-based fungicides has raised concerns regarding the development of resistance, although despite of its extensive use to control the disease worldwide, only limited evidence have been published on the existence of phosphite tolerant or resistant isolates and breakdown of disease control in practice (Cohen and Samoucha, 1984; Vegh *et al.*, 1985; reference within Guest and Grant, 1991; Duvenhage, 1999; Dobrowoski *et al.,* 2008). Widespread phosphonate resistance and a lack of field control has not yet become a reality.

In order to maintain and maximize the effect of phosphonate applications, an understanding of the mechanism of action is very important. Studies on the *in vitro* and *in vivo* phosphite sensitivity of *Phytophthora* isolates can provide valuable information towards knowledge on the mode of action, and the threat and implications of resistance development. The *in vitro* sensitivity of *P. cinnamomi* isolates to phosphite in South Africa has only been studied by Duvenhage (1994, 1999, and 2001) more than a decade ago and only in one orchard. Phosphate has been shown to affect the *in vitro* toxicity of phosphite in a few *Phytophthora* spp. (Fenn and Coffey 1989; Griffith *et al.*, 1989a; Darakis *et al.*, 1997), but this has not been investigated extensively in *P. cinnamomi* populations. Only one study has been published that investigated one *P. cinnamomi* isolate (Fenn and Coffey, 1984). The effect of different *in planta* (*in vivo*) phosphite concentrations in avocado towards *P. cinnamomi* has also not been investigated sufficiently. In Australia a commercial critical root phosphite concentration of 25 - 30 $\mu g/g_{FW}$ is used (personal communication, A.W. Whiley), but it is unclear as to how this value was derived. In South Africa, van der Merwe and Kotze (1994) identified a critical root phosphite concentration in avocado glasshouse seedlings of 9.5 $\mu g/g_{FW}$, where no improved control was achieved at higher phosphite concentrations. In South

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Africa, no commercial analytical laboratory currently supplies a service for measuring phosphite in plant roots, only fruit residues can be quantified. Avocado root samples can be especially problematic in analytical analyses due to the viscosity of samples, and the production of phenolics in roots sampled in orchards. Therefore, investigations are required into modern analytical methods such as LC-MS/MS, for accurately measuring phosphite in avocado roots. Fluorescent enzyme based assays have also been published for quantification of phosphite (Berkowitz *et al.,* 2011), but were reported to only be effective in herbaceous crops. These assays can be very cost effective if it can be optimized for precise quantification in woody host plants. Analytical methods that are precise in quantifying phosphite from avocado roots, will enable growers to monitor the efficacy of their phosphonates applications and also possibly understanding the mode of action.

The overall aim of study was to investigate the phopshite sensitivity of South African *P. cinnamomi* isolates in *in vitro* and *in vivo* studies. Since there are no laboratories that can quantify phosphite in plant roots in South Africa, the first aim was to identify an analytical method with high precision. Three different methods were investigated that included liquid chromatography-mass spectrometry (LC-MS/MS), ion chromatography (IC) and enzymatic fluorescent assay. The *in vitro* phosphite sensitivity of *P. cinnamomi* was investigated using a large population. The population was screened at different phosphite and phosphate concentrations to obtain a greater understanding of the interaction of phosphate and phosphite in inhibiting *P. cinnamomi.* Two isolates that were identified in the aforementioned studies as highly phosphite tolerant and sensitive were investigated for their *in vivo* response to phosphite using a root bioassay (van der Merwe and Kotze, 1994). The phosphite concentrations in roots were quantified using the analytical method that showed the greatest precision. The knowledge gained in this thesis will assist growers in knowing whether *in vitro* phosphite tolerant isolates are wide spread and if a critical root phosphite concentration exists. The analytical methods developed will also be very useful for the avocado industry.

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Table 1. Summary of studies that have evaluated the *in vitro* phosphite sensitivity of *Phytophthora*.

Reference	Phytophthora species	Number isolates evaluated	Basal medium	Phosphite concentrations tested	Phosphate concentration tested[a]	Range of EC50 values reported	Range of % inhibition reported
Griffith *et al.* 1993	*P. palmivora*	3	Ribeiro's minimal (RMM) liquid	0.08 µg/ml	0.01,0.03,0.1,0.3,1,3 mM	1.09-2.2µg/ml	
Coffey and Bower 1984	*P. cinnamomi*	12	RMM soild	5 µg/ml	0.84 mM	5.2-224.4 µg/ml	0-44.8%
	P. citricola	5	RMM soild	5 µg/ml	0.84 mM		48.3-67.6%
	P. citrophthora	7	RMM soild	10 µg/ml	0.84 mM		80.3-89.3%
	P. parasitica	5	RMM soild	10 µg/ml	0.84 mM		27.9-58.8%
	P. megasperma	12	Rye-seed agar	20 µg/ml			-1.5-62.5%
	P. palmivora	4	Corn meal agar (CMA)	10µg/ml	0.38 mM		53-81.4
	P. citrophthora	4	CMA	10 µg/ml	0.38 mM		40.1-56.7%
	P. capsici	3	CMA	10 µg/ml	0.38 mM		-0.85-30.9%
	P. infestans	10	Rye-seed agar	200 µg/ml			30.4-71.2%
Fenn and Coffey, 1984	*P. cinnamoni*	1	RMM soild	47 µg/ml	0.084 mM	4.2 µg/ml	
	P. capsici	2	RMM soild	47 µg/ml	0.084 mM	2.5 -5.4 µg/ml	
	P. cinnamomi	1	RMM liquid	47 µg/ml	0.084,0.84,8.4 mM		67-52%
	P. citricola	1	RMM liquid	47 µg/ml	0.084,0.84,8.4mM		82-84%

Reference	Phytophthora species	Number isolates evaluated	Basal medium	Phosphite concentrations tested	Phosphate concentration tested[a]	Range of EC50 values reported	Range of % inhibition reported
Fenn and Coffey, 1989	*P. capsici*	4	RMM soild	24-780 µg/ml	5,15,45 mM	77µg/ml	
	P.parasitica var.nicotianae	8	0.5% CMA			47-665 µg/ml	
Coffey and Joseph 1985	*P. cinnamomi*	3	RMM	0-5 µg/ml	0.84 mM	4.6 -6.2 µg/ml	
Bashan *et al*., 1990	*P. infestans*	23	Rye-seed agar	0-400 µg/ml		62l->1000 µg/ml	
Wilkinson *et al*., 2001a	*P. cinnamomi*	71	RMM soild	0-160 µg/ml	7.35 mM	4-148 µg/ml	4-100%
Duvenhage,1994	*P. cinnamomi*	5-10	CMA	100 µg/ml	0.38 mM	.	40-70%
Duvenhage,1999	*P. cinnamomi*	30	CMA	100 µg/ml	0.38 mM	9-98µg/ml	79-90%
Garbelotto *et al*., 2009	*P. ramorum*	12	10% V8 agar	1.32,53,2133 µg/ml		ED_{90}=2134 µg/ml	11-97.3%
Ouimette and Coffey, 1989a	*P. cactorum*	4	0.5% CMA		0.38 mM	20.3-24.3 µg/ml	
	P. capsici	3	CMA		0.38 mM	12.2-18.6 µg/ml	
	P. cinnamomi	4	CMA		0.38 mM	1.6-6.5 µg/ml	
	P. citricola	2	CMA		0.38 mM	1.6-2.4 µg/ml	
	P. citrophthora	4	CMA		0.38 mM	6.5-9.0 µg/ml	
	P. cryptogea	1	CMA		0.38 mM	27.5 µg/ml	
	P. megasperma	6	CMA		0.38 mM	7.3-26.7 µg/ml	

Reference	Phytophthora species	Number isolates evaluated	Basal medium	Phosphite concentrations tested	Phosphate concentration tested[a]	Range of EC50 values reported	Range of % inhibition reported
	P. palmivora	4	CMA		0.38 mM	4.0-76.1 µg/ml	
	P. parasitica	3	CMA		0.38 mM	7.3-44.6 µg/ml	
Dolen and Coffey, 1988	*P. palmivora*	5	0.5% CMA	0,30,50,100,200 µg/ml	0.38 mM	mycelium:36.6-130.3 µg/ml; zoospore:4.5-14.8 µg/ml	5-75%
Fenn and Coffey, 1985	*P. capsici*	1	V8 agar			80 µg/ml	
Bashan *et al.*, 1990	*P. infestans*	11	RMM soild agar	0,10,20,50,100,200,400 µg/ml	0.2 mM	4-281 µg/ml	

[a] The phosphate concentration for corn meal agar (CMA) media was not reported by the articles, but is based on the amount of phosphate present in CAM by (Guest and Grant, 1991)

Table 2. Summary of articles that have evaluated the concentration of phosphite in plants.

Reference	Crop and *Phytophthora* Species	Glasshouse or field trials	Phosphote application method and amount applied	Time of analysis after application	Phosphite quantified in plant tissue	Phosphite quantification method
Ouimette and Coffey, 1989b	Avocado *P. cinnamomi*	Glasshouse	Soil drench: 2.1g/l	1,2,4,6,8 week	Root: 213-1399 µg/g_{FW} Stems:221-1561 µg/g_{FW} Leaves: 47-221 µg/g_{FW}	Ion chromatography
			Foilar spray:2.1g/l;		Roots: 11-18 µg/g_{FW} Stems: 21-209 µg/gfw Leaves: 19-118 µg/g_{FW}	
Ouimette and Coffey,1988	Pepper	Glasshouse	Soil drench: 5.4 g/l	24hr	Roots: 342 µg/g_{FW} Stems:186 µg/g_{FW} Leaves: 44 µg/g_{FW}	Ion chromatography
Borza *et al.*, 2014	Potato; *P.infestans*	Glasshouse	Folar spray with 2.4, 4.8 g/l	Leaves: one week Tuber: 4 month	Leaves:167-1111 µg/$g_{FW;}$ Tuber: 77-378. µg/g_{FW}	Ion chromatography
Barrett *et al.*, 2003	*Banksia brownie;* *P. cinnamomi*	Glasshouse	Foilar spray:12,24, 96kg/ha	7 days	Shoots: 286-1223 µg/g_{DW}	Gas chromatography
Fairbanks *et al.*,2000	*Corymbia calophylla*	Glasshouse	Foliar spray :2.5,5,10g/l Misting:100,200,400g/l	7 days	Root tips:789-3561 µg/g_{DW}; Mature roots:487-1863 µg/$g_{DW;}$ Stems:447-1760 µg/g_{DW}; Mature leaves:412-2010 µg/$g_{DW;}$ Young leaves:463-1954 µg/$g_{DW;}$ Shoot tips:442-2844 µg/g_{DW}	Ion chromatography
			Soil drench: 10g/l		Root tips:41095 µg/$g_{DW;}$ Shoots tips:1611 µg/g_{DW}	
Wilkinson *et al.*,2001b	*Banksia grandis Willd; Banksia hookeriana; Dryandra sessilis; P. cinnamomi*		Foilar spray:5, 10 g/l	2 weeks -12 months	Stems: 2 weeks 5g/l:1284-2393 µg/g_{DW}; 10g/l: 1674-4083 µg/g_{DW} 12 months: 5g/l: 209-635 µg/g_{DW} 10g/l:398-1922 µg/g_{DW}	Ion chromatography
Pilbeam *et al.*, 2000	*Adenanthos barbiger; Daviesia decurrens; Xanthorrhoea preissii; P. cinnamomi*	Forest	Foliar spray:2,5,20 g/l	5 weeks	*A.barbiger*: leaves:4-80µg/g_{DW} *D.decurrens*: leaves:18-871µg/g_{DW} *X.preissii*: root:0.5-2.2 µg/g_{DW}	Gas chromatography

Reference	Crop and ***Phytophthora*** Species	Glasshouse or field trials	Phosphote application method and amount applied	Time of analysis after application	Phosphite quantified in plant tissue	Phosphite quantification method
Barrett *et al.*, 2004	*Adenanthos cuneatus; Astartea glomerulosa; Banksia coccinea; Dryandra tenuifolia; Eucalyptus recondite; Jacksonia spinosa; Lysinema ciliatum; Melaleuca thymoides; M. spathulata*	Gull rock site	Foliar spray: 36,72,144kg/ha	5 weeks	*J.spinosa* >1000 µg/g_{DW}; *A.cuneatus* 73-185 µg/g_{DW}; *M.thymoides* 124-402 µg/g_{DW}; *L.ciliatum* 481-1055 µg/g_{DW}; *B.coccinea* 672-590 µg/g_{DW}	Gas chromatography
		Kambalup site			*E.recondita* 146-566 µg/g_{DW}; *D.tenuifolia* 30-292 µg/g_{DW}; *M.spathulata* 44-264 µg/g_{DW}; *A.glomerulos*a 44-380 µg/g_{DW}	
Dalio *et al.*, 2014	Fagus sylvatica; *P. plurivora*	Glasshouse	Foliar spray : 0.5 g/l	4 days	Root: 370-510 µg/g_{DW}	Ion chromatography
Groves *et al.* 2015	Lupine; *P. cinnamomi*	Glasshouse	Foliar spray: 1 g/l	1- 10days	Root: 242.9-382.5 µg/g_{DW}	Gas chromatography
Whiley *et al.* 1995	Avocado	Orchard	Trunk injection: 200 g/l	5 month	Root: 20-30 µg/g_{FW}	Gas chromatography
Nartvarantant *et al.*, 2004	Avocado	Orchard	Foliar spray .5, 10 g/l	2 weeks	pollen during early anthesis : 260-383 g/g_{FW}; summer flush maturity 3-10 µg/g_{FW} floral bud break 20-28 µg/g_{FW}	Gas chromatography
Botha *et al.* 1988	Avocado; *P. cinnamomi*	Glasshouse	Trunk injection :100 g/l	3-21 days	Root: > 200 µg/g_{FW}	Gas chromatography

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CHAPTER 2

Evaluation of bioanalytical methods for the quantification of phosphite in avocado roots

ABSTRACT

Phosphonates are widely used fungicides that effectively control avocado root rot caused by *Phytophthora cinnamomi*. Quantification of phosphite, the breakdown product of phosphonates in plants, is useful for investigating the fungicide mode of action, and for evaluating the efficacy of phosphonate applications. The reliability of three bioanalytical methods for quantification of phosphite in avocado roots was assessed by considering inter-day incurred sample reanalyses (ISR) and recovery rates. The best analytical method was a liquid chromatography-mass spectrometry (LC-MS/MS) method that yielded good recovery rates (78 - 124%) with excellent precision based on coefficient of variation percentages (1.9 - 9.7 CV%). The ISR precision for LC-MS/MS was acceptable for samples with phosphite concentrations equal to or higher than 64 $\mu g/g_{DW}$ (0.4 - 11 CV%; 0.6 - 21% difference [%DF]). However, the ISR precision was unacceptable for samples with phosphite concentrations lower than or equal to 27 $\mu g/g_{DW}$ (~6.75 $\mu g/g_{FW}$) (22 - 29 %CV; 38 – 56 %DF). The ion chromatography (IC) method was less reliable than the LC-MS/MS method, and had low (20.7 - 50.3%) recovery rates with low precision (29.5 - 93.7 CV%), and unacceptably (22 - 124 CV%; 19 – 238 %DF) ISR precission. Furthermore, samples containing high sulfate concentrations was not quantifiable. The evaluated enzymatic fluorescent assay was highly imprecise for ISR (29 - 61 CV%, 33 - 122 %DF). Nonetheless, the root phosphite concentration values measured with this method tended to be comparable to those of LC-MS/MS quantifications for samples with concentrations higher than 27 $\mu g/g_{DW}$. The linearity of standard curves for all three methods was good (R^2 > 0.9986), and within the range of 0.01 to 20 µg/ml, 2 to 50 µg/ml and 1 to 20 µg/ml for the LC-MS/MS, IC and enzyme assay methods repectively.

INTRODUCTION

The quantification of phosphite, the breakdown product of phosphonates in plants, is important in agricultural crops (Coffey and Bower, 1984; Ouimette and Coffey 1989a). Phosphonates are widely used fungicides that effectively control Phytophthora diseases in many crops, including avocado root rot caused by *Phytophthora cinnamomi* (Guest and Grand, 1991; Engelbrecht and van den Berg, 2013). Phosphite quantification data can be used to provide clues as to whether the fungicide has a direct (fungistatic) or indirect (induction of host resistance) mode of action, since the mechanism of action is still controversial (Coffey and Bower, 1984; Fenn and Coffey, 1984; Fenn and Coffey, 1985; Grant *et al.*, 1990; Jackson *et al.,* 2000; Daniel and Guest, 2005). Knowledge on the distribution and concentration of phosphite in plants can also be useful for identifying the optimum time required for fungicide applications during the crop growth cycle, and dosages required for effective control (Bezuidenhout *et al.,* 1987; Whiley *et al.*, 2001; Giblin *et al.*, 2005; Thomas, 2008). More recently, phosphite quantification has also become relevant in the consumption of human food, with maximum residue levels being introduced for several crops in order to comply with good agricultural practices, and food safety (Hernández *et al.*, 2003).

A number of analytical methods have been employed in literature for phosphite quantification in different plant tissues including radiolabelling, gas chromatography, gas chromatography – mass spectrometry (GC-MS), ion chromatography (IC) and liquid chromatography-mass spectrometry (LC-MS/MS) (Fenn and Coffey,1985; Smillie *et al.,* 1988; Roos *et al.*, 1999). Ion chromatography has been used by several research groups for phosphite quantification in plants (Ouimete and Coffey, 1989a; Roos *et al.,* 1999; Jackson *et al.*, 2000; Wilkinson *et al.* 2001b; Whiley *et al.*, 2001; Barrett *et al.*, 2003; Nartvaranant *et al.*, 2004; Orbovic *et al.*, 2008; Thao *et al.*, 2008; Borza *et al.*, 2014; Dalio *et al.*, 2014), as well as GC-MS analyses (Bezuidenhout *et al.*, 1985; Botha *et al.*, 1988; Smillie *et al.*, 1988; Schutte *et al.*, 1988; Shearer and Crane, 2009; Mckay *et al.*, 1992; van der Merwe and Kotze, 1994; Barrett *et al.*, 2003; Shearer *et al.*, 2012; Torres Elguera, *et al.*, 2013). Only a few of the aforementioned

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articles have published methods, or generated phosphite quantification data specifically for avocado roots (Bezuidenhout *et al.*, 1985; Schutte *et al.*, 1988; Botha *et al.*, 1988; Ouimette and Coffey, 1989b; Whiley *et al.*, 2001; Nartvaranant *et al.*, 2004).

Berkowitz *et al.* (2001) published a cost effective enzymatic fluorescent assay for quantification of phosphite in *Arabidopsis thaliana.* The assay is based on the principle of oxidation of phosphite to phosphate by the enzyme phosphite dehydrogenase (PTDH) from *Pseudimonas stutzeri* with nicotinamide adenine dinucleotide (NAD^+) as a co-substrate. Resazurin is included in the reaction and is reduced to resorufin through a cycling reaction where electrons are transferred from NADH via phenazine methosulfate. The reaction product resorufin is highly fluorescent at a wavelength of 590 nm with an excitation wavelength of 535 nm, and the fluorescence of the reaction can thus be monitored and used to infer phosphite concentrations in samples (Berkowitz *et al.*, 2011). The assay is very sensitive and has a detection limit of 0.25 nmol (0.41 µg/ml). However, the published assay is only effective when used in herbaceous plants e.g. lupin, but not in woody plants e.g. avocado roots (personal communication, O. Berkowitz, School of Biological Sciences and Biotechnology, Murdoch University, Murdoch, Australia).

Liquid chromatography-mass spectrometry (LC-MS/MS) is a powerful analytical tool that combines the scope and utility of liquid chromatography with the sensitivity and specificity inherent to mass spectrometry (Black and Read, 1998; Hernández *et al.*, 2003). This method is becoming increasingly popular for testing the presence of highly polar pesticide residues in foods of plant origin, due to the robust selectivity and sensitivity that makes it a good option for residue analyses in foods of plant origin (Hogenboom *et al.*, 2000; Hernández *et al.*, 2001; Pozo *et al.*, 2003). LC-MS/MS has not been reported widely in literature as an analytical method for quantification of phosphite. Hernández *et al.* (2003) was able to determine fosetyl-aluminum (Al) residues in lettuce with excellent recovery rates and a sensitivity level lower than that reported for IC. Fosetyl-Al was the first phoshonate fungicide that was registered for

commercial use. One of the dissociation products of fosetyl-Al is phosphite, which is a strong acid that exists as a mixture of two tautomers, phosphonic acid and phosphorous acid, with phosphonic acid tending to dominate (Karasali *et al.,* 2014). Therefore, the terminology phosphonic acid is sometimes used interchangeable with phosphite.

In South Africa, commercial laboratories (Hearshaw and Kinnes Analytical laboratory (Pty) Ltd, Cape Town South Africa; Hortec, Cape Town, South Africa) only recently started testing edible foods for phosphonic acid residues using LC-MS/MS analyses, but their methods are not publically available and they only test fruit residues not root samples. In New Zealand, Hill Laboratories (Hamilton, New Zealand) uses LC-MS/MS analyses to test edible foods, as well as avocado roots for phosphonic acid residues. In Australia, SGS Australia (Toowoombam Queensland, Australia) is cited in popular literature as providing a service for quantifying phosphite from avocado roots (Thomas, 2008, Smith *et al.*, 2010), but their assay method is unknown.

The reliable quantification of phosphite in plant tissue is important. In literature, there are no clear guidelines as to what the acceptable statistics and parameters are for the validation and reliability of bioanalytical methods in plant tissue. However, clear guidelines have been set by the Food and Drug Administration (FDA) for standards on bioanalytical method validation in clinical samples that include LC-MS/MS analyses methods (Fluhler *et al.*, 2014). Initially, in most studies the reliability of assays was only assessed using analyte negative biological samples spiked with known amounts of analyte (QC) within the range of the calibration curve, after sample extraction and purification. The QC samples were used to determine the accuracy (how close values are to a known value) and precision (how close measured values are too each other) and linearity of standard curves in inter-day (assays conducted on different days) and intra-day assays (assays conducted on the same day, also known as within-run assays) (FDA 2001; Rower *et al.*, 2010, FDA 2103). However, in 2008, the AAPS workshop on Current topics in Good Laboratory Practices bioanalysis introduced the concept of incurred sample reanalysis (ISR) as compulsory for determining the

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reproducibility of bioanalytical methods in pharmacokinetic/toxicokinetic studies (Yadav and Shrivastav, 2011). ISR consist of repeat analysis of naturally occurring test samples containing the molecule of interest, and is used to determine the reproducibility of a method within the biological matrix of samples. This is important, since assay outcomes can be substantially influenced by biological matrices as compared to when the molecule of interest is analyzed in water or after sample extraction and purification (Yadav and Shrivastav, 2011; FDA, 2013; Fluhler *et al.*, 2014; Subramaniam *et al.*, 2015). This is especially important in complex samples such as plant roots that can contain substantial amounts of phenolics and polysaccharides.

In South Africa, avocado root rot is an economically important disease (Engelbrecht and van den Berg, 2013), yet no commercial laboratory can provide a service for quantification of phosphite in avocado roots. Although the South African Bureau of Standards provide a service on a haphazard basis when they have equipment available, the cost is too high for use in research projects. Therefore, the aim of this study was to evaluate the reliability of three bioanalytical methods (IC, LC-MS/MS and enzymatic fluorescent assay) for the quantification of phosphite in avocado roots. The reliability of the methods was determined by focusing on inter- and intra-day ISR analyses using avocado root samples from phosphonate field trial treatments that contained a range of phosphite concentrations. The recovery rates for the different analytical methods were also determined by spiking root samples with known phosphite quantities prior to extraction and purification steps.

MATERIALS AND METHODS

Root sample origin and sample processing

Avocado root samples from orchard trials were used to evaluate three analytical methods for quantification of phosphite. Feeder roots were collected from 2 - 3 year old avocado orchard trees that were treated with different potassium phosphonate concentrations, applied using trunk injections or foliar sprays. This provided root samples containing a range of phosphite concentrations.

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Avocado roots were washed and dried in an oven at 60˚C for 2 days. The dried roots were ground into a fine powder using a coffee grinder (Bosch, Midrand, and South Africa). Samples were sieved with a tea-strainer to remove large particle sizes that remained after grinding. The dried and sieved roots were used in all three analytical methods.

Standard curves

Phosphite standard curve solutions were prepared for all analytical methods in the same manner, except that the concentration range differed for each method. A 200 g/l phosphite stock was prepared by accurately weighing 20 g of phosphorous acid crystals (Sigma-Aldrich-Aldrich, Oakville, ON) and dissolving it in 80 ml deionized water. The pH was adjusted to 6.5 with 10M KOH and the solution made up to 100 ml. The phosphite stock solution was diluted to 1000 µg/ml by adding 50 µl of the stock solution to 9.95 ml of distilled water. This solution was used to make serial dilutions to the desired phosphite concentrations (10 ml each) required for each of the standard curves of the different analytical methods. The standard curve concentrations used for IC analyses were 2, 5, 10, 20, and 50 µg/ml, for LC-MS/MS 0.01, 1, 5, 10 and 20 µg/ml and for the fluorescent enzyme assay 1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5 and 20 µg/ml.

Phosphite extraction and sample analyses using ion chromatography (IC)

Sample extraction and clean-up

Phosphite was extracted from the grounded and sieved roots by combining 500 mg of roots with 5 ml distilled water in a 50 ml falcon tube. The tubes were slowly shaken on a rotary shaking incubator (3082U, Labcon, Midrand, South Africa) at 100 rpm overnight at ~25˚C room temperature, and then centrifuged at 12000 g in a centrifuge (Centrifuge 5810R, Eppendorf, Hamburg Germany) with a fixed rotary head for 10 min at 20˚C. Two millilitre of the supernatant was passed through a 0.22 µm PALL acrodisc ® syringe filter containing a Supor ® membrane (Pall Corporation, Midrand, South Africa), and 1 ml of this filtrate was then added to a Waters Sep-Pak ® C_{18} SPE

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cartridge, 3 cc, 500 mg (Waters Corporation, Milford, USA). Just prior to adding the sample to the C18 cartridge, the cartridge was first conditioned by passing 2 ml methanol, followed by 1ml water through the cartridge. Four hundred microliters of the C18 filtrated sample was added to a 5K Nanosep ® centrifugal device (Pall corporation), and centrifuged at 5000 g for 20 min. The filtrate from the collection tubes was used for phosphite analysis.

Sample analyses

All sample analyses were conducted by the Central Analytical Facility (CAF) at Stellenbosch University. The ion chromatography system consisted of a Waters 717plus Auto sampler (Waters Corporation) equipped with a Waters 432 Conductivity detector (Waters Corporation) and an Agilent 1100 series binary pump. A Waters IC-Pak A anion exchange column (Waters Corporation) was used with a borate-gluconate mobile phase. The borate-gluconate buffer was made by combining 20 ml of borate gluconate concentrate (16 g sodium gluconate, 18 g boric acid and 25 g sodium tetraborate decahydrate added to 500 ml of Milli-Q water and mixed thoroughly until dissolved, followed by the addition of 250 ml glycerin), 20 ml of n-butanol and 120 ml of acetonitrile, which was filtered through a 0.22 µM GHP membrane (Sigma-Aldrich, St Louis, USA) before use. Empower ® Chromatography software (Waters) was used to process the quantitative data obtained from the calibration standards and samples. The separation of phosphite was performed at a flow rate of 1.1 ml/min isocratically at room temperature. Phosphite concentrations were determined by loading 200 µl of each extract or standard solution into a vial and injecting a 100 µl aliquot into the column as described above.

Phosphite extraction and sample analyses using LC-MS/MS

Sample extraction and clean-up

Phosphite extraction from avocado roots was done as described in the IC section except that 500 mg roots were extracted in 10 ml of distilled water within a 15 ml falcon

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tube. Tubes were centrifuged at 4000 g in a swing bucket centrifuge (Eppendorf 5810R) for 10 min at 20˚C. The use of the swing bucket head allowed for a higher throughput of samples, since 36 tubes could be spun at once, as opposed to six tubes in the fixed rotary head. The supernatant (3 ml) was passed through a 0.22 µm PALL acrodisc ® syringe filter containing a Supor ® membrane (Pall Corporation). Subsequently, 400 µl of the filtrate was added to a 10K Nanosep ® centrifugal device (Pall Corporation) and centrifuged at 14000 g for 20 min. The filtrate from the collection tubes was used for phosphite analysis.

Sample analyses

All sample analyses were conducted by CAF at Stellenbosch University. The LC-MS/MS method was based on the European Commission Reference Laboratories for residues of pesticides Single Residue Methods (EURL-SRM): Quick method for the analysis of numerous highly polar pesticides in foods of plant origin via LC-MS/MS involving simultaneous extraction with methanol (QuPPe-Method). The method 1.3 “Glyphosate and Co. AS 11-HC” (http://www.crl-pesticides.eu/library/docs/srm/meth_QuPPe.pdf) within this document was used. The analyses were conducted on a Waters Acquity Ultra Performance liquid chromatography system (UPLC) (Waters Corporation) connected to a Waters Xevo TQ mass spectrometer with electrospray probe (Manchester, UK). The column used in LC separation was a Thermo Hypercarb (100 x 2.1 mm, 5 µM particle size) (Thermo Fisher Scientific, Waltham, USA) at a flow rate of 0.4 ml/min. The mobile phase was a gradient mixture of HPLC-grade water plus 1% acetic acid (Associated Chemical Enterprises, South Africa) (solvent A) and HPLC-grade methanol (Merck, Darmstadt, Germany) plus 1% acetic acid (solvent B) in which the percentages of solvent A and solvent B were changed linearly as follows: 0 min, 98% A and 2% B; 0.5 min, 98% A and 2% B; 5 min, 93% A and 7% B; 5.1 min, 10% A and 90% B; 5.2 min, 98% A and 2% B; 10 min, 98% A and 2% B. The column temperature was held at 40˚C. For operation of MS, the settings on the instrument were optimized for maximum ion

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sensitivity: capillary voltage was 3.50 kV, cone voltage 20 V, source temperature 140˚C, and desolvation temperature 400˚C. Desolvation gas flow was 800 L/Hr and cone gas flow 50 L/Hr. Nitrogen gas was supplied by a nitrogen generator and bottled argon was used as collision gas. Phosphite was detected using multiple reaction monitoring (MRM) mode with the 80.9 > 63 transition at a collision energy of 15 eV. Phosphite concentrations were determined by loading 200 µl of each root extract or standard solution into a vial and injecting a 2 µl aliquot into the column. Masslynx and Targetlynx software (Ver.4.1) was used to process the quantitative data obtained from the calibration standards and from root samples.

Enzyme production, phosphite extraction and sample analyses using an enzymatic fluorescent assay

Transformation of E. coli with plasmid containing a phophite dehydrogenase gene

A thermostable mutant PTDH plasmid (113 ng/µl) containing a recombinant His-tagged phosphite dehydrogenase gene was kindly provided by H. Zhao (University of Illinois, Urnana-Champaign, USA). The plasmid was transformed into *E. coli* BL21 (DE3) cells by first thawing 200 µl of *E. coli* competent cells, which were then added into a pre-chilled 15ml tube on ice containing 7 µl of plasmid. After an incubation period of 10 min, the cells were heat-shocked for 50 s in a water bath at 42˚C and immediately placed on ice for 2 min. Nine hundred microliters of cold Super Optimal broth with Catabolite Repression (SOC) medium was added to the tube and incubated for 60 min at 37˚C with shaking at 225 rpm. The transformation mix (50 µl) was plated onto a Luria-Bertani (LB) agar plate containing 0.1 g/ml ampicillin, and incubated overnight at 37˚C. The presence of the plasmid in the transformed cells growing on the ampicillin plate was confirmed by PCR screening using universal T3 and T7 primers.

Sequencing of the phosphite dehydrogenase plasmid gene

Since the identity of the mutated *phosphite dehydrogenase* gene in plasmid PTHD received from H. Zao was uncertain, the gene was sequenced to determine which

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mutated gene was present on the plasmid. The PTHD plasmid was purified from 10ml of the transformed *E. coli* cells grown overnight in LB broth containing 0.1 g/ml ampicillin. The plasmid was purified from the cells according to the manufacturer's instructions of the GeneJet plasmid miniprep kit (Thermo Fisher Scientific). The plasmid was sent to CAF at Stellenbosch University for sequencing using universal primers T3 and T7. A consensus sequence was generated by aligning the T3 and T7 sequences using Geneious Pro v. 3.6.2 (Bio-matters Ltd., Auckland, New Zealand). BLAST analyses were conducted in GenBank at the nucleotide and protein level.

Expression and purification of phosphite dehydrogenase enzyme

Expression and purification of the phosphite dehydrogenase enzyme from the transformed *E. coli* cells was carried out as described by Koekemoer (2006). Briefly, LB media (500 ml) supplemented with 30 μg/ml kanamycin was inoculated with the *E. coli* BL21 (DE3) starter culture transformed with the PTHD plasmid. The culture was grown until the log phase was reached (OD_{600} = 0.6), and expression was induced using a final concentration of 1.0 mM isopropyl-β-D-thiogalactoside (IPTG). The culture was grown overnight by shaking at 250 rpm at 37˚C. Cells were harvested by centrifugation at 5000 g at 4˚C for 15 min, then re-suspended in a volume of 10 x the pellet weight of binding buffer (20 mM Tris-HCl, 500 mM NaCl and 5 mM Imidazole) and cooled to < 10˚C. Cells were disrupted by sonication and the cell debris collected by centrifugation at 25000 rpm for 20 min at 10˚C. The supernatant was filtered through CAMEO 25 AS acetate filters with a pore size of 0.45 micron before injection into the ÄKTA*prime*-system (GE, Healthcare Life Science, Little Chalfont, UK). The PTHD His-tagged protein was loaded onto a 1.0 ml Amersham Biosciences HiTrap Chelating HP column preloaded with Ni^{2+}. After a wash step (15% elution buffer, 85% binding buffer) to remove any non-specifically bound proteins from the column, the target protein was eluted by stepwise increasing the concentration of imidazole (the functional group of histidine) in the buffer. The elution was monitored by UV absorption at 280 nm. The protein concentration of the fraction containing the protein of interest was determined

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using Bradford reagent (Sigma-Aldrich) according to manufacturer's instructions, which in general yielded a protein concentration of 2.3 mg/ml. The purity of the protein was analyzed by SDS-PAGE.

Sample extraction and purification

Avocado roots were dried, ground and sieved as described in the IC section except that phosphite was extracted from 500 mg of avocado roots (dry weight) in 10 ml of 1% acetic acid and shaken overnight. The slurry was centrifuged at 12000 g in a centrifuge (Eppendorf 5810R) with a fixed centrifuge head for 10 min at 20°C. The supernatant was passed through a 0.22 µm PALL acrodisc ® syringe filter containing a Supor ® membrane (Pall Corporation), 400 µl of the filtrate was added to a 10K Nanosep ® centrifugal device (Pall Corporation) and centrifuged at 14000 g for 20 min. The filtrate from the collecting tubes was used for phosphite analysis.

Sample analyses

Phosphite was quantified as described by Berkowitz *et al.* (2011), where three reactions were set up per sample in Sterilin ® 96- flat well black microtiter plates (Thermo Scientific, Newport, UK). These reactions consisted of (i) root extract sample only (25 µl root extract + 25 µl MQ water), (ii) internal standard control (25 µl root extract + 25 µl 30 µg phoshite standard/ml that resulted in a final phosphite concentration of 15 µg/ml) and (iii) a blank (25 µl root extract + 25 µl MQ water, without enzyme [see below]). The reaction was started by adding 200 µl of assay mix to provide a final concentration of 50 mM MOPS (pH 7.3), 100 µM NAD^+, 100 µM phenazine methosulfate, 100 µM resazurin, and 1 µg recombinant His-tagged phosphite dehydrogenase enzyme per well. All wells containing the standard curve solutions (50 µl) in triplicate also received the aforementioned reaction mixture. For the blank, the same reaction assay mix was added except that the enzyme was omitted to allow for correction of auto-fluorescence in the root extract and non-specific resazurin reduction. The microtiter plate was loaded into a FLUOstar OPTIMA (BMG LABTECH,

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Ortenberg, Germany) machine and incubated at 37˚C for 1 h in the dark, and product formation was directly monitored in real time. The fluorescence of the end product resorufin was quantified with a fluorescence reader at 535 nm excitation and 595 nm emission wavelengths.

The phosphite concentration in root samples were calculated by subtracting the fluorescence of the blank from the fluorescence of the sample only. For calculation of the recovery rate of the added phosphite standard the following formula was used: ((fluorescence of the internal standard control - fluorescence of the sample only) ÷ 15) x 100. The adjusted phosphite concentration for each sample was calculated by: Phosphite concentration of the root extract as derived from the standard curve / recovery rate for the added standard. The final root concentration was calculated by correcting for root sample dilution during root extraction, by multiplying the adjusted phosphite concentration by 20.

Validation and reliability of analytical methods

Validation and reliability of the analytical methods was focused on incurred sample reanalysis (ISR) on an inter-day (different days) and/or intra-day (within-run) basis. Depending on the analytical method, ISR was determined for four to six avocado field root samples (Table 1, 3 and 4). The samples were selected to represent low, medium and high phosphite root concentrations, which were identified in pilot analytical analyses. Intra-day analyses were only conducted for LC-MS/MS and fluorescent enzyme assays, with samples being analyzed in duplicate. Inter-day analyses were conducted on three different days for both IC and LC-MS/MS except for one sample in the LC-MS/MS analyses that was only conducted on two different days. For intra-day data, precision was assessed by calculating the coefficient of variation percentage (CV% = standard deviation/mean x 100) (Lalitkumar and Gemzell-Danielsson, 2013). The precision of the inter-day data was determined by calculating the CV% and percentage difference (%DF = (repeat – original) / (mean repeat and original) x 100) (Yadav and Shrivastav, 2011). Precision was deemed acceptable if the CV% was

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below 15% and the %DF below 20% (FDA, 2013; Yadav and Shrivastav, 2011; Subramaniam *et al.*, 2015).

Recovery rates were calculated for IC and LC-MS/MS analyses. IC root samples were spiked to yield final root phosphite concentrations of 20, 30, 50, 100 µg/g, and for LC-MS/MS analyses concentrations of 5, 10, 20, 40 µg/g were used. Spiking experiments were conducted three times. Root samples were spiked by adding the required phosphite stock solution to centrifuge tubes, each containing 500 mg of dried roots and 10ml of distilled water. For example, to determine the recovery rate of a 20 µg/g root phosphite concentration, the tube was spiked to a final concentration of 2 µg/ml or 1 µg/ml for IC and LC-MS/MS samples respectively, which took the different dilution factors into consideration for these methods. The samples were processed further as described previously for each sample.

RESULTS

Sample analyses using ion chromatography

A linear standard curve was obtained within the concentration range of 2 to 50 µg/ml (Fig. 1A). The linearity of the standard curve was excellent (R^2 = 0.9995), and the equation of the curve was $y = 1.0434X - 0.5375$. The IC chromatogram of phosphite in avocado roots showed the absence of spectrophotometric interference of the root matrix (Fig 2A, B). The only exception was for samples that contained relative high sulfate concentrations, since the chromatographs contained a very large sulfate peak overlapping the phosphite peak (Fig. 2C). For these samples, phosphite could not be quantified. The retention time of phosphite in the sample was around 9.2 min (Fig. 2).

Sample analyses using LC-MS/MS

Regression analysis of the analyte peak area response versus phosphite concentration exhibited an exellent linear relationship (R^2 = 0.9993) within the concentration range of 0.01 to 20 µg/ml. The regression equation for the standard curve was $y = 1.004X - 0.1031$ (Fig. 1B). Representative LC-MS/MS chromatograms

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for standard curve samples and root sample are shown in Fig. 3. The average retention time of phosphite for avocado root samples was 2.15 min.

Enzyme production and sample analyses using an enzymatic fluorescent assay

Sequencing of the phosphite dehydrogenase plasmid gene

Blast analyses at the nucleotide and protein level showed that the gene within the plasmid received from H. Zhao contained mutations in 16 amino acids of the wild type *Pseudomonas stutzeri* phosphite dehydrogenase gene (Gi 3127074; Metcalf and Wolfe, 1998). The protein sequence had the highest similarity to GenBank accession Gi 388604286 (pdb 4E5P), which contains 17 mutated amino acids (Zou *et al.*, 2012) in the wild type gene. The only difference between the two proteins sequences was that our plasmid gene contained a Glu-130 → Lys mutation instead of the Glu-130 → Gln mutation in 4E5P, and it further did not contain the Ala-176 → Arg mutation in 4E5P.

Sample analyses

Evaluation of the fluorescence signal in real time for a range of phosphite concentrations from 1 to 20 µg/ml over a 2 hour period, showed an initial linear increase in fluorescence until approximately 30 min. for most concentrations, except for 1 and 2.5 µg/ml. Thereafter, for all samples except the two aforementioned, a gradual plateauing of the formation of the reaction product resorufin and consequently fluorescence occurred. At the maximum assay concentration of 20 µg/ml phosphite, the formation of resorufin reached saturation within 45 min (Fig. 4).

The standard curve was linear over the phosphite concentration range of 1 to 20 µg/ml (Fig. 1C). The regression equation for the curve was $y = 152.23X - 22.287$, and the correlation coefficient indicated good linearity ($R^2 = 0.9986$).

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Validation and reliability of analytical methods

Ion chromatography

The precision for inter-day ISR was measured as the CV% and percentage difference (DF%), which should be below 15% and 20% respectively (FDA, 2013). None of the six root samples had CV% and %DF values that were within the acceptable ranges except one %DF value for B6.5D that was within the acceptable range. The two samples (B5.2D and B6.5D) that contained the highest mean phosphite concentrations (67 and 121 µg/g_{DW}) tended to have lower CV% and %DF values, but most (87.5%) of these values were still not within the acceptable range (Table 1).

The average percentage recovery rates for the spiked avocado root samples at all four concentrations (20, 30, 50 and 100 µg/g) were low (20.7 to 50.3%). It was furthermore concerning that the precision, as measured by the CV%, for all the concentrations were poor and unacceptable, ranging from 29.5 to 93.7% (Table 2).

LC-MS/MS analyses

For the intra-day ISR precision analyses, only one of the samples (B5.6F) that were used as a medium range phosphite concentration, and one high concentration sample (B6.4E) each contained one unacceptable CV% (23) and/or %DF (22 and 33) value (FDA, 2013), which were slightly above the acceptabel 15 CV% and 20 %DF limits. The remaining samples representing high and low phosphite concentrations were all within the acceptable range (Table 3).

The inter-day analyses identified three samples (B4.6E, M4.4B and B5.6F) with with unacceptable precisions (22 - 29 CV%; 38 – 56 %DF) (Table 3). These were all within the low (B4.6E and M4.4B) and medium (B5.6F) range of phosphite concentrations analyzed, containing phosphite concentrations of 11 to 27 µg/g_{DW}. The three samples (B5.4E, B6.4E and B5.1E) that contained phosphite concentrations equal or higher than 64 µg/$g_{Dry\ Weight}$ (µg/g_{DW}) had unacceptable precission levels (0.4 and 11 CV%; 0.6 – 21 %DF). Although sample B6.4E resulted in one %DF value that was just above the limit (21%) that was above the acceptable 20% level, we considered

the precission of this sample acceptable since the other two %DF values for the sample were well below (10 and 11 %DF) the acceptable limit (Table 3).

The recovery rates of the spiked avocado root samples at all four phosphite concentrations (5, 10, 20 and 40 µg/g) were high (78 to 124%). The precision of the recovery rates for all four concentrations was very good (≤ 9.7 CV%) (Table 2).

Enzymatic fluorescent assay

The ISR precission (CV% and %DF) of the fluorescent enzyme phosphite assay quantifications from avocado root samples, was sample specific for the intra- and inter-day precision. Sample B5.6F, containing a medium phoshite concentration (36 $\mu g/g_{DW}$), was the only sample that contained precession statistics that were relatively low (≤ 17%) and acceptable (Table 4). The other roots samples, whether representing low (M4.4B) or high (B4.6E and B5.1E) phosphite concentration samples were imprecise, with most (77%) of the CV% (29 - 61) and %DF (33 - 122) values not being below the acceptable range of 15% and 20% respectively (Table 4).

The adjusted phosphite concentrations in avocado root samples were calculated as described by Berkowitz *et al.* (2011). For these calculations the value of a 15 µg/ml phosphite spiked sample is used to determine the recovery values (Table 5), which also gives an indication of inhibition. The recovery values showed that the samples differed in recovery values from 0.29 to 0.65 (i.e. 29 to 65%).

The quantification of phosphite using the fluorescent enzyme assay and LC-MS/MS analyses was compared in five avocado root samples. The phosphite concentrations in root samples determined by the fluorescent enzyme assay were generally higher than those of LC-MS/MS quantifications (Fig.5). For samples that represented low phosphite containing samples (B4.6E, M4.4B, and B1.4C), the enzyme assay overestimated (~30%) the phosphite concentration when compared to LC-MS/MS quantifications. For samples that represented medium (B5.6F; 36 $\mu g/g_{DW}$) and high (B5.1E; 104 $\mu g/g_{DW}$) phosphite containing root samples, the differences in

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phosphite quantification for the two methods were more comparable and within a range of 15% (Fig.5).

DISCUSSION

Although IC analysis is one of the most published method for the analysis of phosphite in plant tissues (Ouimette and Coffey, 1988; Ouimette and Coffey, 1989b; Fairbanks *et al.*, 2000; Wilkinson *et al.*, 2001b; Borza *et al.*, 2014), in our study the method was not deemed reliable for quantification of phosphite from avocado roots. The first problem encountered, was with a lack of robustness of the Waters IC-Pak A anion exchange column (Waters Corporation) that was used. The column was very sensitive to impurities in root sample extracts, which resulted in the breakdown of the expensive column. Therefore, root extracts had to be purified through a C_{18} cartridge and a 5K Nanosep centrifugal device, otherwise the column broke down and had to be replaced. For the IC analyses, care had to be taken to not run samples with a brown colour or viscous appearance. These samples had to be put through a second round of the clean-up process, which created problems with different recovery rates for these samples. The clean-up method that had to be used for the IC method not only increased the cost and labour associated with the method, but likely also contributed towards the low recovery rates (20.7 - 50.3%) that had unacceptable precision levels (29.5 - 93.7 CV%).The precision of inter-day ISR was also unacceptable (22 - 124 CV%; 19 – 238 %DF). Another significant problem associated with IC analyses was that some root samples contained high sulfate concentrations, due to the application of various sulfate based fertilizer by growers in avocado orchards, which yielded a huge sulfate peak that overlapped and interfered with the phosphite peak chromatograms, preventing phospite quantification.

Different IC columns have been used in published literature for quantification of phosphite in plant material through IC analyses. Since all IC columns are not compatible with all IC analysing systems, researchers are often limited to the specific column that can be used. For example, the Waters IC-Pak A anion exchange column

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(Waters Corporation) that was used in the current study, was the only column that was suitable for phosphite analyses on the IC Waters Conductivity detector and Auto sampler (Waters Corporation) system available in our laboratory. The columns from literature were not compatible with this system. A few publications have used the Vydac 302IC4.6 silica-based non-suppressed ion chromatography column (The Nest Group, Inc., Southborough, MA) and method published by Roos *et al.* (1999) to quantify phosphite from various plant hosts including *Corymbia calophylla* (Fairbanks *et al.*, 2000), *Banksia grandis Willd, Banksia hookeriana, Dryandra sessilis* (Wilkinson *et al.*, 2001b) and *Fagus sylvatica* (Dalio *et al.*, 2014). The Vydac column was reported to be robust for analyses of plant samples (Roos *et al.,* 1999), but it is unfortunately no longer manufactured. Studies using the method of Roos *et al.* (1991) only used a simple 0.45 µm nylon Acrodisc® (Gelmann Sciences) to clean their plant extracts, proving the robustness of the column to plant extract samples. A few studies have also used a Dionex Ionpac AS4A separator column of the IC method first published by Ouimette and Coffey (1988). This method has been used for phosphite quantification in pepper (Ouimete and Coffey, 1988), avocado (Ouimete and Coffey, 1989b), citrus (Orbovic *et al.*, 2008), tomato and tobacco tissue (Fenn and Coffey, 1989). Most of these studies, with the exception of Fenn and Coffey (1989), used one Sep-Pak C_{18} cartridge followed by filtration through a GS-type filter pore size 0.22 µm to clean up their plant extract samples. Fenn and Coffey, (1989) used two Sep-Pak C_{18} cartridges for each sample, suggesting that the Dionex sample was less robust for analysing plant extracts. Recently, Borza *et al.* (2014) used a Metrosep A Supp 7 - 250 column (MetrohomUSa, Riverview, FL) for their phosphite ion chromatography analyses of potato tissue, where sample extracts were cleaned using a 3K Amicon Ultra-4 centrifugal device followed by filtration through a 0.2 µm polyethersulfone filter. This sample clean-up approach did not work for avocado root extracts since the 3K device got clogged and the samples were still brown and viscous, which resulted in breakdown of our IC column. Considering the relative simple sample clean-up method used by Borza *et al.* (2014) it is likely that the Metrosep column is more robust for

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analyzing plant samples, or that the potato tissues (leaves and tuber) are less problematic for analyses by IC than our perennial tree roots.

The LC-MS/MS method was the best analytical method for quantifying phosphite from avocado roots, and had several advantages over the other evaluated methods. The first advantage of the method was that it was not sensitive to the presence of sulfate in samples, since high sulfate containing samples that could not be analysed by IC, was successful analysed using LC-MS/MS. The LC-MS/MS method further yielded a very good linear standard curve (R^2 = 0.9993) within the concentration range (0.01 to 20 µg/ml) encountered in orchards trials (unpublished data) The root extract sample clean-up method for the LC-MS/MS analyses was simple and rapid, only consisting of a 0.2 µm syringe filtering and a 10K Nanosep centrifugal device, which yielded high recovery rates (78 to 124%) with good precision (CV% < 10) at all four concentration ranges evaluated (5, 10, 20 and 40 µg/g). The LC-MS/MS showed good precision for intra-day ISR analyses (0.4 – 10 %CV; 0.5 – 14 %DF), with only two samples having slightly higher than acceptable CV% (23) and %DF (22 and 33) values. The inter-day analyses for samples with a mean concentration lower or equal to 27 µg/g_{DW}, had unacceptable precisions (22 - 29 %CV; 38 – 56 %DF). Samples containing concentrations equal or higher than 64 µg/g_{DW} had acceptable precision levels (0.4 – 11 %CV; 0.6 – 21 %DF). Although 21% is slightly above the 20% FDA (2012) level, we considered this value acceptable since the other two DF% values for the sample were well below (10 and 11 %DF) the acceptable limit. If 27 µg/g_{DW} is converted to a fresh weight concentration it is equivalent to 6.75 µg/g_{FW} (assuming a moisture content of 75% used by Hills laboratory; personal communication Jill Rumney, Hills laboratory, Hamilton, New Zealand). The value of 6.75 µg/g_{FW} is below phosphite concentrations that have been hypothesized by van der Merwe and Kotze, (1994) (9.5 - 53.2 µg/g_{FW}), Chapter 3 (9.82 - 19.3 µg/g_{FW}) and the Australian commercial avocado industry (25 - 30 µg/g_{FW}; personal communication, A.W. Whiley) for being biologically relevant for the suppression of *P. cinnamomi* in avocado roots. Thus, the imprecision for the sampes lower than or equal to 27 µg/g_{DW} was not a concern. In future studies, more

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samples at the low phosphite concentration range should be analysed to ensure that the intra-day ISR of the LC-MS/MS method is acceptable.

ISR analyses for all analytical methods is especially important for avocado root samples that can often be viscous and brown, thus having a high possibility of matrix interference. ISR also takes into account extraction efficiency and consistency from samples, which are not reflected by recovery rate analyses.

Although the enzymatic fluorescent assay can provide a cost effective alternative to LC-MS/MS analyses, evaluation of the method revealed several unacceptable characteristics. The ISR based inter- and intra-day analyses showed that the method was not precise, since except for sample B5.6F that had relative low and acceptable CV% and %DF (≤ 17%) values, all the other samples had unacceptable CV% (> 19%) and %DF (> 27%). Since plant extracts can contain some compounds that quench fluoresence at the excitation and emission wavelenghts of the assay, and/or contain inhibitors of the phosphite dehydrogenase enzyme used in the assay, it is important to calculate the adjusted phosphite concentration for each sample by spiking each root sample with 15 µg/ml phosphite as an internal standard for each root sample as reported by Berkowitz *et al.* (2011). The calculation of the adjusted phosphite concentrations includes a calculation for recovery rate. In the current study, the root extracts had a wide range of recovery rates from 29 to 65% emphasizing the importance of including the internal control phosphite spike in all samples, which must then be used for final phosphite concentration calculations. The wide and low recovery rate range observed in the current study could also be indicative of the fact that the sample clean-up method consisting of only a 0.2 µm syringe filter and 10K centrifugal device is not sufficient for avocado root samples. Berkowitz *et al.* (2011) reported recovery rates of 52.7 to 89.5% when analyzing *Arabidopsis thaliana* fresh tissue extracts and 19.2 to 24.6% for samples extracted after being stored at -20˚C for a long period. Since our dried root samples were stored for extened periods at 4C, this could have also contributed to our low recovery rates. Future studies should thus investigate the potential of quantifying fresh root extracts with the enzyme assay. The phosphite

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quantification concentrations with the enzymatic fluorescent assay generally had a higher value than that determined by LC-MS/MS for the samples that had low phosphite concentrations. At the phosphite concentrations higher than 36 - 104 µg/g_{DW}, the results are comparable to the results from LC-MS/MS and within a range of 15%. When converting these dry weight phosphite root concentration to fresh weight, it would be 9 - 26 µg/g_{FW}, which are of specific interest because they are within the range of biological relevant concentrations for root rot management as previously mentioned. However, since only two samples had root phosphite concentrations above 9 µg/g_{FW}, more samples must be analyzed to determine the reliability of the method for samples with lower concentrations.

This study showed that a LC-MS/MS method was the most reliable and robust method for quantifying phosphite in avocado orchard roots, especially for roots containing phosphite concentrations higher than 6.75 µg/g_{FW}. The precision of the LC-MS/MS method at low phosphite concentrations might be improved by adjusting the volume of water used in extractions from 10 ml to 5 ml. Compared to the IC and enzymatic fluorescent assay methods, the LC-MS/MS analyses exhibited much higher precision and is suitable for high throughput analyses since minimal sample preparation is required and some steps can be automated. It remains to be determined if the enzymatic fluorescent assay has potential to be used as an alternative for measuring phosphite in avocado root samples, using fresh root extracts and also lowering the extraction volume from 10ml to 5ml during sample preparation. For the method to be acceptable, however, these modifications should not only result in root concentration values that are comparable to LC-MS/MS quantifications, but it should also have acceptable inter- and intra-day ISR precision. Further investigation of the enzymatic assay is of interest, since it is a very cost effective method for analyzing root samples, and requires very basic equipment (fluorescence meter) available in most laboratories. In conclusion, a LC-MS/MS method for quantification of phosphite in avocado roots was developed, which is currently the only method available for this purpose in South Africa. The method has already been useful for investigating the

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mode of action of phosphonates (Chapter 3), and can also be used in future for optimizing root rot management strategies with phosphonates applications in South Africa.

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Table 1. Inter-day incurred sample reanalysis of phosphite concentrations in avocado roots by ion chromatography (IC).

	Root sample name[a]					
Statistical variable	**B2.1D**	**B6.1C**	**B6.3B**	**B6.2E**	**B5.2D**	**B6.5D**
Mean (µg/g_{DW})	5	15	18	17	67	121
CV (%)[b]	114	124	99	122	53	22
% Difference[c]	149, 75, 224	176, 62, 238	98, 101, 199	172, 64, 236	78, 22, 101	43, 19,25

[a] Root sample values that are underlined were not within the acceptable range for precision based on CV% and %DF. Each sample was evaluated on 3 different days.

[b] Percentage coefficient of variation = (standard deviation/mean) x 100. Values above 15% were considered as unacceptable with regards to precision (FDA, 2013).

[c] Percentage difference = (repeat – original) / (mean repeat and original) x 100. % Difference was calculated in all possible combinations for the 3 days, i.e. day 1 vs day 2, day 2 vs day 3 and day 1 vs days 3. Values above 20% were considered as unacceptable with regards to precision.

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Table 2. Recovery rates of phosphite spiked avocado root samples quantified through ion chromatography (IC) and liquid chromatography-mass spectrometry (LC-MS/MS) analyses.

IC			LC-MS/MS		
Amount added (µg/g)	**% Recovery ± STD[a]**	**CV (%)[b]**	**Amount added (µg/g)**	**% Recovery ± STD[a]**	**CV (%)[b]**
20	34.1±29.5	86.6	5	124±8	6.5
30	33.4±9.86	29.5	10	106±2	1.9
50	20.7±19.4	93.7	20	78±7.5	9.7
100	50.3±39.6	78.8	40	95.5±6.3	6.6

[a] Values are the mean recovery rate for three independent experiments.

[b] Percentage coefficient of variation = (standard deviation/mean) x 100 (FDA, 2013).

Table 3. Intra-day and inter-day precision of incurred sample reanaylsis (ISR) of phosphite in avocado roots by liquid chromatography-mass spectrometry (LC-MS/MS) analyses**.**

Statistical variable	Root sample name[a]					
	B4.6E	**M4.4B**	**B5.6F**	**B5.4E**	**B6.4E**	**B5.1E**
Mean ($\mu g/g_{DW}$)	13	11	27	64	148	96
Intra-day (within day)[b]						
CV (%)[c]	0.4, 6. 8	2, 3, 10	4, 6, 23	0.8, 9	3, 5, 5	1, 2, 2
% Difference (DF)[d]	0.5, 9, 12	2, 4, 14	9, 6, 33	1, 13	4, 7, 22	2, 2,3
Inter-day (between days)						
CV (%)[c]	29	29	22	0.4	11	7
% Difference[e]	17, 39, 56	7, 46, 53	1, 38, 38	0.6	10, 11, 21	2, 11, 13

[a] Root sample values that are underlined were not within the acceptable range for precision based on CV% or %DF. Each sample was evaluated on 3 different days, except for sample B5.4E that was only evaluated on 2 days.

[b] For intra-day analyses, each sample was analysed in duplicate on 3 different days, therefore columns contain three values for each of the duplicate samples that were analyzed within a day.

[c] Percentage coefficient of variation = (standard deviation/mean) x 100. Values above 15% were viewed as unacceptable with regards to precision (FDA, 2013).

[d] Percentage difference = (repeat – original) / (mean repeat and original) x 100. Values above 20% were viewed as unacceptable with regards to precision (FDA, 2013).

[e] Percentage difference calculated as for the intra-day assays. Each column contains three values, since each samples was analysed on 3 different days. %DF was calculates in all possible combinations for the three days, i.e. day 1 vs day 2, day 2 vs day 3 and day 1 vs day 3.

Table 4. Intra-day and inter-day precision of incurred sample reanalysis of phosphite concentrations in avocado roots as measured by an enzymatic fluorescent assay.

Statistical variable	**Root sample name[a]**			
	B4.6E	**M4.4B**	**B5.6F**	**B5.1E**
Mean ($\mu g/g_{DW}$)	54	48	36	100
Intra-day (within day)[b]				
CV (%)[c]	91, 19,13	42, 26, 11	4, 11	53, 20, 5
% Difference (DF)[d]	129, 27, 18	59, 37, 15	6, 15	75, 28,7
Inter-day (between days)				
CV (%)[c]	29	61	12	37
% Difference[e]	45, 53, 7	122, 50, 72	17	41, 33, 74

[a] Root sample values that are underlined were not within the acceptable range for precision based on CV% or %DF. Each sample was evaluated on 3 different days, except for sample B5.6F that was only evaluated on 2 days.
[b] For intra-day analyses, each sample was analysed in duplicate on 3 different days, therefore columns contain three values for each of the duplicate samples that were analyzed within a day.
[c] Percentage coefficient of variation = (standard deviation/mean) x 100. Values above 15% were considered as unacceptable with regards to precision (FDA, 2013).
[d] Percentage difference = (repeat – original) / (mean repeat and original) x 100. Values above 20% were considered as unacceptable with regards to precision (FDA, 2013).
[e] Percentage difference calculated as for the intra-day assays. Each column contains three values, since each samples was analysed on 3 different days. %DF was calculated in all possible combinations for the three days, i.e. day 1 vs day 2, day 2 vs day 3 and day 1 vs day 3.

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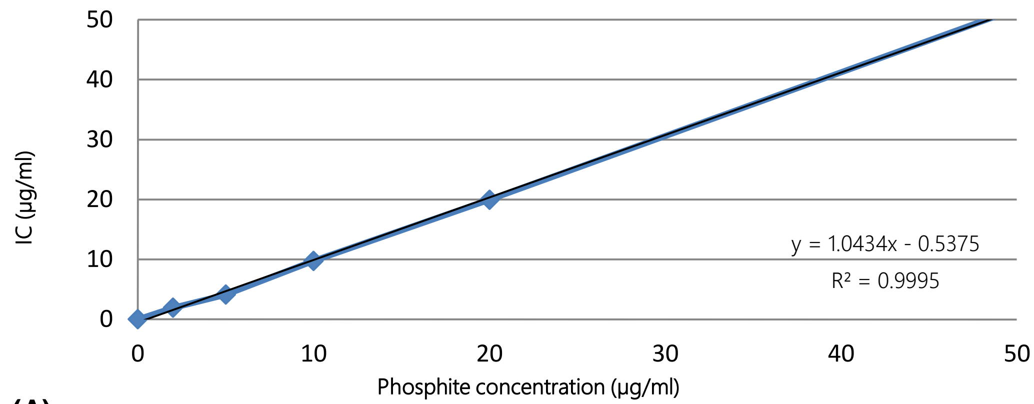
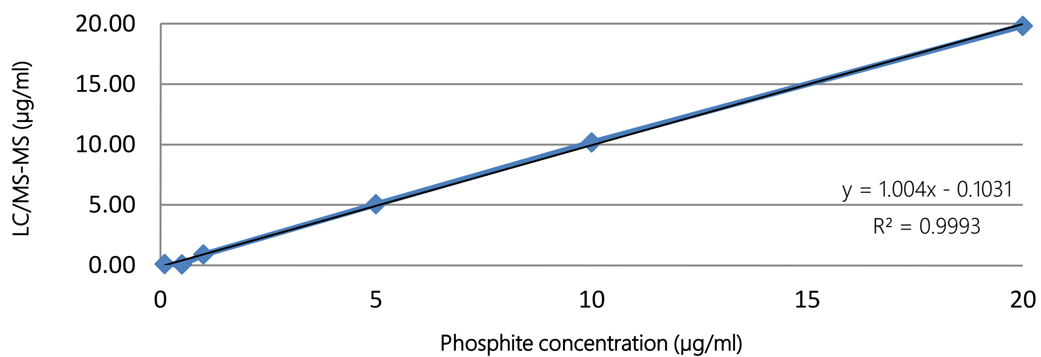
Table 5. Phosphite concentrations in spiked and unspiked avocado root samples quantified using an enzymatic fluorescent assay, which were used to calculate the adjusted and final root phosphite concentrations.

	Phosphite concentrations (µg/g_{DW}) [a]				
Sample name	**Unspiked sample**	**15 µg/ml internal spike[b]**	**Recovered internal spike[c]**	**Adjusted phosphite[d]**	**Final root phosphite[e]**
B4.6E	1.22 ±0.3	7.01±0.2	0.46±0.014	2.7±1.8	54±15.9
M4.4B	0.7±0.7	4.4±3.6	0.29±0.24.	2.4±2.0	48±29.9
B5.6F	1.15±0.006	9.8±1.4	0.65±0.09	1.7±0.3	36±4.2
B5.1E	1.5±0.5	4.5±0.7	0.30±0.05	5.0±2.6	100±38.6
B1.4C	0.7±0.1	7.7±0.7	0.50±0.07	1.4±0.5	28±9.1

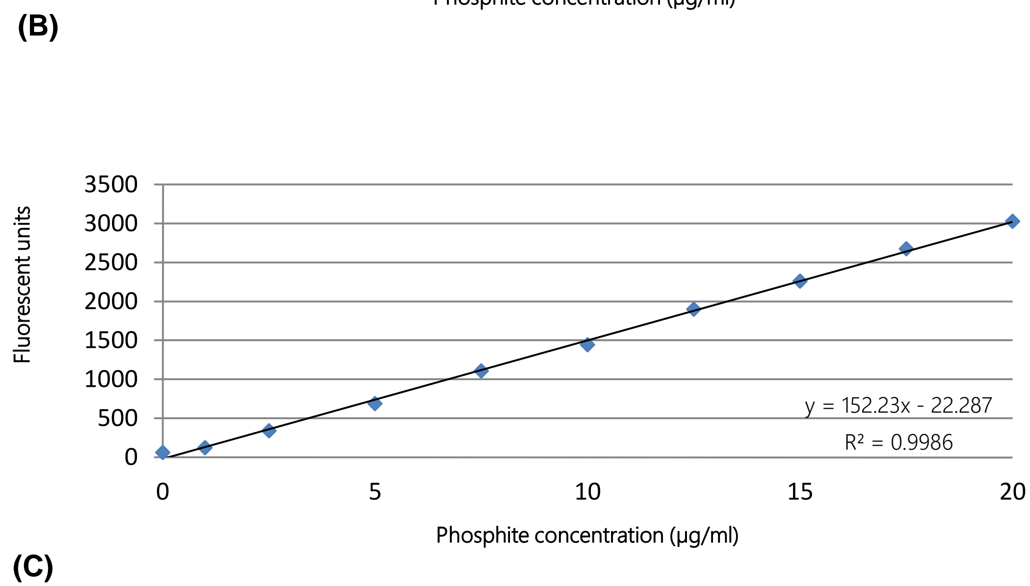
[a] Each value is the mean of three experiments each containing two replicates, except for sample B1.4C that is the mean of three experiments with one replicate each and sample B5.1E that is the mean of two experiments with two replicates each.
[b] The recovered internal spike value of each sample was used to calculate the adjusted phosphite concentration for the sample as described by Berkowitz *et al.* (2011), by first spiking each root extract sample with 15 µg/ml phosphite that served as an internal control spike.
[c] The recovered internal spike value of each sample was calculated by subtracting the unspiked sample value from the 15 µg/ml internal spike sample value, which was then divided by 15 µg/ml.
[d] The value of the unspiked sample divided the recovered internal spike value to obtain the adjusted phosphite sample concentration.
[e] Since 500 mg of roots were used in 10 ml extraction buffer, the final root phosphite concentration was obtained by multiplying the adjusted phosphite concentration with 20.



(A)



(B)



(C)

Fig. 1. Representative standard curves for phosphite concentrations diluted in water for (A) high performance ion chromatography, (B) LC-MS/MS, and (C) an enzymatic fluorescent assay (Berkowit *et al.*, 2011).

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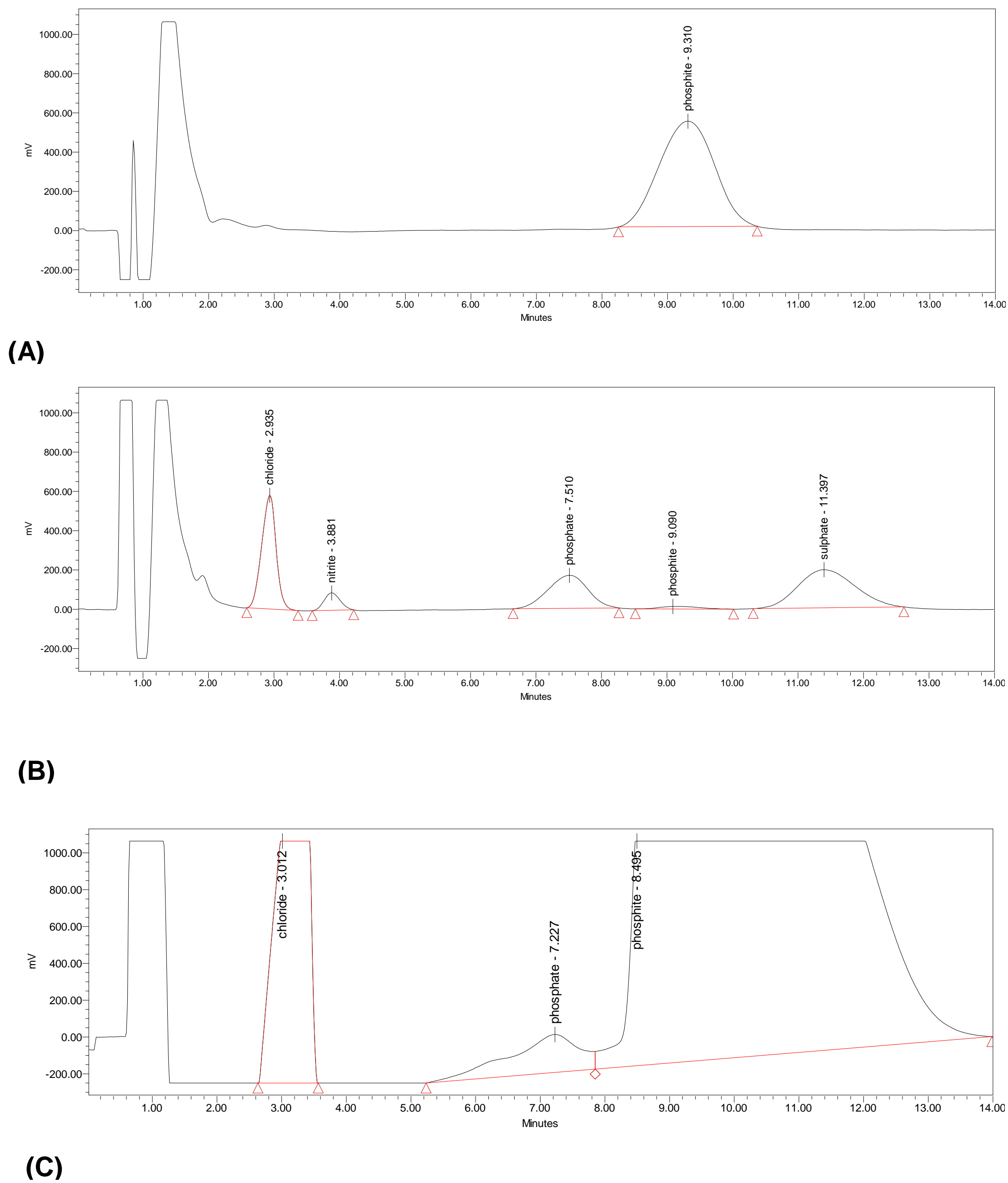

Fig. 2. Representative chromatograms of (A) a standard curve 10 µg/ml phosphite sample; (B) an avocado orchard root sample and (C) an avocado root sample containing a high sulfate concentration that resulted in an overlap of sulfate and phosphite peaks.

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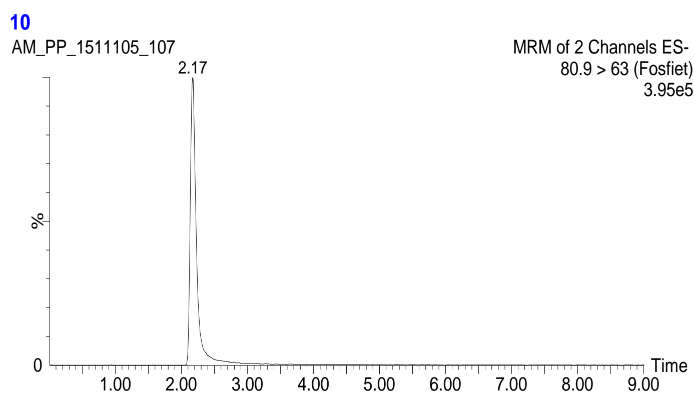
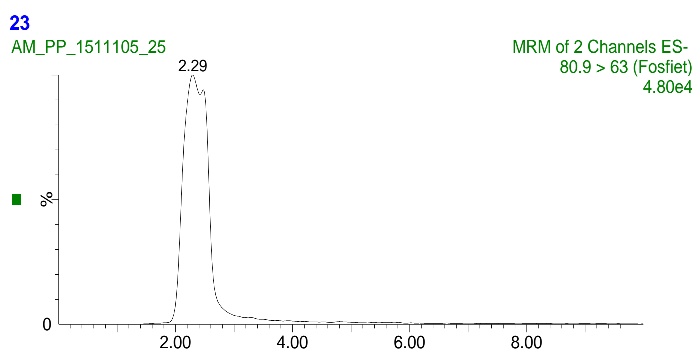


(A)



(B)

Fig. 3. Representative chromatograms of (A) a standard curve 10 µg/ml phosphite sample and (B) an avocado root sample obtained in LC-MS/MS analyses.

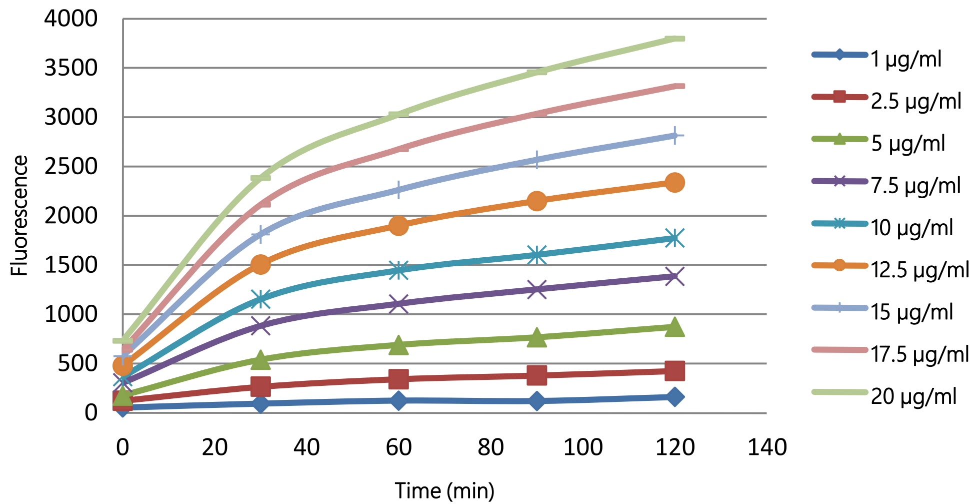


Fig. 4. Real-time detection of resorufin production over a 2 hour time period, using 1 to 20µg/ml of phosphite. Each value is the mean of three replicates.

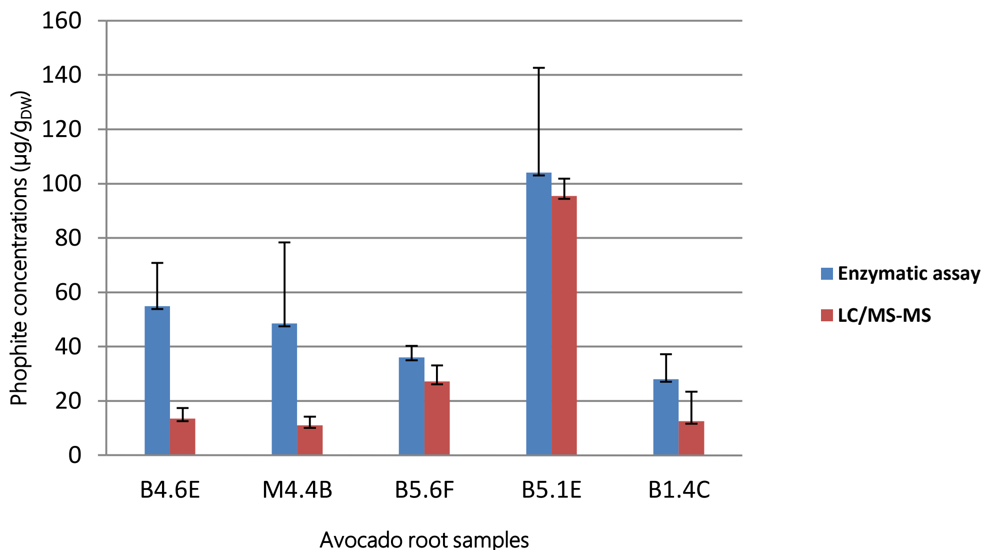


Fig. 5. Comparative phosphite in avocado field sample roots, quantified using an enzymatic fluorescent assay and LC-MS/MS analyses. For LC-MS/MS analysis, each value is the mean of three independent experiments with three replicates per experiment, expect sample B5.6F that is the mean of two experiments each containing two replicates. For the enzymatic assay, each value is the mean of three independent experiments with two replicates per experiment, expect for sample B1.4C that is the mean of three experiments with one replicate each and sample B5.1E that is the mean of two experiments, two replicates per experiments. Error bars represent standard deviations.

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CHAPTER 3

In vitro and *in vivo* sensitivity of *Phytophthora cinnamomi* from avocado to phosphonates

ABSTRACT

Phytophthora root rot caused by *Phytophthora cinnamomi* threatens the production of avocado worldwide, but the disease is currently effectively managed using phosphonate-based fungicides. The study investigated the *in vitro* and *in vivo* phosphite sensitivity of *P. cinnamomi* isolates from avocado, and if *in vitro* phosphite sensitivities of isolates are influenced by phosphate concentration. *In vitro* screening of 42 isolates at three phosphate concentrations (1, 7 and 15 mM) and two phosphite concentrations (30 and 100 µg/ml) revealed the presence of three sensitivity groups. The sensitive, intermediate and tolerant groups represented 28.6%, 28.6% and 42.9% of the isolates respectively. Percentage radial growth inhibition, considering all phosphite and phosphate concentrations, varied for the sensitive (11 - 89%), intermediate (11 - 67%) and tolerant (4 - 19%) groups. The sensitive group showed a significant decrease in inhibition by phosphite at increasing phosphate concentrations at 30 µg/ml phosphite. However, at 100 µg/ml phosphite, inhibition was only significantly higher between 1 mM phosphate versus 7 and 15 mM. The intermediate group was inhibited significantly less with increasing phosphate concentrations at both phosphite concentrations. In contrast, for the tolerant group there was no significant difference in inhibition by phosphite as phosphate concentrations increased, except at 100 µg/ml for the 1 mM versus 7 and 15 mM phosphate concentrations. The *in vivo* sensitivity of one isolate from each of the sensitive and tolerant groups was investigated using an excised root bioassay. The roots in the assays were harvested from seedlings that were soil drenched with a range of phosphonate concentrations (0.125 g/l to 2 g/l) in two experiments. The two isolates responded similar to all treatments, but the tolerant isolate was more virulent causing significantly higher percentage of root lengths colonized than the sensitive isolate. Only the roots of seedlings that received the 1 or 2 g/l phosphonate soil drench had a significantly lower percentage of root length colonized than the untreated control, and also contained root phosphite concentrations of 9.82 µg/g_{FW} or higher, whereas the remaining treatments contained lower root phosphite concentrations. The only exception was the 0.25 g/l soil drench treatment in experiment 2, which unexpectedly caused a significant reduction in root length colonized relative to the control at a very low root phosphite concentration of 1.92 µg/g_{FW}. Altogether, the data supports an indirect mode of action involving host defence responses.

INTRODUCTION

Phytophthora root rot, caused by the oomycete pathogen *Phytophthora cinnamomi,* is one of the main limiting factors in avocado (*Persea americana*) production in South Africa. However, since the introduction of phosphonates, losses have been curbed, especially with the introduction of trunk injections in the 1980s by Darvas *et al.* (1984). Currently, phosphonate trunk injections or foliar sprays remain pivotal in preventative and curative root rot management strategies (Duvenhage and Köhne, 1995; Whiley *et al.*, 2001; Duvenhage 2001; Thomas, 2008). Once phosphonates are taken up by plants, at a physiological pH the fungicide is rapidly hydrolysed to phosphonic acid, which is then ionized to phosphite anions (HPO_3^{2-} and/or $H_2PO_3^{1-}$) that include some of the key molecules active against *P. cinnamomi* (Coffey and Bower, 1984; Ouimette and Coffey 1989a).

Several studies have investigated the *in vitro* phosphite sensitivity in *Phytophthora* (Fenn and Coffey, 1984; Fenn and Coffey, 1985; Dolan and Coffey, 1988; Ouimette and Coffey, 1989b). The sensitivity of *Phytophthora* species to phosphite varies between species, but also for isolates from the same species (Coffey and Bower, 1984; Ouimette and Coffey, 1989a; Komorek and Shearer, 1997; Jackson, 1997a; Wilkinson *et al.*, 2001a). The first reports on the sensitivity of *P. cinnamomi* showed that it was a species that is very sensitive to phosphite with the EC_{50} values for sporangial formation being 1.8 µg/ml, for zoospore release 6 µg/ml and for mycelia 4.1 to 6.2 µg/ml (Coffey and Joseph, 1985). However, subsequent studies reported much higher EC_{50} values of 4 to 148 µg/ml for radial growth inhibition of mycelia (Wilkinson *et al.*, 2001a). In South Africa, the only *in vitro* phosphite sensitivity studies that have been conducted was reported by Duvenhage (1994, 1999, 2001). The isolates analysed in these studies all originated from one orchard, since the study was aimed at determining if phosphite sensitivity increased in isolates collected from phosphonate treated versus untreated trees over several years (1993 to 2000). The research concluded that there was a shift in sensitivities of *P. cinnamomi* isolates towards resistance after long term exposure of isolates to phosphonates (Duvenhage, 1999).

Although concerns have been raised regarding the development of resistance to phosphonates due to their intensive use in avocado production, major failures in disease control have not been observed under field conditions. To date, only a few studies have reported the occurrence of phosphonate resistant isolates 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 approximately five years (Vegh *et al.*, 1985; reference within Guest and Grant, 1991). Cohen and Samoucha (1984) reported naturally occurring *Phytophthora infestans* isolates that were resistant to fosetyl-Al. Dobrowoski *et al.* (2008), similar to Duvenhage (1999), reported that long term use of phosphonates in orchards tends to select for *P. cinnamomi* isolates that are less sensitive to phosphite, although their

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study was conducted *in planta* unlike the *in vitro* studies of Duvenhage (1994; 1999). However, subsequently, no failures in disease control under native field or orchard conditions have been reported in these regions.

Aside from the uncertainty as to the significance of the identification of *in vitro* phosphite tolerant *Phytophthora* isolates, another aspect that requires further elucidation is the influence of phosphate (HPO_4^{2-}) on the *in vitro* sensitivity of isolates. This effect has been shown to vary between *Phytophthora* species (Fenn and Coffey 1989; Griffith *et al.*, 1989a; Darakis *et al.*, 1997). Fenn and Coffey (1984) reported that phosphate concentration (0.084 to 8.4 mM) had no significant effect on radial growth inhibition in *P. cinnanomi* and *P. citricola.* However, in *P. capsici* and *P. nicotianae* it was reported that phosphite radial growth inhibition is significantly higher at 5 mM phosphate than at 15 and 45 mM (Fenn and Coffey, 1989). Since the studies on phosphate interaction with phosphite *in vitro* were all conducted using only one to four isolates of a specific species (Fenn and Coffey, 1984; Fenn and Coffey, 1989; Griffith *et al.* 1993), it is unknown if responses are similar for isolates within a species. Most phosphite *in vitro* studies in *Phytophthora* used media containing very low levels (< 1 mM) of phosphate or complex media where the phosphate concentration is unknown (Coffey and Bower 1984; Fenn and Coffey, 1984; Coffey and Joseph 1985; Ouimette and Coffey, 1989a; Dolen and Coffey, 1988; Duvenhage,1994, 1999;). These low phosphate concentrations, however, is not representative of phosphate levels occurring in plants. The phosphate concentration occurring in plants is typically between 0.5 to 20 mM (Bieleski, 1973). The influence of phosphate on *in vitro* phosphite sensitivity also makes it difficult to compare the results of studies that have used different phosphate concentrations in media.

The lack of phosphonate 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 and Grant, 1991; McDonald *et al.*, 2001). It has long been debated as to whether the mode of action consists of a direct fungistatic and/or indirect plant host response and which is most important. The research group of Coffey conducted extensive research to support a direct fungistatic mode of action (Coffey and Bower, 1984; Fenn and Coffey, 1984; Fenn and Coffey, 1985; Dolan and Coffey, 1988; Fenn and Coffey, 1989; Ouimette and Coffey, 1989). On the other hand, several other studies have provided supporting evidence for an indirect mode of action, where disease is suppressed by host defense responses activated in phosphonate-treated plants when pathogen attack takes place (Grant *et al.*, 1990; Jackson *et al.,* 2000; Daniel and Guest, 2005).

Studies that have attempted to investigate the mode of action of phosphonates in controlling Phytophthora diseases have used various approaches. Several studies compared the *in vitro* phosphite with *in vivo* sensitivities of isolate and concluded that if the same phosphite concentration is required for *in vitro* than *in vivo* suppression, then a direct mode of

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action is involved. This has been found true in several studies (Fenn and Coffey, 1984; Fenn and Coffey, 1985; Dolan and Coffey, 1988; Saindrenan *et al.*, 1988; Smillie *et al.*, 1989). However, this approach is difficult to interpret due to the unresolved issues of the role of phosphate in *in planta* inhibition by phosphite, especially considering the significant effect that phosphate can have on mycelial growth inhibition percentages *in vitro*. A second approach has been the generation of mutated strains that are tolerant to phosphite *in vitro,* which were shown to be inhibited less *in planta* than the parental sensitive strain, also supporting a direct mode of action (Fenn and Coffey, 1985; Dolan and Coffey, 1988; Fenn and Coffey, 1989). It can, however, not be excluded that the mutated isolates used in these studies were affected in some other gene regions than phosphite response. The use of naturally occurring isolates differing in *in vitro* sensitivity would thus be better, but this have not been investigated. The third approach involves the use of gene and enzyme expression studies along with plants mutated in some defense gene pathways known to be involved in phosphite host responses. This approach was used elegantly by Massoud *et al.* (2012) to show that when low phosphonate concentrations are applied, pathogen suppression is achieved through the induction of host defense genes (indirect mode of action), but that when high phosphonate concentrations are applied a direct mode of action prevails. Jackson *et al.* (2000) also came to the same conclusion in that the mechanism of control is phosphite concentration dependent, by investigating host defense gene expression over time. Lastly, an *in vivo* approach can also be used for investigating mode of action. Plant materials containing a range of *in vivo* phosphite concentrations are exposed to the pathogen, and disease severity is determined to see if disease suppression correlates with phosphite concentration, which would support a direct mode of action as being most important (Fenn and Coffey, 1985, 1989; Smillie *et al.*, 1989).

The *in vivo* response of *Phytophthora* to different phosphite concentrations has only been studied by a few researchers, with reports being controversial. A direct correlation between phosphite tissue concentration and disease has been found in several host-pathogen systems including the tomato/*Phytophthora capsici*, tobacco/*Phytophthora nicotianae* and lupine and *Dryandra sessilis/P. cinnamomi* systems (Fenn and Coffey, 1985, 1989; Smillie *et al.*, 1989; Wilkinson *et al.*, 2001b). However, other studies involving the interaction of *P. cinnammomi* with several hosts including eucalyptus (*Eucalyptus marginata*)*, Lupinus angustifolius* (lupin), hairy glandflower (*Adenanthos brabiger)* and avocado (van der Merwe and Kotze, 1994; Jackson *et al.*, 2000; Pilbeam *et al*, 2000; Wilkinson *et al.,* 2001c) found no correlation between these parameters, thus rather supporting the involvement of host defenses. Both van der Merwe and Kotze (1994) and Pilbeam *et al.* (2000) found that on avocado and hairy glandflower respectively, even though phosphite tissue concentrations increased, the level of disease suppression did not change. This suggests that there is a critical value or plateau that

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is reached above which higher phosphite concentrations do not yield additional control. The existence of such a plateau has been shown experimentally by Massoud *et al.* (2014) for the foliar oomycete pathogen *Hyaloperonospora arabidopsidis* in *Arabidopsis thaliana*. The application of a concentration range of phosphonate soil drenches yielded a dose response curve showing a bi-phasic response where in the first phase a linear increase in pathogen spore inhibition was observed, followed by a plateau showing no differences in spore inhibition as phosphonate dosage increased, which was followed by a second linear phase. It was hypothesized that in the first linear phase plant host response are involved, whereas in the second linear phase a direct fungistatic effect is involved (Massoud *et al.*, 2012). The existence of two linear phases could explain the controversial reports on the *in vivo* effect of phosphite tissue concentrations on disease control for Phytophthora diseases.

The aims of this chapter were to first evaluate the *in vitro* phosphite sensitivity of 42 *P. cinnamomi* isolates from different avocado orchards in South Africa, using radial growth inhibition on agar media. The effect of three phosphate concentrations (1, 7 and 15 mM) was investigated on radial growth inhibition at two phosphite concentrations (30 and 100 µg/ml). Subsequently, one of each of the most sensitive and phosphite tolerant isolates identified *in vitro* were selected to conduct *in vivo* phosphite sensitivity studies. An excised root bioassay described by van der Merwe and Kotze, (1994) was used for this purpose, by evaluating the tolerance of avocado seedling roots obtained from seedlings that were treated with a concentration range of phosphonate soil drenches, to *P. cinnamomi* infections.

MATERIALS AND METHODS

Phytophthora cinnamomi isolate collection

Forty-two *P. cinnamomi* isolates were obtained from various sources. One isolate was provided by Westfalia Technological Services (Tzaneen, South Africa) and was collected in the 1990's from an avocado orchard in the Tzaneen region. Thirteen isolates were obtained from a culture collection at Stellenbosch University. These isolates were collected in 2007 from four avocado orchards in the Tzaneen and Modjadjiskloof regions. No information was available on the phosphonate usage in the aforementioned orchards. Twenty-eight *P. cinnamomi* isolates were collected in the current study in 2013 in ten different orchard blocks in the Tzaneen, Mooketsi and Modjadjiskloof regions. The 2013 isolates were collected from orchards that were all treated with phosphonates for more than 10 years.

The 2013 isolates were isolated from soil using a soil baiting technique (Tsao, 1983) with lupine seeds, pear fruit (Greenhalgh, 1978) and citrus leaf disks (Linderman and Zeitoun, 1977). In each orchard, a bulk soil sample was collected from the root zone of three to five trees, which were thoroughly mixed. Approximately 250 ml of soil was sieved through a 2 to 3 mm sieve, placed in plastic containers and 250 ml of distilled water was added to form a soil

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slurry. Lupine seeds were surface sterilized in 80% ethanol for 1 min and rinsed with distilled water followed by soaking overnight in distilled water. The seeds were then sown and germinated in moist vermiculite for 3 days, and germinated seeds with radicles of 2 - 3 cm long were selected for baiting. Citrus leaves were surface sterilized with 70% ethanol and cut into small blocks (1 cm x 1 cm) that were used as baits. Packham pears were also surface sterilized in 70% ethanol, rinsed in sterile distilled water and used as baits. The three different bait types were all floated onto the soil slurries in plastic containers and were incubated under natural light conditions at 25˚C. After 3 to 4 days, symptomatic leaf disks, lupine radicals and pears were rinsed in 70% ethanol for 30 s and dried in the laminar flow. Citrus leaf disks, 1 cm lupine radicals and small blocks from the edge of pear lesions were plated onto PARPH (Jeffers and Martin, 1986) medium in 90 cm dia. petri plates. The plates were incubated for 3 to 5 days at room temperature in the dark. Mycelia with the morphology of *P. cinnamomi* (coralloid-type) emerging from the plated tissues were hyphal tip transferred to potato dextrose agar plus streptomycin (PDA^+). The hyphal tipping was repeated once more to fresh PDA^+ media to obtain pure cultures. All isolates used in the study were stored in small glass vials containing 7 ml of distilled water and a few citrus leaf disks that were autoclaved. The cultures were stored at 15˚C.

Confirming the species identity of isolates

DNA extraction and PCR amplification

Isolates were grown on V8 agar plates (Galindo and Gallegly, 1960) for 5 days at 25˚C. Mycelia were harvested from the plates and used for the extraction of DNA according to Lee and Taylor (1990). A conventional *P. cinnamomi*-specific PCR targeting the ras-related protein gene *Ypt1* was used to confirm the identity of the isolates as previously described (Schena *et al.*, 2008), with slight modifications. The PCR reaction contained 2 µl of template DNA, 100 µM dNTPs, 1 mM $MgCl_2$, 50 µg bovine serum albumin (BSA) (Roche Diagnostics South Africa, Randburg, South Africa), 1 Unit Taq DNA polymerase (Taq DNA polymerase, Bioline, London, United Kingdom), 1x reaction buffer (Bioline) and 3 µM of each forward (Ycin3F) and reverse (Ycin4R) primer in a total volume of 25 µl. The touch down PCR amplification was conducted at an initial denaturation of 94˚C for 5 min, followed by several cycles where the annealing temperature was decreased step-wise by 1˚C from 64˚C to 60˚C. Each of the step-wise cycles consisted of 5 cycles of 94˚C for 30 s, 64˚C - 60˚C for 30 s, 72˚C for 30 s, and a final extension step at 72˚C for 5 min. The PCR products, 5 µl per gel lane, were electrophoresed in a 3% agarose gel, and then stained with ethidium bromide for visualization under a UV transilluminator. A100 bp DNA ladder (Thermo Scientific, Waltham, USA) was included on all gels.

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Phytophthora species closely related to *P. cinnamomi, Phytophthora niederhauseri* and *Phytophthora parvispora* (syn. *Phytophthora cinnamomi* var *parvispora*; Scanu *et al*., 2014) was used as negative controls. The aforementioned two species are the only *Phytophthora species* in South Africa that also belong to *Phytophthora phylogenetic* clade 7, to which *P. cinnamomi* also belongs (Kroon *et al*., 2004). The positive control consisted of a *P. cinnamomi* isolate (C3) of which the identity was confirmed through sequence analyses of the internal transcribed spacer region as previously described (Tewoldemedhin *et al*., 2011).

***In vitro* phosphite sensitivity testing**

The phosphite sensitivity of the 42 *P. cinnamomi* isolates was determined at two phosphite (30 and 100 µg/ml) and three phosphate concentrations (1, 7 and 15 mM), by first growing each of the isolates on three PDA^+ agar plates (90 mm dia.) for 5 days at 25˚C. Small mycelia agar plugs (0.5 cm dia.) were pierced from the edge of the growing colony using a cork borer, and were used to inoculate Ribeiro's medium agar plates (90 mm dia.), one plug per plate. Ribeiro's medium is a defined mineral salts medium and was made according to Ribeiro *et al*. (1975) as modified by Fenn and Coffey (1984), except that the potassium phosphate concentrations were adjusted to three different concentrations (1, 7 and 15 mM). Prior to autoclaving the pH of the medium was adjusted to 6.2 using 0.03 M MES hydrate buffer (Sigma-Aldrich, St Louis, USA). Bacteriological agar (Merck, Darmstadt, Germany) was added to the medium to yield a final concentration of 1.4% (Fenn and Coffey, 1984). After autoclaving, a commercial potassium phosphonate fungicide Phytex (200 g/l phosphorous acid equivalent, Villa Crop Protection, Kempton Park, South Africa), was filter sterilized through a 0.22 µm PALL acrodisc ® syringe filter containing a Supor ® membrane (Pall Corporation, Washington, USA), and added to the cooled media to a final concentration of 30 or 100 µg/ml phosphite. Control plates did not receive any phosphite addition, only the three different phosphate concentrations. For each specific phosphite and phosphate concentration, three replicate plates were inoculated per isolate, along with the same number of control plates. The inoculated plates were incubated at 24˚C in the dark in a sealed black plastic bag for 9 days. The mycelial radial growth of each plate was determined by measuring the colony diameter at two random points and calculating the average diameter. The percentage mycelial growth inhibition for each isolate at each phosphite and phosphate concentration was calculated using the formula: growth on unamended medium – growth on amended medium/growth on unamended medium x 100. Each isolate was tested in two independent experiments, and for those treatments that showed large variations (> 20%) between experiments, the treatment was repeated in a third experiment and the two most representative values were included in analyses.

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Statistical analyses

Multivariate analyses were conducted with XLStat (Version 2014, Addinsoft, New York, USA). Isolates were first clustered by means of Ward's method based on the percentage mycelial growth inhibition at two different phosphite (Phi) concentrations (30 and 100 µg/ml) and three phosphate (Pi) concentrations (1, 7 and 15 mM). Principal component analysis (PCA) was also done to visualize the relationship between isolates and their inhibition of radial growth at different phosphite (Phi) and phosphate (Pi) concentrations. To further elucidate observed associations, other classification factors for the isolates were also plotted as factor labels, namely the Ward's grouping of isolates, orchard number from which isolates were collected and the year of isolation. Discriminant analysis (DA) was conducted to confirm that the groups of *P. cinnamomi* isolates classified according to Ward's clustering could be mathematically distinguished based on the inhibition of radial growth at different phosphite (Phi) and phosphate (Pi) concentrations.

Univariate analysis of variance (ANOVA) was performed on the percentage mycelial growth inhibition using the GLM (General Linear Models) Procedure of SAS statistical software (Version 9.2; SAS Institute Inc, Cary, USA). For the ANOVA, individual isolates were considered as random replicates for the resistance clusters (identified based on multivariate analyses results), each tested at different phosphite and phosphate concentration combinations. The Shapiro-Wilk test was performed to test for normality (Shapiro and Francia, 1972). Fisher's least significant difference was calculated at the 5% level to compare means (Pilcher and Ott, 1998). A probability level of 5% was considered significant for all significance tests.

***In vivo* phosphite sensitivity testing using the excised root bioassay**

Phosphonate treatment of avocado seedlings

Degania seedlings in 5 L bags that were 6 months old were kindly provided by ZZ2 avocado nursery (Mooketsi, South Africa). Seedlings were watered approximately three times a week with ammonium sulphate (20 µg/ml) to supply nitrogen, and once a month with iron sulphate (20 µg/ml). The seedlings were grown in a glasshouse at temperatures ranging from 20 to 28°C.

Avocado seedlings were drenched with a range of phosphonate concentrations including 0, 0.125, 0.25, 0.5, 1 and 2 g phosphorous acid/L. Phytex (200 g/l phosphorous acid equivalent) solutions were made to contain the desired final concentration of phosphorous acid, and each seedling was drenched with 250 ml of the solution. Controls consisted of seedlings drenched with the same volume of water. In the first experiment, three replicates (each consisting of one seedling) were included per treatment, whereas in the second experiment, four replicates were included. The trial was a completely randomized design.

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Preparation of excised roots and root phosphite analyses

Two weeks after the avocado seedlings received their phosphonate soil drench treatment, the bag of each seedling was carefully cut open in order to cause minimal root damage. For each replicate (one seedling) 24 roots tips (12 per *P. cinnamomi* isolate), 40 mm in length, were removed with a scalpel and placed in a petri plate (90 mm dia.) containing a moist filter paper to prevent drying of the roots. Harvested roots were placed in ice boxes and transported to the laboratory. The remaining part of the root system was removed from the plant, washed, weighed and dried for 2 days at 60˚C. Phosphite was extracted and quantified from the roots using LC-MS/MS analyses, as described in Chapter 2.

The roots in each petri plate containing roots from each respective treatment, was carefully washed four times with sterile distilled water. The roots were then packed in plastic containers (29 cm x 21 cm) containing three layers of moist paper towels. The containers were closed with plastic lids to prevent drying of the roots until inoculation was conducted. This facilitated the simulations inoculation of roots in a short period of time.

Inoculum production

Two *P. cinnamomi* isolates characterized in *in vitro* studies (see Results section) as phosphite tolerant (isolate A5) and phosphite sensitive (isolate 12C2D) were used in the inoculation studies. Zoospores were produced using a slightly modified method of Londsdale *et al.* (1988). The isolates were grown for 7 days on PDA^+ agar plates (90 mm dia.) in the dark at 24˚C. Pea agar (200 g peas autoclaved, strained and made up to 1 L with water plus 17 g agar) plates (90 mm dia.) were overlaid with a moist miracloth (EMD Millipore Corporation, St Charles, USA) circle, slightly smaller than 90 mm dia. The miracloth was prepared by rinsing the circles in distilled water, followed by autoclaving. Each miracloth plate was inoculated with 10 pieces (2 x 2 mm) of 7 day old PDA cultures, and was incubated at 25˚C in the dark. After 7 days, each miracloth circle was transferred to a sterile 250 ml flask containing 100 ml of 1:8 diluted pea broth (similar to pea agar, except that no agar was added), and were shaken overnight in the dark at 22˚C at 160 rpm. The pea broth was poured off, and the mirracloth was washed four times for 30 min. at 160 rpm with 75 ml of mineral salt solution [0.01 M $Ca(NO_3)_2$, 0.005 M KNO_3, 0.004 M $MgSO_4$, and 1 ml l^{-1} of a solution containing 10 M $FeSO_4$, 7.5 M KOH and 20 M EDTA] (Byrt and Grant, 1979). After the final wash, 40 ml of mineral salt solution was added and the flasks were shaken incubated for 18 h at 22˚C in the dark at 160 rpm. The miracloth was rinsed in 100 ml cold double-distilled water, where after 20 ml of cold double-distilled water was added. The flasks were incubated at 19ºC in an incubator containing lights for 2 h to induce sporangial production and zoospore release. The zoospore suspensions were poured through sterile mirracloth to remove mycelial fragments. Zoospore suspensions were

left at 19°C in the incubator until the excised roots were ready for inoculation, and a subsample was quantified using a hemocytometer. Zoospore solutions were adjusted to a final concentration of 10^4 zoospores/ml, just before inoculation commenced. Dilution of zoospore solutions prior to the inoculation process resulted in higher encystment rates.

Inoculation of excised roots and infection quantification

The zoospore inoculum was aliquoted into 0.2 ml polypropylene PCR 12-tube strips, 50 µl per tube, and each of the washed excised roots were immediately placed into one PCR tube. Zoospores were prone to encystment once placed into the PCR tubes, and therefore the placement of roots into the zoospore containing PCR tubes was done promptly. A water control was also included that consisted of the control roots that were not treated with phosphonate being placed in 50 µl water in PCR tubes. The PCR tubes containing roots were placed back into the plastic containers lying flat (Fig. 1A). The lids of the containers were closed and placed in an incubator at 25°C for 2 h. Subsequently, the roots were removed from the tubes and placed back into the containers of which the lids were then closed (Fig. 1B). The inoculated roots were incubated for another 44 h at 25°C to allow colonization to take place.

Following the incubation period, the roots were surface sterilized for 3 s in 70% ethanol, and dried on sterile paper towels. The sterilized roots were plated on PARP agar medium (Jeffers and Martin, 1986). The percentage root length colonized was calculated after 2 days of growth using the formula: root length colonized ÷ root total length (40 mm) x 100.

The percentage control was calculated using the formula: (% root length colonized control - % root length colonized phosphonate treatment) ÷ % root length colonized control) x 100.

Statistical analyses

All analyses were conducted in Statistica 12 (Dell Software). Data was assessed for normality using the Shapiro-Wilk test. The data on the phosphite root concentrations, percentage root length colonized and relative percentage root length colonized were subjected to analyses of variance. The Student's least significance difference post hoc test was conducted, and a probability level of 5% was considered significant. Correlation analyses between the percentage root length colonized and phosphite concentration was conducted using Spearmans correlation analyses.

RESULTS

Phytophthora cinnamomi isolate collection

In the current study, 28 *P. cinnamomi* isolates were obtained from avocado soils collected in 2013 through baiting with different plant baits. Most isolates were obtained from pears (47%),

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followed by citrus leaf disks (30%) and lupine radicles (23%). The pear isolations were more feasible than the other plant baits, since the cultures were less contaminated with *Pythium* spp. that were able to grow on the PARP medium. Almost all of the orchard soils contained *Pythium* spp. that could tolerate hymexazol, and which could also infect citrus leaf disks and lupin radicles, unlike for the pear fruit.

***Phytophthora cinnamomi* isolate collection and confirmation of species identity**

The 28 *P. cinnamomi* isolates from 2013, along with the 14 isolates obtained from culture collections all yielded a single 250 bp amplicon when their DNA was amplified with *P. cinnamomi*-specific primers. This was the same amplicon size observed for the positive control *P. cinnanmomi* isolate (C3). The two negative control isolates, *Phytophthora niederhauseri* and *Phytophthora parvispora,* which are phylogenetically most closely related to *P. cinnamomi* in South Africa, did not yield any amplification products.

***In vitro* phosphite sensitivity testing**

Ward's cluster analyses identified three sensitivity groups based on the response of the 42 isolates to the two phosphite concentrations (30 and 100 µg/ml) and three phosphate concentrations (1, 7 and 15 mM) (Fig. 2). The three sensitivity groups will hereafter be referred to as the sensitive, intermediate and tolerant groups. Most isolates (42.9%) were classified as tolerant, whereas the percentages of intermediate and sensitive isolate were similar (28.6%). The Ward's cluster analysis graph showed that the response of the sensitive and intermediate isolates to phosphate was similar at 30 and 100 µg/ml phosphite, with inhibition by phosphite decreasing as phosphate concentration increased. The response of the tolerant group isolates was notably different since inhibition at 30 and 100 µg/ml was depicted as an almost flat line showing limited response at different phosphate concentrations (Fig. 2).

ANOVA analyses showed that there was a significant interaction ($P < 0.01$) between Ward's sensitivity group x phosphite concentration x phosphate concentration (Table 1). Each of the three groups differed significantly from each other in percentage inhibition at each of the three phosphate concentration. For the tolerant group, the lack of influence of phosphate concentration on inhibition by phosphite, as observed in the Ward's cluster graph (Fig. 2), was also supported statistically with posthoc testing. With one exception, there were no significant differences in the mean inhibition of radial growth for the tolerant group at 30 and 100 µg/ml phosphite for the three phosphate concentrations, with inhibition ranging from 9.16 to 19.09% at 100 µg/ml phosphite and 3.90 to 8.62% at 30 µg/ml phosphite. The exception was that at 100 µg/ml phosphite the tolerant group was inhibited significantly more at 1 mM than at 7 and 15 mM (Table 2). In contrast, the sensitive group was inhibited significantly less (11.04 to 41.29%) as the three phosphate concentrations increased from 1 to 15 mM at 30 µg/ml

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phosphite, but at 100 µg/ml significant less inhibition was only evident at 1 mM (89.21%) compared to 7 and 15 mM phosphate (68.98 to 70.30% inhibition). The intermediate group also showed significantly decreasing inhibition with increasing phosphate concentration at 30 µg/ml (11.26 to 30.6%) and 100 µg/ml (39.51 to 66.75%) between all three phosphate concentrations (Table 2).

PCA analyses showed that the first two principal components explained 91.76% of the variation in percentage mycelial growth inhibition, and these components were thus used in all analyses (Fig. 3). Principal Component 1 (PC1) explains 82% of the variation in percentage mycelial growth inhibition and separates isolates differing in sensitivity. Isolates located on the negative (left) part of PC1 (e.g. A5 and 12C2BL) exhibited low inhibition in their growth at all phosphite concentrations (30 and 100 µg/ml) and phosphate concentrations (1, 7 and 15 mM) and thus represent tolerant isolates, whereas those (e.g. pcin39 and 12C2DL) on the positive (right) part of PC1 showed high inhibition of growth at all phosphite and phosphate concentrations and thus represent sensitive and intermediate isolates (Fig. 3A). This was also supported by using the Ward's cluster number as input variable (Fig. 3B). PCA analyses based on orchard identity showed that isolates differing in sensitivity were often found within the same orchard (orchards 1, 4, 7, 8, 10, 12, and 14). The remaining orchards contained either only tolerant isolates (orchards 5, 6 and 13) or sensitive and intermediate isolates (Fig. 4A). Isolates representing the sensitive group was located in 38% of the orchards and the intermediate and tolerant isolates in 56% and 62% of the orchards respectively. When considering the year of isolation, there was a more or less equal distribution of the three sensitivity groups for 2013 isolates, whereas the 2007 isolates were mostly located on the positive part of PC1 indicating sensitivity. However, fewer isolates from 2007 were included in the study than from 2013. The only isolate (Westfalia) representing a collection in the 1990s was sensitive (Fig. 4B).

Principal Component 2 (PC2) only explained a further 10% of the variation in percentage mycelial growth inhibition and mainly separates the 30 µg/ml phosphite and 15 mM phosphate treatment from the rest (Fig. 3). Table 2 confirms that for the sensitive and intermediate isolate groups the significantly lowest growth inhibition was observed at 30 µg/ml phosphite and 15 mM phosphate.

Discriminant Analysis (DA) based on the inhibition of radial growth at different phosphite (Phi) and phosphate (Pi) concentrations showed that 97.62% (41) of the 42 *P. cinnamomi* isolates could by correctly classified into the sensitivity groups created using Ward's clustering. The DA scores plot for the first two discriminant functions confirmed that the groups observed in the Ward's cluster analysis were very well discriminated. The only exception was isolate Pcin42 that was identified as intermediate by the Ward's cluster analyses whereas it grouped with the sensitive isolates in DA (Fig. 5).

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The *in vitro* sensitivities of two isolates, A5 and 12C2D, are important since these isolates were used in the *in vivo* study. Isolate A5 was identified as resistant, and its radial growth inhibition varied according to phosphate concentration resulting in -9 to 0.23% inhibition at 30 µg/ml, and -2 to -8% inhibition at 100 µg/ml. Isolate 12C2D was identified as sensitive and its radial growth inhibition varied according to phosphate concentration and resulted in 14 to 42% inhibition at 30 µg/ml, and 74 to 100% inhibition at 100 µg/ml.

***In vivo* phosphite sensitivity testing using the excised root bioassay**

Root phosphite concentrations

The water control was not included in ANOVA and post hoc testing, since all the replicates had a value of 0, thus showing no variance. ANOVA showed that for both trials there was a significant effect ($P < 0.01$) of the different phosphonate soil drench concentration treatments (0.125 to 2 g/l) on the wet weight root phosphite concentrations in both experiments. In both trials, the 2 g/l application yielded significantly higher root phosphite concentrations (15.50 and 19.30 $\mu g/g_{FW}$) relative to the rest of the soil drench phosphonate concentrations (Table 3). In experiment 1, the 1 g/l application also resulted in a significantly higher phosphite concentration than the 0.125 g/l application, but not in trial 2 (Table 3). Similar findings were obtained for the dry weight root phosphite concentration analyses, than for the wet weight analyses (data not shown).

Percentage root length colonized

Analyses of variance on the percentage root length colonized showed that there was a significant experiment × treatment (concentration) interaction ($P > 0.05$) (data not shown), and therefore results of each experiment was considered separately. For both experiments, there were no significant isolate × concentration interactions ($P > 0.16$) in both experiments. Thus, both isolates responded in a similar manner to the different phosphonate soil drench concentrations. However, in both trials, isolate A5 caused a significantly ($P < 0.05$) higher percentage root length colonized and showed higher virulence than isolate 12C2D for all the treatments (Table 4). In both trials, the 2 g/l applications resulted in significantly lower percentage root lengths colonized than the control treatment. In experiment 1, the 1 g/l treatment also resulted in significant less root length colonized than the control. Unexpectedly, in experiment 2, the 0.25 g/l treatment also resulted in significantly less root length colonized than the control (Table 3). The treatments that thus had a significant effect on colonization by both isolates due to the presence of root phosphite, were the 1 and 2 g/l treatments in experiment 1, and 0.25 and 2 g/l in experiment 2. The root phosphite concentrations that corresponded to these treatments were larger than 9.82 $\mu g/g_{FW}$ in both experiments, except for the 0.25 g/l treatment in experiment 2 that had a very low root phosphite concentration

(1.92 µg/g_{FW}) that did not differ significantly from the root phosphite concentrations of phosphonate drench treatments that did not differ from the control. The root length colonized at 1 and 2 g/l did not differ significantly from each other in experiment 1, even though the 2 g/l treatment had almost twice the amount of root phosphite (19.30 µg/g_{FW}) than the 1g/l treatment (9.82 µg/g_{FW}) (Table 3).

Percentage control

Analyses of variance on percentage control of the root length colonized by the two isolates in the different treatments showed that there were no significant isolate x treatment interactions ($P \geq 0.61$) in both experiments (Table 5). Therefore, the main effects of isolate and treatment could be considered. In experiment 1, there was a significant difference (P < 0.001) between the two isolates in percentage control, with isolate 12C2D being controlled significantly better than isolate A5 across all phosphonate drench treatments. However, in experiment 2 there was no significant difference ($P = 0.23$) between the control of the two isolates, although there was a slight trend for the percentage control of isolate 12C2D being higher than for A5 (Table 5). For both isolates the percentage control in experiment 1, at 1 and 2 g/l did not differ significantly from each other, but were significantly higher than the rest of the treatments. This was true, even though in experiment 1, the root phosphite concentration was almost double the amount for the 2 g/l treatment (19.30 µg/g_{FW}) than for the 1 g/l treatment (9.82 µg/g_{FW}) (Table 3). In experiment 2, the percentage control for the two isolates was significantly higher for the 2 g/l and 0.25 g/l treatments than for the other treatments (Table 3). In this case, the role of root phosphite concentration was less clear since although the 2 g/l root phosphite concentration was high (15.50 µg/g_{FW}), the value for the 0.25 g/l treatment was low (1.92 µg/g_{FW}) and comparable to the rest of the treatments.

Correlation analyses between root phosphite concentration and percentage control

Correlation analyses between the wet weight root phosphite concentration and percentage control showed that in experiment 1 there was a significant correlation for isolate 12C2D (r = 073, $P < 0.001$) and A5 ($r = 0.59$; P =0.02). For both isolates the percentage control was highest (12C2D ≥ 58%; A5 ≥ 22%) for data points above 10 µg/g_{FW} (Fig. 6). The results from experiment 2 were less clear, due to the unexpected high percentage control for the 0.25 g/l soil drench concentration even though phosphite concentration was low (1.92 µg/g_{FW}). This also contributed to there being no significant correlation between percentage control and phosphite concentration for isolate 12C2D ($r = 0.33$; $P = 0.17$) and A5 ($r = 0.14$; P =0.56). For both isolates, providing that the four data points from the 0.25 g/l is not considered, then data points that had more than 9 µg/g_{FW} resulted in more than 20% control for both isolates (Fig. 6).

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Comparison between *in vitro* and *in vivo* phosphite sensitivities of two *P. cinnamomi* isolates

The *in vitro* and *in vivo* sensitivity of two *P. cinnamomi* isolates, A5 and 12C2D was compared. The *in vitro* inhibition of the two isolates was considered at the more physiological relevant phosphate concentrations of 7 and 15 mM, rather than at 1 mM phosphate, and at a phosphite concentration of 30 µg/ml rather than at 100 µg/ml, since 19.30 µg/g_{FW} was the highest phosphite concentration quantified from avocado seedling roots in the *in vivo* study. The *in vivo* percentage control of the isolates was considered only for the 1 and 2 g/l soil drench treatments in experiment 1, and 2 g/l treatment in experiment 2, since these treatments resulted in significantly lower percentage root length colonized and percentage control of the isolates (Table 3). At the relevant *in vitro* phosphate (7 and 15 mM) and phosphite (30 µg/ml) concentrations isolate A5 was inhibited *in vitro* by -6 to 0.23 %, and isolate 12C2D by 14 to 37% (data not shown). At the relevant *in vivo* treatments both isolates were inhibited more than 40% *in vivo* than *in vitro. In vivo* isolate A5 was inhibited by 13.6 to 27.06%, and isolate 12C2D by 48 to 62.4% (Fig. 6).

DISCUSSION

The study investigated a collection of 42 *P. cinnamomi* isolates in South Africa, of which 28 were isolated in the current study, with the remaining isolates obtained from culture collections. The 28 isolates were isolated through soil baiting using different plant baits. Lupin radicles were least effective in yielding *P. cinnamomi* isolates (23%), with citrus leaf disks (30%) only performing slightly better. Several studies have used blue lupin successfully as baits for isolation of *P. cinnamomi* (Chee and Newhook, 1965; Podger, 1968; Pratt and Heather, 1972). The low isolation efficacy of lupin radicals and citrus leaf disks in the current study was most likely due to the high incidence of *Pythium* species that were insensitive to hymexazol, and that grew faster than *P. cinnamomi* on the PARP plates. This often resulted in failures in dissecting *P. cinnamomi* hyphae among *Pythium* hyphae on culture plates, yielding mixed cultures that could not be purified, even in repeated baiting experiments. This problem was solved by using pear fruit, which was most successful in yielding *P. cinnamomi* isolates (47% of isolates). The pathogen caused hard dark brown lesions on pears, which can easily be discriminated from water soaked lesions caused by organisms such as *Penicillium* or *Pythium.* The *P. cinnamomi* pear fruit lesions also yielded pure cultures that were not contaminated with *Pythium.*

Several novel *Phytophthora* spp. are being described on a continuous basis using DNA based taxonomy, but the species are morphologically almost indistinguishable, for example *P. cinnamomi* and the recently described *P. parvispora* (Scanu *et al.*, 2014). The *P. cinnamomi*

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specific primer pair (Ycin3F-Ycin4R) of Schena *et al.* (2008) that targets the ras-related protein gene, was successfully used in the current study to differentiate *P. cinnamomi* from *P. parvispora,* and also from *P. nierderhauserii*, another recently described species. This showed that all 42 isolates used in the current study belonged to *P. cinnamomi.* The primers of Schena *et al.* (2008) have only been used by Dempsey *et al.* (2012) for confirming the presence of *P. cinnamomi* root infections in artificially inoculated Eucalyptus roots.

The 42 South African *P. cinnamomi* isolates showed a large range in phosphite sensitivities in *in vitro* agar assays based on radial growth inhibition. The phosphite sensitivity data was somewhat difficult to interpret since phosphite sensitivity was influenced by phosphate concentration (1, 7 and 15 mM) and two different phosphite concentrations (30 and 100 µg/ml) were evaluated (see section below). However, Ward's cluster analyses were able to identify three sensitivity groups (sensitive, intermediate and tolerant), which was also supported using a multivariate approach. Discriminatory analysis was further able to predict the designation of all isolates to the correct sensitivity group using all the phosphite and phosphate concentrations, with the exception of one isolate that was incorrectly assigned. The existence of the groups was also supported statistically since each of the three groups differed significantly from each other in percentage inhibition at each of the three phosphate concentrations for both phosphite concentrations, except at 15 mM phosphate and 30 µg/ml phosphite where the groups had similar percentage inhibitions. The sensitive group contained 28.6% of isolates and was inhibited by 11.04 - 89.21%, the intermediate group contained 28.6% of isolates and was inhibited by 11.26 - 66.75% and the tolerant group contained 42.9% of isolates and was inhibited by 3.9 - 19.09%.

Phosphate was found to selectively influence the phosphite sensitivity of the three *P. cinnamomi* phosphite sensitivity groups. The sensitive group showed a significant increase in inhibition at all three phosphate concentrations at 30 µg/ml (11.04 - 41.29%), but at 100 µg/ml inhibition was only significantly less between 1 mM phosphate (89.21%) versus 7 and 15 mM (68.98 - 70.30%). The intermediate group was inhibited significantly less (11.26 - 66.75%) with increasing phosphate concentrations at both phosphite concentrations. In contrast, for the tolerant group there was no significant difference in inhibition by phosphite as phosphate concentrations increased at 30 µg/ml (3.90 - 8.62%), but at 100 µg/ml a significant difference was only present between 1 mM (19.09%) versus 7 and 15 mM (9.16 - 11.97%) phosphate concentrations. The inhibition by phosphite of the tolerant group was thus in general less responsive to phosphate concentration, than the other two sensitivity groups. It has been hypothesized that the influence of phosphate on phosphite toxicity *in vitro,* is due to competition between phosphite and phosphate anions for the phosphate transport system, since both ions are taken up by the same transport system (Bompeix and Saindrenan, 1984; Fenn and Coffey, 1984; Griffith *et al.*, 1993). It is possible that our tolerant group has

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phosphate transport systems that can discriminate between phosphite and phosphate thus resulting in less phosphite being taken up by the pathogen as suggested by Griffith *et al.* (1993). There is likely also differential sensitivity to phosphite at one or more internal sites in the isolates (Griffith *et al.*, 1993). Griffith *et al.* (1993) also found that for a *P. palmivora* sensitive isolate, inhibitions occurred at all levels of phosphate, but that for two tolerant isolates inhibition only occurred if phosphate was limited in growth. Unlike these findings, Fenn and Coffey (1989) found that the influence of phosphate on phosphite sensitivity did not differ between a phosphite tolerant (generated through mutagenizes) and sensitive isolate of *P. capsici*. It is possible that the mutated tolerant isolate was also affected in gene regions other than those only influencing phosphite sensitivity, and that the response to phosphate in the presence of phosphite is not representative of what occurs in natural populations. Their results, did however correspond to our finding for the sensitive and intermediate groups in that their lower phosphate concentration (5 mM) caused more inhibition than the higher phosphate concentrations (15 and 45 mM) for two phosphite sensitive *P. parasitica* var. *nicotianae* (syn. *P. nicotianae*) isolates (Fenn and Coffey, 1989).

Since it is clear that phosphate concentration can influence the *in vitro* phosphite sensitivity in *Phytophthora* spp., it is difficult to compare the results of different *P. cinnamomi in vitro* studies due to the phosphate concentration present in the medium being inconsistent. The study of Coffey and Bower (1984) and Duvenhage (1994, 1999, 2001) used low phosphate concentrations of 0.84 mM phosphate and 0.38 mM respectively, whereas Wilkinson *et al.* (2001a) used 7.38 mM. Early studies by Coffey and Bower (1984) found limited inhibition by phosphite (0 - 48.8% radial growth inhibition at 5 µg/ml phosphite) for 12 *P. cinnamomi* isolates from California, USA. In Australia, a large population of 66 isolates exhibited a range of phosphite sensitivities that were investigated. The isolates could be grouped subjectively into three sensitivity groups based on their percentage radial growth inhibition at 5 and 50 µg/ml phosphite in a medium containing 7.35 mM phosphate. At 50 µg/ml, the sensitive isolates were inhibited 94 - 100%, the intermediate group 73 - 94% and the tolerant group 31 - 76% (Wilkinson *et al.*, 2001a). Comparing our results at 7 mM phosphate and 30 and 100 µg/ml phosphite, which is somewhat speculative since we did not evaluate the exact same concentrations than they did, suggests that our tolerant isolates were less sensitive since at 30 µg/ml the inhibition ranged from -6.8% to 14% and at 100 µg/ml was -17 to 35%. The only reports on the *in vitro* sensitivity of South African *P. cinnamomi* isolates to phosphite consisted of work conducted by Duvenhage (1994, 1999 and 2001). The isolates had a range of phosphite sensitivity showing 40 - 90% inhibition at 100 µg/ml and 0.38 mM phosphate. Comparing our results at 1 mM phosphate and 100 µg/ml phosphite the inhibition of all 42 isolates ranged from 19.09 and 89.21%, which is comparable to the inhibition reported

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by Duvenhage (Duvenhage 1994, 1999, 2001), although it is unknown how much inhibition by phosphite in *P. cinnamomi* will differ at 0.38 mM and 1 mM phosphate.

In the current study, three approaches were used to investigate the *in vivo* mode of action of phosphite to *P. cinnamomi* in avocado. In the first approach the *in vitro* and *in vivo* sensitivity of two isolates were compared. This approach is not that feasible due to the effect of phosphate on *in vitro* phosphite sensitivity and a lack of knowledge on whether phosphate also has an influence on inhibition by phosphite *in vivo.* Nonetheless, considering *in vitro* inhibition of the isolates at physiological relevant phosphate concentrations (7 and 15 mM) at 30 μg/ml phosphite, isolate A5 was inhibited *in vitro* by -6 to 0.23 % and isolate 12C2D by 14 to 37%. *In vivo,* both isolates were inhibited to a greater extent (> 40%) than *in vitro*, especially isolate A5 that was inhibited by 13.6 to 27.06% at even lower phosphite concentrations (9 to 19.3 μg/g_{FW}) than the *in vitro* 30 μg/ml. Isolate 12C2D was also inhibited more *in vivo* (48 to 62.4%) than *in vitro*, at drench treatments where significant control occurred. This would suggest an indirect mode of action. In the second approach, the virulence of a phosphite sensitive (12C2D) and tolerant (A5) isolate identified in *in vitro* studies was compared. The results of this approach was difficult to interpret since the phosphite sensitive isolate 12C2D was controlled significantly better than isolate A5 across all phosphonate drench treatments, including those that did not differ significantly from the control treatment in experiment 1, but not in experiment 2, although the same trend was present in experiment 2. Therefore, it is likely that isolate A5 is more virulent than isolate 12C2D which makes it difficult to know if the significant differences in percentage control at drench treatments where significant control were achieved, was due to the inherent higher virulence of isolate A5 that makes it more difficult to control *in vivo*. Therefore, in future studies more isolates representing the sensitive and tolerant group should be evaluated. In the third approach for investigating mode of action, avocado roots containing a concentration range of phosphites (0.58 - 19.30 μg/g_{FW}) was generated by soil drenching seedlings with different phosphonate concentrations. The tolerance of the roots to infection by two isolates (A5 and 12C2D) was evaluated using an excised root bioassay in two experiments. Only roots containing concentrations above 9.82 μg/g_{FW} yielded a significant reduction in percentage root length colonized. The exception was the unexpected higher percentage control obtained in the second experiment at a soil drenching of 0.25 g/l (root phosphite correspond to 1.92 μg/g_{FW}), which is most likely due to sample mislabelling, and is considered to be faulty since two subsequent excised root bioassay in our laboratory supported trends of the first experiment (unpublished data). It was further important to note that no significant differences were found in protection of roots against *P. cinnamomi* at root phosphite concentration of 9.82 and 19.30 μg/g_{FW} (equivalent to 1 and 2 g/l soil drench application), i.e. control did not improve if phosphite concentration increased. This agrees with finding by van der Merwe and Kotze (1994), also for *P. cinnamomi* in

avocado, where no significant improvement in control was obtained at root phosphite concentrations from 9.5 to 53.2 $\mu g/g_{FW}$. A similar finding was also made by Pilbeam *et al.* (2000) for *P. cinnamomi* on *A. barbiger* where the percentage infected stems did not differ significantly when phosphite concentrations were 7 or 80 $\mu g/g_{DW}$ in leaves. It is not clear why phoshite concentrations were measured in leaves by Pilbeam *et al.* (2000) rather than the stem, but it is likely that concentrations in leaves were representative of those in stem. Altogether, these findings support the hypothesis that at low phosphite concentrations in these hosts, *P. cinnamomi* is suppressed by host plant defences. Considering the bi-modal model of Massoud *et al.* (2012) for suppression of oomycetes in plants, the aforementioned studies would have monitored suppression in the first linear phase of suppression that is followed by a plateau where no significant improvement of control is achieved even though phosphite concentration increases, which are all mediated by host defence responses rather than a direct fungistatic effect according to Massoud *et al.* (2012).

This research has improved our knowledge on the *in vitro* and *in vivo* response of *P. cinnamomi* isolates from avocado. *Phytophthora cinnamomi* isolates were shown to respond differentially to phosphite, but this response was influenced by phosphate concentration. The inhibition response of isolates to phosphite *in vitro* depended on whether the isolate was classified as phosphite resistant or sensitive, since sensitive and intermediate group isolates in general were inhibited significantly less as phosphate concentration increased, whereas the tolerant group isolates were mostly unaffected. Given the fact that phosphate concentration influences the *in vitro* phosphite sensitivity of isolates, it will be important to determine phosphate concentrations in plants, and conduct *P. cinnamomi* disease control experiments with plants varying in phosphate concentration to determine if the *in vitro* observations are also relevant *in vivo*. Of the three approaches used in the current study to investigate the mode of action of phosphonates, one yielded inconclusive results, but the other two supported an indirect mode of action mediated by host defence responses. The study also, using an improved excised root bioassay of van der Merwe and Kotze (1994) found evidence that *P. cinnamomi* is suppressed at root phosphite concentration above 9.82 $\mu g/g_{FW}$, which will be useful for interpretation of the efficacy of orchard phosphonate trials. However, due to contradictory resuts of experiment 2, and only one treatment having a phosphite concentration above 9.82 $\mu g/g_{DW}$, more excised root bioassay trials will have to be conducted. The assays should also be used to evaluate more *in vitro* phosphite sensitive and tolerant isolates to further evaluate the inconclusive result of the *in vivo* inhibition of tolerant versus sensitive isolates, due to our tolerant isolates having higher virulence than the sensitive isolate. Our improved excised root bioassay fortunately facilitates larger experiments with higher throughput than the published assays (Botha *et al.,* 1980; van der Merwe and Kotze, 1992, 1994) since (i) PCR tubes are used for inoculating roots in large plastic containers (96 roots

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per container), rather than roots having to be balanced on glass rods imbedded within 90 cm water agar petri plates, where at most 10 roots can be inoculated per plate, (ii) it is not required to cut the 40 mm roots into 3 - 4 mm segments before plating, since leaving roots intact did not results in much different results than cutting roots into segments (data not shown) and (iii) infection is more consistent with roots being inserted into the zoospore supsension rather than having to inoculate a root tip with a zoospore suspension droplet that sometimes falls off the root.

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Table 1. Analysis of variance on the effect of different phosphite and phosphate concentrations on the inhibition of radial mycelia growth of *Phytophthora cinnamomi* isolates.

Source of variation	**Degrees of freedom**	**Means square**	**F value**	***P* value**
Group	2	40510.931	113.23	<0.001
Isolate(Group)=Error (a)	39	357.784		
Phosphite concentration	1	47712.402	770.93	<0.001
Group x fungicide concentration	2	10562.952	170.68	<0.001
Phosphate concentration	2	5811.527	93.90	<0.001
Group x phosphate concentration	4	912.876	14.75	<0.001
Phosphite concentration x phosphate concentration	2	19.460	0.31	0.730
Group x phosphite concentration x phosphate concentration	4	376.532	6.08	<0.001
Error (b)	195	61.889		

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Table 2. Effect of phosphite and phosphate concentration on the mycelial growth inhibition of 42 *Phytophthora cinnamomi* isolates.

Sensitivity group	**Phosphite concentration**	**Phosphate concentration**	**N**	**Mean percentage mycelial growth inhibition**
Sensitive	30 µg/ml	1 mM	12	41.29 d
Sensitive	30 µg/ml	7 mM	12	25.94 e
Sensitive	30 µg/ml	15 mM	12	11.04 gh
Intermediate	30 µg/ml	1 mM	12	30.60 e
Intermediate	30 µg/ml	7 mM	12	18.97 f
Intermediate	30 µg/ml	15 mM	12	11.26 gh
Tolerant	30 µg/ml	1 mM	18	8.62 ghi
Tolerant	30 µg/ml	7 mM	18	3.90 i
Tolerant	30 µg/ml	15 mM	18	5.49 hi
Sensitive	100 µg/ml	1 mM	12	89.21 a
Sensitive	100 µg/ml	7 mM	12	68.98 b
Sensitive	100 µg/ml	15 mM	12	70.30 b
Intermediate	100 µg/ml	1 mM	12	66.75 b
Intermediate	100 µg/ml	7 mM	12	60.02 c
Intermediate	100 µg/ml	15 mM	12	39.51 d
Tolerant	100 µg/ml	1 mM	18	19.09 f
Tolerant	100 µg/ml	7 mM	18	11.97 g
Tolerant	100 µg/ml	15 mM	18	9.16 ghi

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Table 3. Effect of different phosphonate soil drench concentrations on the root phosphite concentration of avocado seedlings, colonization and control in roots of two *Phytophthora cinnamomi* isolates inoculated in an excised root bioassay.

Phosphite concentration (g a.i./ml) [a]	Root phosphite concentration (µg/$g_{fresh\ weight}$)[b]	% Root length colonized[c]	% Control[c]
Experiment 1			
0 (control)	0.00	82.87 a	-
0.125	0.58 c	71.94 a	12.86 b
0.25	2.37 bc	84.19 a	- 1.88 b
0.5	3.05 bc	79.79 a	3.28 b
1	9.82 b	49.58 b	39.57 a
2	19.30 a	48.85 b	40.70 a
Experiment 2			
0 (control)	0.00	65.01 a	-
0.125	0.82 b	61.12 ab	6.06 b
0.25	1.92 b	48.17 cb	25.80 ab
0.5	3.03 b	61.00 ab	6.40 b
1	3.88 b	58.62 ab	9.95 b
2	15.50 a	39.98 c	38.74 a

[a] Avocado seedlings were drenched with 250 ml of phosphonate at different concentrations, in two independent experiments. The control treatment only received 250 ml of water.
[b] Root phosphite concentrations measured through LC-MS/MS analyses, two weeks after phosphonate soil drenching. Values are the mean of three (experiment 1) or four (experiment 2) replicates per treatment. Means in a column followed by the same letter do not differ significantly at $P < 0.05$.
[c] Percentage root length colonized by two *P. cinnamomi* isolates in an excised root bioassay, 2 weeks after phosphonate soil drenching. The percentage control of the root length colonized was calculated as: (Control-treatment/control) x 100. The mean for the two isolates are shown since there was no significant treatment x isolate interaction. Values are the mean of three (experiment 1) or four (experiment 2) replicates, with each replicate containing 12 roots. Means in a column followed by the same letter do not differ significantly at $P < 0.05$.

Table 4. Analysis of variance on the effect of phosphonate treatment of avocado seedlings on the percentage root length colonized by two *Phytophthora cinnamomi* isolates in two repeat experiments.

	Experiment 1				Experiment 2		
Source of variation	**DF**	**MS**	**F**	**P**	**MS**	**F**	**P**
Isolate	1	3432.10	20.35	< 0.001	750.10	3.95	0.05
Treatment	5	1595.6	9.46	< 0.001	730.40	3.85	0.01
Isolate x treatment	5	298.7	1.78	0.16	73.10	0.39	0.86
Error	36	168.70			189.80		

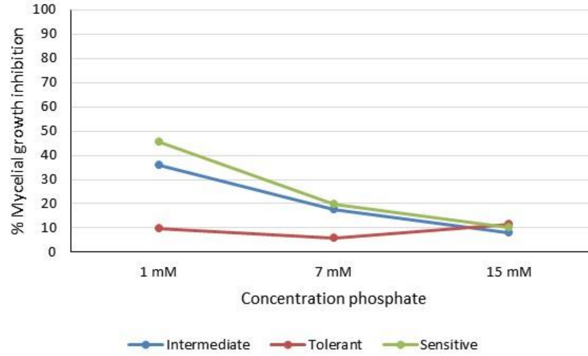
Table 5. Analysis of variance on the effect of phosphonate treatment of avocado seedlings on the percentage control of two *Phytophthora cinnamomi* isolates in two repeat experiments.

	Experiment 1				Experiment 2			
Source of variation	**DF**	**MS**	**F**	**P**	**DF**	**MS**	**F**	**P**
Intercept	1	10720.63	44.97	< 0.001	1	12098.3	24.35	< 0.001
Isolate	1	8254.33	34.62	< 0.001	1	740.27	1.49	0.23
Treatment	4	2421.96	10.16	< 0.001	4	1662.28	3.34	0.02
Isolate x treatment	4	162.52	0.68	0.61	4	189.54	0.38	0.82
Error	20	238.42			30	496.95		

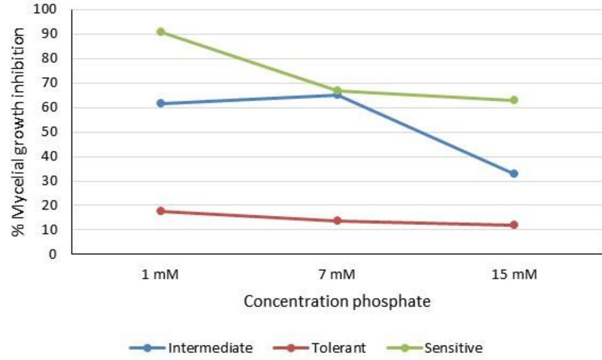
(B)

(A)

Fig. 1. Inoculation of avocado roots with two isolates (A5 and 12C2D) of *Phytophthora cinnamomi* using an excised root bioassay for assessing percentage root length colonized in phoshonate experiments. (A) Plastic container containing avocado seedling roots inserted into 12-strip PCR tubes each containing 50µl of zoospores suspension during the 2 hour inoculation period, (B) avocado roots after the 44 hour inoculation and colonization period, showing lesion development (right).



(A



(B

Fig. 2. Mycelial growth inhibition of three Phytophthora cinnamomi phosphite sensitivity groups at (A) 30 µg/ml phosphite and (B) 100 µg/ml phosphite as influenced by three different phosphate concentrations (1, 7 and 15 mM). The groups were identified using Ward's cluster analyses on a data set containing the growth inhibition data for all phosphite and phosphate concentrations of the 42 evaluated isolates. Mycelial growth inhibition was determined on phosphite and phosphate amended Ribeiro's medium after 10 days of growth. Percentage mycelial growth inhibition was calculated relative to growth of isolates on Ribeiro's medium lacking phosphite.

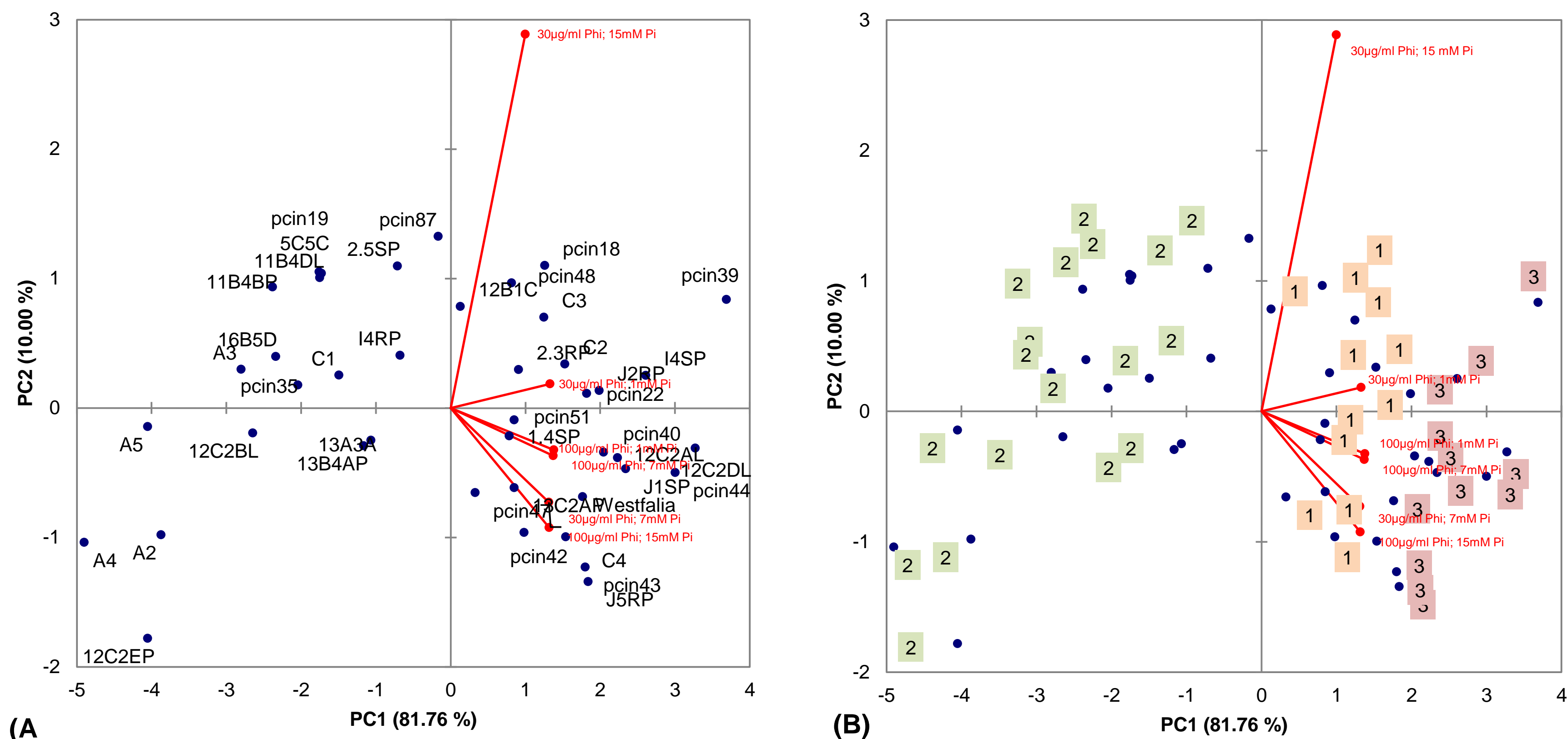


Fig. 3. Plot of the first and second principal components (PC1 and PC2) of 42 *Phytophthora cinnamomi* isolates according to (A) inhibition of their radial growth at different phosphite (Phi) and phosphate (Pi) concentrations and (B) the Ward's grouping of isolates where nr. 1 represents intermediate isolates, nr. 2 tolerant isolates and nr. 3 sensitive isolates. The percentage of variation accounted for by each principal component is indicated in brackets.

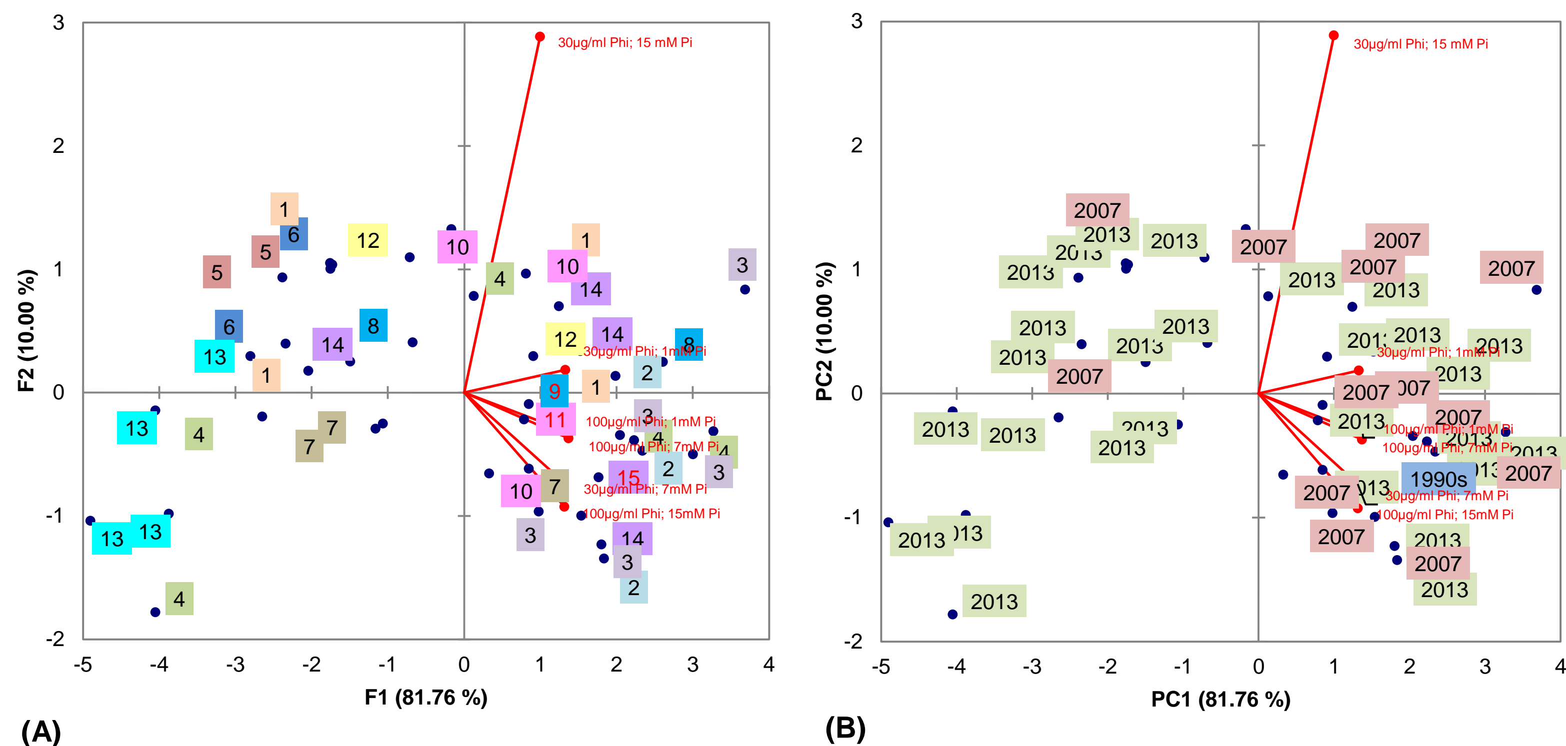


Fig. 4. Plot of the first and second principal components (PC1 and PC2) of 42 *Phytophthora cinnamomi* isolates according to their inhibition of radial growth at different phosphite (Phi) and phosphate (Pi) concentrations. (A) Numbers on the plot indicate the orchard number from which isolates were collected (nr. 1 to 15), whereas in (B) the year of isolation of each isolate is indicated. Isolates located on the negative part of PC1 were classified as phosphite tolerant and those on the positive part of PC1 as sensitive or intermediate isolates (see Fig. 4B). The percentage of variation accounted for by each principal component is indicated in brackets.

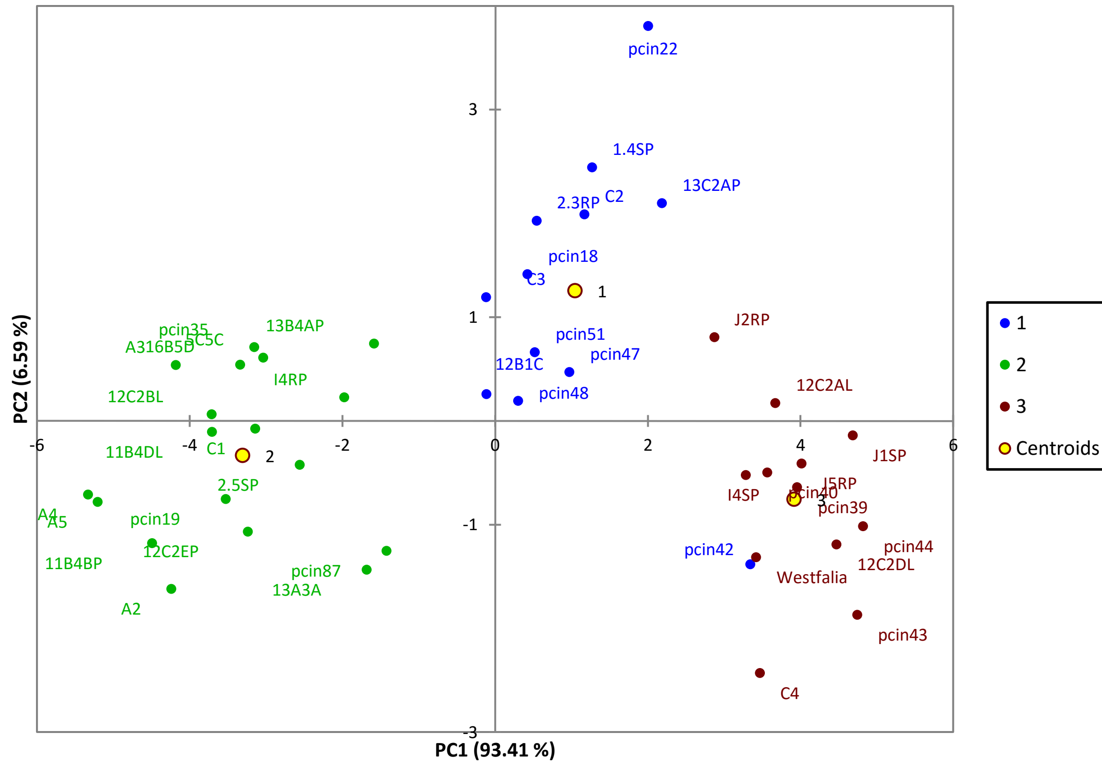
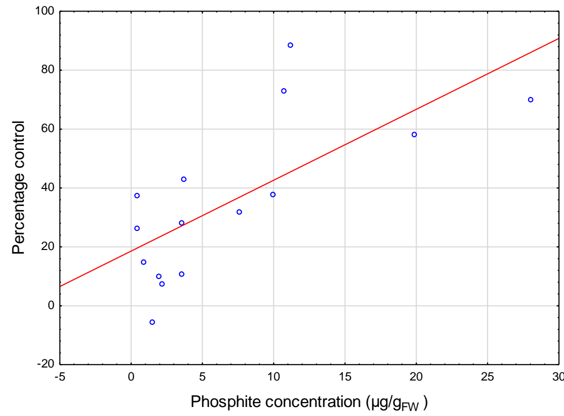
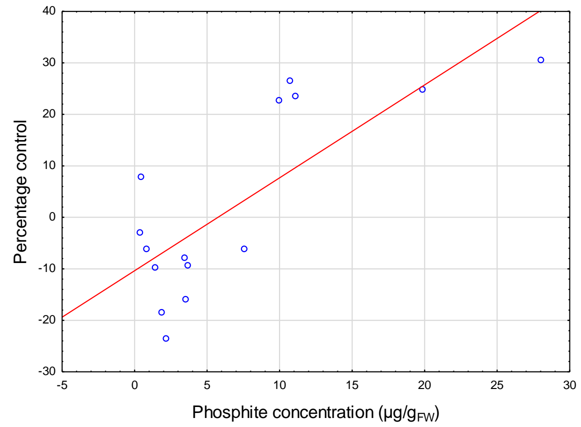


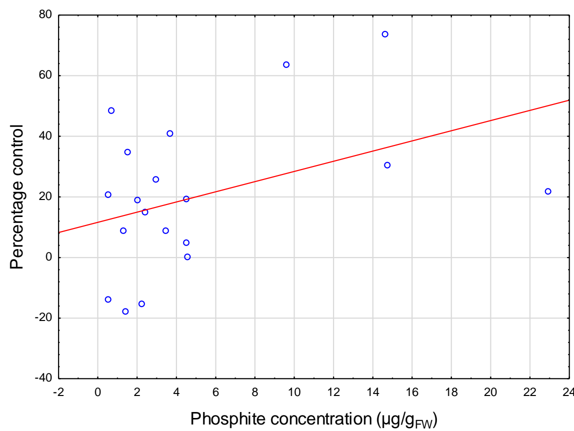
Fig. 5. Discriminant Analysis (DA) scores plot for the first two discriminant functions on the inhibition of radial growth by phosphite at different phosphate concentrations for the three sensitivity groups using 42 *Phytophthora cinnamomi* isolates as observation labels. Isolates labelled in green, blue and brown were identified as tolerant, intermediate and sensitive respectively by Ward's cluster analysis.



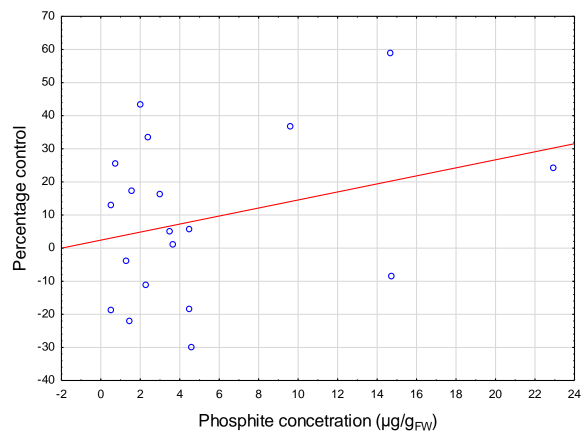
(A)



(B)



(C)



(D)

Fig. 6. Scatters graphs of Spearman's correlation between the association of percentage control and root phosphite concentration in two experiments (experiment 1: A and B); experiment 2: C and D), where avocado seedlings were drenched with a concentration range of phosphonates and excised roots were inoculated with *Phytophthora cinnamomi.* The percentage control of root length colonized was calculated for a *P. cinnamomi* isolate A5 (B and D) that was tolerant to phosphite *in vitro* and 12C2D (A and C) that was sensitive.