SPECTRUM OF IN-VITRO ACTIVITY AND EFFICACY OF PHOSPHONATES FOR MANAGEMENT OF APPLE REPLANT DISEASE AND OOMYCETE ROOT ROT PATHOGENS IN SOUTH AFRICA

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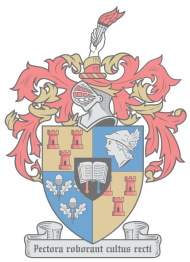

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DECLARATION

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SUMMARY

In South Africa, apple replant disease (ARD) and Phytophthora root rot are two soilborne diseases that are important in apple production. ARD occurs when old apple orchards are replanted, causing a reduction in tree growth. Several biotic agents are involved. In South Africa, a few *Pythium* spp. and *Phytophthora cactorum* were previously shown to be prominent in the development of ARD, with *Pratylenchus* spp. occasionally being involved. Phytophthora root rot, caused by *P. cactorum,* most often becomes problematic in the 2nd or 3rd year post-plant causing tree death and reduced tree growth. The pathogen is most likely introduced through nursery trees, irrigation water and residual soil populations (unfumigated inter-row strips). Management of ARD mainly consists of preplant fumigation of tree rows with chloropicrin/1,3-dichloropropene. Phytophthora root rot can be controlled using phosphonate fungicides, but these are not registered for apples in South Africa. Phosphonates, which breaks down to phosphite in plants, are highly mobile in plants and can reduce disease through a direct toxic effect towards pathogens or the induction of host plant defences.

Three orchard trials were conducted to determine whether ARD can be managed using semi-selective chemicals and different chloropicrin formulations. In all three trials, tree growth (trunk diameter and shoot growth) was improved significantly relative to the control by preplant fumigation with either of two formulations of chloropicrin/1,3-dichloropropene (formulations containing chloropicrin at 60.8% or 57.0%), or with a postplant semi-selective treatment programme that included applications of fenamiphos, phosphite, imidacloprid and metalaxyl. Yield increases did not always accompany the tree growth increases. In one orchard, yield was only increased significantly by combining semi-selectives with a fumigation treatment, whereas in the other two orchards all fumigation treatments significantly increased yield. *Phytophthora cactorum* and *Pratylenchus* spp. likely interacted synergistically and were important ARD pathogens.

In a second set of two orchard trials, the temporal nature of root phosphite concentrations in asymptomatic apple trees [trees where oomycete pathogens were present in roots, but no foliar symptoms were evident] was examined following different methods of application of phosphonates (foliar sprays, stem sprays, soil drenching and trunk paints) applied in summer and fall. A trunk paint application, was the best application method based on root phosphite concentrations. Foliar sprays, which were only applied in summer, also showed potential based on root phosphite concentrations. *Phytophthora cactorum* and *Pythium irregulare* DNA quantities in the roots of trees receiving phosphonate treatments were significantly lower than the quantities in the control treatment. *In vitro* studies showed

that medium type (liquid or solid) and phosphate concentration significantly influenced the percentage mycelial growth inhibition of *P. cactorum* and *P. irregulare* by phosphite. This made it problematic to assess the relative effect of root phosphite concentrations as a determinant of pathogen suppression in orchard tree roots.

A third set of trials were conducted, aimed at evaluating the curative efficacy of phosphonates in three apple orchards with Phytophthora root symptoms. Different phosphonate application methods (foliar sprays, trunk sprays and trunk paints), yielded similar levels of shoot growth in trees, which was significantly better than the control in two trials in the Grabouw region after 11-months, but not in the Koue Bokkeveld trial. Yield data could only be obtained in the latter trial, which was also not significantly increased by phosphonate applications. In the two Grabouw trials, all application methods yielded relative high root phosphite concentrations for fall phosphonate applications 13-weeks post-application, but not in the Koue Bokkeveld trial. In all three trials, *P. cactorum* root quantities were not reduced by any of the phosphonate treatments.

The study showed that phosphonates have potential for managing Phytophthora root rot in apple orchards. Phosphonates combined with other semi-selective chemicals (fenamiphos, imidacloprid and metalaxyl), can also be used to manage ARD. The relationship between phosphite concentrations required in tree roots for suppression of *P. cactorum* and *P. irregulare,* and phosphite concentrations required for pathogen suppression *in vitro* is unclear due to (i) various factors influencing the *in vitro* sensitivity of isolates and (ii) the seasonal fluctuation of root phosphite concentration in apple trees. Future work should focus on determining whether root phosphite concentrations are important for direct pathogen suppression by co-quantification of root phosphite and pathogens in time course studies in orchard trials. Furthermore, the effect of root phosphite concentrations on host plant defence induction must be investigated.

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OPSOMMING

In Suid-Afrika is appel herplantsiekte (AHS) en Phytophthora wortelvrot twee grondgedraagde siektes wat belangrik is in appelverbouing. AHS kom voor wanneer ou appelboorde herplant word, en veroorsaak ‘n vermindering in boomgroei. Verskeie biotiese agente is betrokke. In Suid-Afrika was ‘n paar *Pythium* spp. en *Phytophthora cactorum* voorheen aangewys as prominent in die ontwikkeling van AHS, met *Pratylenchus* spp. soms betrokke. Phytophthora wortelvrot, veroorsaak deur *P. cactorum,* word meestal problematies in die tweede en derde jaar ná plant, en veroorsaak boom-afsterwing en verminderde boomgroei. Die patogeen word heel waarskynlik deur kwekerybome, besproeiingswater en oorblywende grondpopulasies (nie-berookte tussen-ry stroke) ingebring. Bestuur van AHS bestaan hoofsaaklik uit vóór-plant beroking van boomrye met chloropikrien/1,3-dichloropropeen. Phytophthora wortelvrot kan beheer word deur die gebruik van fosfonaat fungisiedes, maar dit is nie vir appels in Suid-Afrika geregistreer nie. Fosfonate, wat in plante na fosfiet afbreek, is hoogs beweeglik in plante en kan siekte verminder deur ‘n direk toksiese effek teenoor die patogene, óf deur die induksie van gasheer plantverdedigings.

Drie boordproewe is uitgevoer om te bepaal of AHS bestuur kan word deur die gebruik van semi-selektiewe chemikalieë en verskillende chloropikrien formulasies. In al drie proewe is boomgroei (stamdeursnit en lootgroei) betekenisvol verbeter relatief tot die beheer deur vóór-plant beroking met enige van twee formulasies van chloropikrien/1,3-dichloropropeen (formulasies bevattende chloropikrien teen 60.8% of 57.0%), of met ‘n ná-plant semi-selektiewe behandelingsprogram wat toedienings van fenamifos, fosfiet, imidacloprid en metalaksil insluit. Opbrengs toenames het nie altyd met die boomgroei toenames saamgegaan nie. In een boord is opbrengs slegs betekenisvol verhoog wanneer semi-selektiewe chemikalieë met ‘n berokingsbehandeling gekombineer is, terwyl in die ander twee boorde alle berokingsbehandelings opbrengs betekenisvol verhoog het. *Phytophthora cactorum* en *Pratylenchus* spp. het moontlik sinergisties op mekaar gereageer en was belangrike AHS patogene.

In ‘n tweede stel van twee boordproewe, is die temporele aard van wortelfosfietkonsentrasies in asimptomatiese appelbome [bome waar oömiseet patogene in wortels teenwoordig was, maar geen blaarsimptome sigbaar was nie] ondersoek, volgende op verskillende metodes van toediening van fosfonate (blaarspuite, stamspuite, gronddrenkings en stamverwe) toegedien in die somer en herfs. ‘n Stamverf toediening was die beste toedieningsmetode gebaseer op wortelfosfietkonsentrasies. Blaarspuite, wat slegs in die somer toegedien is, het ook potensiaal getoon gebaseer op wortelfosfietkonsentrasies. *Phytophthora cactorum* en *Pythium irregulare* DNS hoeveelhede in die wortels van bome

wat fosfonaat behandelings ontvang het, was betekenisvol laer as die hoeveelhede in die kontrole behandeling. *In vitro* studies het getoon dat mediumtipe (vloeistof of vastestof) en fosfaatkonsentrasie betekenisvol die persentasie miseliumgroei inhibisie van *P. cactorum* en *P. irregulare* deur fosfiet beïnvloed. Dit maak dit moeilik om die relatiewe effek van wortelfosfietkonsentrasies as ‘n determinant van patogeen onderdrukking in boord boomwortels vas te stel.

‘n Derde stel proewe is uitgevoer met die doel om die kuratiewe effektiwiteit van fosfonate in drie appelboord met Phytophthora wortelsimptome te evalueer. Verskillende fosfonaat toedieningsmetodes (blaarspuite, stamspuite en stamverwe) het soortgelyke vlakke van lootgroei in bome opgelewer, wat betekenisvol beter was as die kontrole in twee proewe in die Grabouw area ná 11 maande, maar nie in die Koue Bokkeveld proef nie. Opbrengs data kon slegs in die laaste proef verkry word, wat ook nie betekenisvol deur fosfonaat toedienings verhoog is nie. In die twee Grabouw proewe, het alle toedieningsmetodes relatiewe hoë wortelfosfietkonsentrasies vir herfs fosfonaat toedienings 13 weke ná toediening opgelewer, maar nie in die Koue Bokkeveld proef nie. In al drie proewe is *P. cactorum* wortel hoeveelhede nie deur enige van die fosfonaat behandelings verminder nie.

Die studie het getoon dat fosfonate potensiaal het om Phytophthora wortelvrot in appelboorde te bestuur. Fosfonate, gekombineer met ander semi-selektiewe chemikalieë (fenamifos, imidacloprid en metalaksil), kan ook gebruik word om AHS te bestuur. Die verhouding tussen fosfietkonsentrasies nodig in boomwortels vir die onderdrukking van *P. cactorum* en *P. irregulare,* en fosfietkonsentrasies nodig vir patogeen onderdrukking *in vitro* is onduidelik weens (i) verskeie faktore wat die *in vitro* sensitiwiteit van isolate beïnvloed en (ii) die seisoenale fluktuasie van wortelfosfietkonsentrasie in appelbome. Toekomstige werk moet daarop fokus om vas te stel of wortelfosfietkonsentrasies belangrik is vir direkte patogeen onderdrukking deur ko-kwantifisering van wortelfosfiet en patogene in tydsverloopstudies in boordproewe. Verder moet die effek van wortelfosfietkonsentrasies op gasheerplant verdediging induksie ondersoek word

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CONTENTS

CHAPTER 1

Causes and intergrated management of apple replant disease.

INTRODUCTION

Apple (*Malus domestica* Borkh.) is a deciduous tree belonging to the Rose family, and is one of the oldest cultivated trees (Zohary and Hopf; 2000, Harris *et al*. 2002; Jackson 2003; Brown 2012). Its origins are still being debated, but the tree is thought to be native to the northern mountains and eastern Kazakhstan in China (Juniper, 1999). Cultivated apple (*Malus domestica* Borhk) is among the most widely grown orchard fruit crops on the globe (Janick *et al.,* 1996; Brown, 2012). Therefore, it has obtained the status of fourth most important fruit crop in the world after citrus, grapes and banana (FAOSTAT, 2008). Several commercial cultivation requirements have resulted in trees being modified in their growth behaviour (Juniper, 1999; Zohary and Hopf, 2000).

South Africa (SA) ranks sixteenth (FAOSTAT, 2008) amongst the world's apple producing nations. This accounts for 819 000 metric tonnes of apples per annum (WAPA Press release, 2013). At least 40% of these apples are exported to the northern hemisphere nations in spring and winter months. Commercial apple cultivation in SA consists of more than 21553 hectares (Hortgro Tree Census, 2010). The key apple producing areas are centered in the Western Cape (FIP, 2004) Groenland, (33° 52' 60" S, 18° 43' 60" E), Ceres (33° 22' 0" S, 19° 19' 0" E) and Villiersdorp (33° 59' 0" S, 19° 17' 0" E), which comprise 60% of the total production output of the country (Hortgro, 2012). The remaining production takes place in the Langkloof East (Eastern Cape), Northern Cape, Free State, Kwazulu Natal and Mpumalanga (Hortgro, 2012). Since apple production is export driven in South Africa, it is fundamental for the industry to grow premium quality varieties to maintain its apple production competitiveness.

For best yields and economic viability, orchard sites with optimum soil and environmental conditions must be selected for growing apples. This is important, since orchards must be economically viable for growers. Depending on several factors, apple tree durability within the orchard is estimated to be in the range of 12 to 25 years, even though apple trees can live for up to a century (Pereira-Lorenzo, 2009). The lifespan of commercial apple trees is hampered by a decrease in fruit quality and increasing disease pressure, possibly brought about by tree ageing (Pennell, 2006). Moreover, the economical life span of orchards is impacted by the need for new cultivars on the current market, together with improved rootstocks and changes in planting densities. Most of these market dynamics can only be satisfied when orchards are replanted with the demanded cultivars, which helps to achieve superior prices in the different market segments (Ndou, 2012).

It is thus crucial to replant and modernize apple orchards with highly viable apple varieties planted at optimally spaced high planting densities. At present, about 33% of the apple orchards are over 25 years old in South Africa (Hortgro Tree Census, 2012). Therefore, large hectares of orchards will require replanting in the near future, to secure market access in the longterm. However, replanting of apples is highly constrained by apple replant disease (ARD), a soilborne disease.

ARD has been reported to occur world-wide including South Africa (Van Schoor, 2009; Teweldhomedhin *et al.,* 2011b, c), North America (Jaffee, 1982a,b; Braun, 1991; Mazzola, 1998), the European Union (Hoestra, 1968; Savory, 1969; Manici 2013), China, New Zealand and Tasmania (Fullerston, 1999; Utkhede and Smirle, 2001; Wilson, 2004). The disease results in stunting of apple trees when apples are planted onto old orchard soils of closely related crops (Mazzola, 1998; Leinfelder and Merwin, 2006; St. Laurent *et al.,* 2008). This phenomenon has been in existence since as early as the 17th century (Shannon and Christ, 1954). In monetary terms, a study conducted by Scientific Horticulture Pty Ltd (Tasmania) estimated a loss of Australian $180,000/ha for the first 7-years, in ARD mismanaged orchards (Apal.org.au, 2018)**.**

Several studies have shown that ARD is caused by multiple biological agents (Merwin *et al.,* 1995; Mazzola 1998; Leinfelder and Merwin 2006; Van Schoor 2009; Tewoldemedhin *et al.,* 2011a,b,c). The biological agents primarily attack the apple root system. This results in smaller root systems (Mazzola and Brown, 2010), discoloured and decayed roots, root tip necrosis, and fewer fibrous and lateral roots (Mazzola, 1997). Root invasion by ARD pathogens hampers water and nutrient uptake assimilation. This translates into stunted growth, short internodes, rosetting foliage and a delay in fruit bearing. The delay in fruit production associated with ARD, results in 5- to 7-years of non-bearing time after planting, compared to the 3-year non-bearing time for healthy orchards (Merwin *et al.,* 1995; Mazzola, 2002; Wilson *et al.,* 2004., Leinfelder and Merwin, 2006).

The biological agents involved in ARD have been characterized in a few studies world-wide, and consist of a wide spectrum of organisms including fungi, nematodes and oomycetes. *Pratylenchus penetrans* is known to be involved in ARD world-wide, although in South Africa species other than *P. penetrans* most often occur (Tewoldemedhin *et al.,* 2011c). The fungal causative agents include multi- and binucleate *Rhizoctonia* spp., with the multinucleate *R. solani* AG-5 and AG-6 being highly virulent oomycetes (Mazzola, 1997; Manici *et al*., 2003). However, in South Africa, multinucleate *R. solani* AG-5 and AG-6 have not been identified, only a few bi-nucleate *Rhizoctonia* species (Tewoldemedhin, 2011b). Another group of fungi involved in ARD is the *Cylindrocarpon*-like spp. fungi, for which isolates within species are known to range from non-pathogenic to mildy virulent (Strzelczyk

and Pokojska-Burdziej,1982). The specific species involved are currently not well defined due to major taxonomic changes in this group of fungi. The '*Cylindrocarpon*-like spp.' known to be involved in ARD have been transferred to the genera *Neonectria, Thelonectria, Ilyonectria* and *Dactylonectria*. Several species have furthermore been split into multiple species (Chaverri *et al*., 2011; Jaffee *et al*., 1982b; Braun, 1991; 1995; Dullahide *et al*., 1994; Mazzola, 1998; Manici *et al*., 2003; Tewoldemedhin *et al*., 2011c; Cabral *et al.*, 2012; Lombard *et al*., 2014). In South Africa, oomycetes seem to play a major role in ARD based on their wide-spread occurrence in orchards and high virulence. The oomycetes involved include *Phytophthora cactorum, Phytopythium vexans* and several *Pythium* spp. (*P. irregulare* and *P. sylvaticum*) (Tewoldemedhin *et al.*, 2011b,c). These pathogens have also been reported in the USA (Mazzola *et al*., 2002).

Apart from ARD, another soilborne disease that is problematic in South African apple production is *Phytophthora* root rot. Symptoms of tree death and stunted growth usually become evident one year after planting, even when soils have been fumigated pre-plant. In South Africa, *Phytophthora* root rot is especially problematic on young non-bearing apple trees. The most likely source of inoculum is planting material, since apple nursery trees are produced in open fields where rootstock are cut from mother layer blocks. Contamination with *Phytophthora* spp. may easily occur and spread within nurseries. Alternatively, irrigation water may contribute to the introduction of the pathogen into fumigated soil, since *Phytophthora* spp. are known to be spread through irrigation water (Pettit *et al*., 1998, Werres *et al*., 2007). *Phytophthora cactorum* is the main cause of *Phytophthora* collar-, root - and crown rot on apple, although a few other species have also been identified as root rot pathogens (Mazzola *et al*., 2002).

In South Africa, crown rot symptoms are seldom seen on young trees (personal communication J.P.B. Wessels, ProCrop, Wellington, South Africa). Phosphonates are used extensively on several crops for the management of *Phytophthora* diseases, especially for the management of crown- and root rot diseases (Jackson *et al*., 2000). This is due to the mobile nature of phosphite, the breakdown product of phosphonates in plants, which can be translocated basipetal and acropetal to plant tissues that are affected by the pathogen. Phosphonates can be applied using various application methods including stem injection, stem sprays, trunk paints, foliar sprays and soil drenches (Hardy *et al*., 2001). Phosphite is known to translocate to plant tissues in a source sink manner (Whiley *et al*., 1995). The mode of action of phosphite in suppressing pathogens can be due to direct toxic effect or indirect through the induction of host plant defence responses. However, there is still a lot of controversy regarding the relative importance of the direct versus indirect mode of action of phosphite (Fenn 1984; Grant and Guest, 1991; Massoud et al., 2012).

Aspects that will be covered in this literature review are first of all the etiology of ARD and the management of ARD. The use of phosphonates for the management of soilborne oomycete pathogens is furthermore discussed including phosphonate translocation, different methods of application and the mode of action of phosphonates. The literature review also elaborates on the use of phenylamides for managing oomycete pathogens and how phenylamides are translocated, problems with resistance and its mode of action. Although information is limited, the specific use of phosphonates, phenylamides and fenamiphos for the management of ARD and Phytophthora root rot on apple are also discussed. The literature review ends with information regarding fenamiphos as a nematicide.

ROOT GROWTH IN APPLE TREES

Currently, most commercial apple trees consist of a combination of a scion and a rootstock (Jackson 2003; Webster 2005). Rootstocks hold several benefits for apple tree productivity, including the control of vegetative growth, promotion of flower-bud formation, cropping efficiency and quality of the fruits. In some cases, the rootstock can also provide disease resistance and winter hardiness (Hanke *et al*., 2007; Wertheim and Webster 2005). Roots are also involved in anchorage, water uptake, and the perception and integration of environmental signals (Brenner, 2006). Therefore, the presence of a poor root system, due to biotic and abiotic stresses, will hamper apple productivity.

The developmental events of apple trees are interrelated and synchronized, and influenced by the translocation of assimilates. Stages of development include budburst, flower-bud formation, flowering, extension growth, fruit set and development, extension growth cessation, leaf abscission, root growth and winter dormancy (Morrow, 1950, Lyr and Hoffman, 1967; Reich *et al.,* 1980). These growth events occur in episodes (Lyr and Hoffman 1967; Reich *et al.,* 1980). Assimilate partitioning occur in a sink source relation, i.e. carbohydrates are translocated to areas of active growth such as the roots.

Apple root growth studies are currently a subject of debate. Particularly root growth timing, since this has been shown to vary due to various factors. These factors include rootstock genotype, age, planting density, edaphic factors, and management and cultural practices (Shengrui, 2006). Some of the controversies regarding root growth is due to the fact that the available root detection methods have some limitations. Examples of indirect root growth evaluation methods include carbon and nitrogen budget or carbon isotope methods (Gill and Jackson, 2000; Madji *et al.,* 2005). These methods can result in fewer root dynamics and demography being evident. Direct root detection methods such as soil coring address problems associated with indirect methods, but the methods have a lack of sensitivity for the detection of fine root death and decomposition. This can result in an under

or over estimation of fine root production (Kurz and Kimmins, 1987; Publicover and Vogt, 1993). More over, soil coring is a destructive method that poses difficulties in longterm research. A direct root monitoring method that has shown promise, is the use of minirhizotron tubes. It has fewer limitations compared to the other methods including for example that it is a non-destructive method of root detection (Taylor, 1987; Madji, 1996; Johnson *et al.,* 2001). The method involves mounting a transparent tube, usually 1 m long and 12 cm wide into the soil surrounding the root system. The white root growth is then detected using a digital camera and quantified using image analyses (Patena and Ingram, 2000).

EFFECTS OF CONTINUOUS MONOCULTURE ON SOIL MICROBIAL POPULATIONS

Crop monoculture is a practice of replanting identical crops on the same land, with no interruption with another different crop species. The term can also be used to describe large areas under cultivation of one species (Cook and Weller, 2004). Considering financial benefits, apple monocropping forms a profitable practice, since the advantage under monoculture is that an ideal crop can be cultivated under conditions known to be suitable and productive for its growth (Bronk and Jacoby, 2013). Above all, due to a scarcity of suitable land, monoculture is becoming the method of choice in several annual and perennial crops (Zhu *et al.,* 2014), including apple.

For apples, the impaired growth of trees planted in a successive generation of monoculture has been given several names. These include replant disease, soil sickness, replant problem and replant disorder. In apple, the poor growth during orchard establishment on replanted soil, is known as apple replant disease (ARD) when growth reductions are caused by biological factors (Sewell, 1981). Abiotic factors have been reported to contribute to the severity of ARD, including for example of soil fertility (Dullahide, 1994).

The negative effect of monoculture, from a biological point of view, is due to the fact that planting one crop year after year, changes soil biological stability (Szajdak, 2003) through the modification of soil species composition (Barabasz, 1998). It often happens that beneficial microbes are reduced, with a concurrent build-up of pathogenic and parasitic soil pathogens. In replant sites, parasitic species such as *Pythium* spp., *Rhizoctonia* spp., '*Cylindrocarpon'*-like spp. and *Fusarium* spp. have been observed to increase in populations (Mazolla and Manici, 2012). Although the mechanism is not well understood, monoculture of apple results in the composition of microbial species in the rhizhosphere becoming dominated by parasitic and pathogenic spp. The build up of these pathogens has been attributed to several factors that include a steady supply of food such as root exudates containing chemical substances including carbohydrates, carboxylic and amino acids

(Graystone *et al*., 1998). Recently, it has been shown that apple rootstock rhizodeposits are genotype specific, and that it can also affect soil chemistry, by lowering the pH, which could impact microbial communities. Although, the rhizodeposit quantities correlated with the quantities of culturable rhizosphere bacteria, it did not correlate with the ARD tolerance of the rootstocks (Leisso *et al*., 2017).

ETIOLOGY OF APPLE REPLANT DISEASE

Biological agents have been cited by several workers, as the causal agents of ARD using isolation and pathogenicity studies (Mazzola, 1998; Browne *et al.,* 2006; Tewoldemedhin *et al.,* 2011a, b, c). Fungi, oomycetes and several nematodes have mostly been implicated in these studies. However, these pathogens can vary in composition and incidence from location to location. This could be due to soil type and cropping practices (Hoestra, 1968; Mazzola 1998). The specific agents involved have been isolated from roots using selective and semi-selective growth mediums, or colony growth counts from soil. Alternatively qPCR can be used to confirm the presence of known ARD pathogens (Yao *et al.,* 2006; Van Schoor *et al* 2009; Tewoldemedhin *et al.,* 2011a,b,c). The known ARD pathogens include species of *Pratylenchus, Phytophthora, Pythium, Phytopythium, Rhizoctonia* and '*Cylindrocarpon*'-like fungi (Dullahide *et al.*, 1994; Mazzola, 1998; Manici, 2001, Tewoldemedhin *et al.,* 2011a,b,c). The involvement of other parasites and pathogens, however, can not be excluded. Therefore, *Pratylenchus, Phytophthora, Pythium, Phytopythium, Rhizoctonia* and '*Cylindrocarpon*'-like fungi are referred to as ARD marker pathogens. The ARD marker genera will all be discussed in more detail in the following sections.

The biological nature of ARD has been confirmed using several approaches (Mazzola and Manici, 2012). Chemical fumigants including methyl bromide and chloropicrin mixtures have been shown to increase tree growth in replant orchards (Mai and Abawi, 1981; Slykhuis and Li, 1985; Browne *et al.,* 2006). The application of soil pasteurisation under glasshouse conditions, and the improvement of apple seedling growth in pasteurized versus non-pastuerized soils, has also implicated biological agents as causing ARD (Hoestra, 1968; Jaffee *et al*., 1982a; Utkhede and Thomas, 1988; Yim *et al.,* 2013). Another approach that has been used for proofing the involvement of biological agents is the amendment of pasteurized soil with 10% (v/v) of the original untreated soil (Hoestra, 1968; Jaffee *et al*., 1982a). These glasshouse studies showed that overall seedling growth performance (shoot height, root dry and -fresh weight, shoot dry and –fresh) was reduced in

the amended soil versus unamended soil (Jaffee, *et al.,* 1982a; Utkhede and Smith, 1991, Tewoldemedhin, *et al.,* 2011a, b).

***Pratylenchus* spp.**

Pratylenchus spp. are migratory endoparasitic worm-like organisms, that are translucent and small, about 0.3 to 0.9 millimeters long (Siddiqi, 2000). The genus *Pratylenchus* contains about 68 species that parasitize and harm numerous plants (Duncan and Moens, 2006; Castillo and Vovlas, 2007). The genus has been associated with diseases in many crops including corn, potato (Dickerson, 1964) and turf grasses (Troll and Rohde, 1966). Species of *Pratylenchus* have a cosmopolitan distribution and is common in monoculture soils of low fertility. Light textured soils, low in nitrogen, phosphorous, calcium and organic matter are known to favour *Pratylenchus* spp*.,* often resulting in high population levels (Florini *et al.,* 1987). These parasitic nematodes are also considered to be the initial organisms in habitat occupation, after disturbance (Palomares *et al*., 2010).

Pratylenchus spp. parasitize plant roots, causing brown and extended lesions, root pruning and root size reductions (Bao and Neher, 2011). Their feeding, and the consequent lesion development, are mainly due to host-plant glycoside interactions as well as enzymes released during feeding (Haegeman *et al*, 2012). The level of host-parasite interaction is determined by the concentration and location of specific glycosides in the root tissue, as well as the concentration of enzymes released during feeding. Nematodes can also promote fungal root diseases since they are able to disseminate fungal spores during their migration (Kurppa and Vrain, 1989; Koenning *et al*., 1999).

Plant spp. differ in their tolerance to lesion nematodes, and the threshold levels that are required for plant damage is likely influenced by various factors. In certain plants, low populations are generally considered to be harmful, while in others higher populations are required for plant growth reductions to occur (Melakeberhan *et al*., 1997). Thresholds associated with nematode damage, can be influenced by soil factors. Soil factors may modify root tissue plasticity and alter the ability of nematodes to penetrate roots (Inserra *et al*., 1979).

Epidemiology and life cycle of *Pratylenchus* spp.

The *Pratylenchus* spp*.* life cycle begins with the laying of eggs by the females. Even in the absence of males, females continue to produce eggs through the process of parthenogenesis. Parthenogenesis is when eggs are laid by females in the absence of males (Duncan and Moens, 2006), this process is mostly dependent on temperature. Temperatures of 23°C allows adult females to lay 1 to 2 eggs a day, which yield a total of 16

to 35 eggs within a generation (Castillo and Vovlas, 2007). Adult females deposit their eggs into the cortical root tissue cells, proximate to the root surface, or even outside infected plants, alongside the length of the root (Zunke, 1990). The first molt and second stage larva develops in the egg and undergoes three more molts before morphing into an adult (Davis and MacGuidwin, 2005). All *Pratylenchus* spp life stages that are formed outside the root are generally infective (Davis and MacGuidwin, 2005). Lesion nematodes overwinter as eggs, larvae as well as adults inside root tissue or soil (Duncan and Moens, 2006; Castillo and Vovlas, 2007).

Depending on the presence of ideal soil temperatures, *Pratylenchus* spp. can complete their life cycle within 3- to 7 weeks (Duncan and Moens, 2006; Castillo and Vovlas, 2007). Temperature has been reported to affect the life cycle of *Pratylenchus* spp. The optimum temperature for completing their life cycle is usually 25°C (Duncan and Moens, 2006; Castillo and Vovlas, 2007). For the lesion nematodes to attack plant roots, they are naturally drawn to the region of root hair production and root tips (Pudasaini *et al*., 2008). The greatest root penetration ensues in the region behind the root elongation region. Lesion nematodes briefly feed on the root surface ectoparasitically, prior to entering the root system (Putten and Stoel, 1998). The nematode then forces its way through root epidermal or between root epidermal cells (Pudasaini *et al*., 2008). Nematode ingress is usually aided by their feeding structure, a stylet, and through cell wall enzymes secreted in oesophageal glands such as cellulases and pectinases (Castillo and Vovlas, 2007). Inside the root, the nematode feeds on cortical cells creating cavities while the tissue will be destroyed. This leads to a loss in cell turgor pressure by shrinking of the feeding cell tonoplast and gradual increase in the size of the nucleus. Subsequently, root cell death occurs on the nematode's route, where cells exhibit nuclear hypertrophy, vacuole formation, condensed cytoplasm and deteriorated organelles (Castillo and Vovlas, 2007). When roots become extremely necrotic, it will be unfavourable for feeding and reproduction. Therefore, the nematode will subsequently migrate through the cortex to healthier areas of the root or even through the soil (Duncan and Moens, 2006; Castillo and Vovlas, 2007). If nematode damage is exceedingly high, plants lose their leaves and infected plants die due to a lack of a healthy root system to nourish the plant (Pudasaini *et al*., 2008). The lesion nematode is generally spread by motile larvae and adults that migrate between infested and uninfested areas of the roots via surface drainage, irrigation water and tillage implements (Reddy, 2016).

Role of *Pratylenchus* spp. in apple replant disease (ARD)

Only a few studies have investigated the role of *Pratylenchus* spp in ARD. *Pratylenchus* spp have been found to have a role in ARD in a few countries such as the United States America

(Jaffee *et al*.,1982a, Mazzola *et al*., 2015), in British Columbia (Canada) (Utkhede *et al*, 1992) and in Australia (Dullahide 1994). However, the findings until now have been unclear as to how important the lesion nematode is in inciting ARD. For example, Manici *et al.* (2013) concluded that *Pratylenchus* spp. were not important in ARD in Eastern Europe (Germany, Austria and Italy), since there were no correlations between the frequency of *Pratylenchus* and plant growth in ARD soils. Furthemore, *Pratylenchus* numbers were overall higher in uncultivated soil (driving lanes or uncultivated strips at the edge of the orchards) than in ARD soil taken from the tree rows (Manici *et al*., 2013). Although Utkhede *et al.* (1992) reported that *Pratylenchus* spp*.* were associated with the ARD complex in apple orchards, the study could not show a correlation between lesion nematode populations size and ARD severity.

In South Africa, Tewoldemedhin *et al.* (2011a) investigated the role of *Pratylenchus* spp*.* in ARD soils under glasshouse conditions through the amendment of ARD soil with fenamiphos. The effect of fenamiphos was variable in soils that were classified as having low or severe ARD. *Pratylenchus* spp. were present in only three of the six investigated soils, with fenamiphos application resulting in an increase in seedling growth in two of the soils (severe and low ARD severity soils). Yet, the nematicide did not improve seedling growth in the soil with the highest *Pratylenchus* spp. numbers. This suggests that *Pratylenchus* spp. populations can be high or low in ARD soils and that their resulting damage depends on their synergistic interactions with other ARD pathogen/s (Tewoldemedhin *et al*., 2011a). Parasitic nematodes were thus regarded as a relatively weak component of ARD, but that it can certainly function synergistically together with various other pathogens including oomycetes and fungi (Tewoldemedhin *et al.,* 2011a). World-wide it has also been reported that the role of *Pratylenchus* spp is site-specific in ARD (Mazzola and Manici, 2012), with their effect depending upon other microorganisms. The *Pratylenchus* spp. associated with ARD vary in different countries. In Washington State, USA, only *Pratylenchus penetrans* has been identified (Mazzola *et al*., 2009). In South Africa, *P. penetrans* only occasionally occurs, along with several other species including *P. delattrei* and *P. scribneri.* In the Greenbelt orchards of Queensland, *Pratylenchus jordanensis* was demonstrated to incite ARD, since it effectively and consistently multiplied on apple trees. *Pratylenchus jordanensis* lowered root and shoot weights by 25 to 35% (Dullahide *et al*., 1994). The decrease in shoot and root weights were directly proportional to an increase in nematode inoculum density. Contrary to this report, was a study by Colbran (1979), who concluded that a *Pratylenchus* spp. now regarded as *P. jordanensis* was not pathogenic to apples. The lack of infectivity of *P. jordanensis* in the studies by Colbran (1979) was likely related to differences in environmental conditions (higher temperatures) (Dullahide, 1994).

Although the economic threshold for damage caused by *Pratylenchus* spp. on apple have not been investigated specifically in ARD soils, some information is available for general apple production. *Pratylenchus* spp numbers of $104g^{-1}$ soil were determined by Barker and Olthof (1976) as being sufficient to incite host injury. *Pratylenchus* spp have been observed to oscillate from season to season on apple (Zimmerman and Miller, 1991).

Rhizoctonia solani Kuhn and bin-nucleate _Rhizoctonia_ spp.

The genus *Rhizoctonia* is an anamorphic genus that contains a large range of fungi belonging to the family Ceratobasidiaceae (Cantharellales, Basidiomycota). *Rhizoctonia* spp. are characterized by the absence of conidia, broad main runner hypha, which initially is colourless, but later turn to buff coloured to dark brown. The hyphae consists of multinucleate cells or binucleate cells, with dolipore septa. Irregular sclerotia can be produced, which can be light to dark brown and typically lacks differentiation (Ceresini, 1999; Sharon, 2006). The genus *Rhizoctonia* includes fungi with a diverse role in nature ranging from saprotrophs, orchid mycorrhiza- and ectomycorrhizal symbionts, non-mycorrhizal endophytes and plant pathogens. The soilborne pathogens can have a wide host range (Cubeta and Vilgalys, 1997)

The taxonomy of *Rhizotonia* fungi is still not well resolved due to their diverse ecological niches and the lack of fruiting bodies in most of the groups (Veldre *et al.*, 2013). The sexual genera that are associated with *Rhizoctonia* include the genera *Thanatephorus, Waitea* and *Ceratobasidium* (Sharon *et al.,* 2008; Veldre *et al.*, 2013; Gonzalez *et al*., 2006).

Plant pathologists first introduced the concepts of differentiating *Rhizoctonia* isolates based on (i) the number of nuclei per cell and (ii) anastomosis compatibility (Parmeter, 1969). The latter refers to the ability of the hyphae of two isolates to recognize each other and fuse when co-cultured, also known as anastomosis, and it indicates some degree of relatedness (Cubeta and Vilgalys, 1997; Veldre *et al.*, 2013; Gonzalez *et al*., 2006). Based on number of nuclei per cell, uninucleate, multinucleate and bi-nucleate groups can be differentiated (Sharon *et al*., 2008). Based on the anastomosis groupings (AGs), multinucleate *Rhizoctonia solani* isolates are divided into 13 AGs (AG-1 to AG-13) and binucleate *Rhizoctonia* spp. into 16 AGs (AG-A to AG-I, AG-K, AG-L and AG-O to AG-S). In some of the AGs a few subgroups (AG-1, -2, -3, -4, -6, -8, and -9) are further differentiated using various criteria such as anastomosis compatibility, morphology and pathogenicity (Carling, 2011; Sharon *et al*., 2008). *Thanatephorus cucumeris* (Frank) Donk is the sexual stage of the multinucleate anamorphic species *Rhizoctonia solani.* The sexual stage of the bi-nucleates *Rhizoctonia* spp. has not been resolved for all bi-nucleates, but most belong to *Ceratobasidium* spp. However, a few bi-nucleate species have a *Thanatephorus* teleomorph

based on molecular data including AG-E, AG-F, AG-P, AG-U, AG-R and AG-S. It is thus confusing that the latter AGs are often still referred to as *Ceratobasidium* spp. (Sharon *et al*., 2008, Veldre *et al.*, 2013; Gonzalez *et al*., 2016). It is known that *Ceratobasidium cornigerum* is the sexual stage of AG-B(o), AG-D, AG-P and AG-Q, and *Ceratobasidium setariae* that of AG-Ba and AG-Bb (Sharon *et al*., 2008; Verder *et al*., 2013).

The inoculum sources of all *Rhizoctonia* spp. include natural soil, weeds, rotational crops, plant debris and contaminated seeds (Parmeter, 1970). The infection potential and persistence of *Rhizoctonia* inoculum relies on soil factors such as soil temperature, moisture, pH and competitive activity with related organisms (Jones *et al*., 1997). *Rhizoctonia* fungi can be active from 24 to 32˚C (Jones *et al*., 1997). *Rhizoctonia* sclerotia may persist for prolonged periods of time within the soil or even plant tissue and -debris (Ceresini, 1999). This is due to the fact that *Rhizoctonia* can survive on organic matter as saprophytes (Olsen and Young, 1998). Under favourable conditions, sclerotia germinate and form mycelia that will attack the host plant (Ceresini, 1999).

Due to the labour intensive nature of identifying *Rhizoctonia* isolates through conventional anastomosis testing, molecular methods have been implemented for identifying AGs based on sequence data of the internal transcribed spacer (ITS) regions (Sharon *et al.*, 2008). Veldre *et al.* (2013) were one of the first to use the ITS region to differentiate, *R. solani, Rhizoctonia oryzae* and *Rhizoctonia oryzae-sativae.* Subsequently, it was further shown that the ITS regions can be used to identify different anastomosis groups (Sharon *et al*., 2008). The taxonomic relevance of anastomosis groups was also shown through the use of multi-locus phylogenies, which showed that the monophyletic groups identified corresponded to anastomosis groups (Gonzalez *et al*., 2016). Gonzalez *et al*. (2016) also showed that *Thanatephorus* and *Ceratobasidium* are not well supported monophyletic groups in the Ceratobasidiaceae, but that they are paraphyletic.

Role of *Rhizoctonia* solani and bi-nucleate *Rhizoctonia* spp. in ARD

Only a few studies have provided evidence that *Rhizoctonia solani* is associated with ARD and that it is important in ARD (Mazzola, 1998; Mazzola and Gu, 2002) *Rhizoctonia solani* AG-5 has been associated with ARD in Washington state, and AG-6 in Washington state and South Tyrol in Italy (Mazzola, 1997; Manici *et al*., 2003). Subsequent studies in Italy, Germany and Austria did not identify any *R. solani* isolates as being associated with ARD soils (Manici *et al.,* 2013). Similarly, in South Africa, *R. solani* has also not been associated with ARD (Tewoldemedhin *et al.*, 2011b). Pathogenicity studies have confirmed the involvement of *R. solani* AG-6 (Manici *et al.*, 2003; Mazzola, 1997) and AG-5 (Mazzola, 1997) in ARD. These AGs were observed to range in virulence and can be highly virulent

(Mazzola, 1997; Manici *et al.*, 2003). Apple trees of up to 20-weeks-old were shown to be susceptible to AG-5 and AG-6 (Mazzola, 1997).

The role of binucleate *Rhizoctonia* spp. have been investigated in several ARD studies, but has not been clearly elucidated. However, in general it seems as if these species only play a minor role in ARD having low virulence, and sometimes also being associated with an increase in plant growth in apple seedling assays. Manici *et al*. (2013) concluded, based on the incidence of isolating binucleate *Rhizoctonia* spp. (mostly AG-A, AG-G and AG-P) in apple seedling bioassays planted in untreated ARD soil and gamma-irradiated ARD soils, that binucleates were not associated with a reduction in seedling growth in Central Europe (Austria, Germany and Italy). The poor correlation between binucleates AG-A, AG-G, AG-P and apple seedling suggested a minimal role and low virulence of these binucleates in ARD (Manici *et al*., 2003, 2013).

The bi-nucleate *Rhizoctonia* spp. AGs that have been most widely associated with ARD include AG-A and AG-G that have been identified in central Europe (Austria, Germany and Italy), South Africa and Washington State in the USA (Mazzola, 1979; Tewoldemedhin *et al*., 2011b; Manici *et al*., 2013). A few other binucleate AGs have also been reported. AG-I has been identified in South Africa and Washington state (Mazzola, 1997; Tewoldemedhin *et al*., 2011b), and AG-J and AG-Q in only in Washington state (Mazzola, 1997). In South Africa, other bi-nucleate AGs associated with ARD were AG-F, AG-K, AG-L and AG-R. AG-A was the most widely distributed in South Africa, occurring in five of the six investigated orchards, followed by AG-I and AG-L that occurred in four of the orchards (Tewoldemedhin *et al*., 2011b).

Only two studies have investigated the pathogenicity of binucleate *Rhizoctonia* spp. by conducting pathogenicity studies (Mazzola, 1997; Tewoldemedhin *et al*., 2011b). Tewoldemedhin *et al*. (2011b) reported that only one of each of the two evaluated isolates of AG-I and AG-F had low virulence towards apple seedlings. AG-A, AG-G, AG-K, AG-L and AG-R were non-pathogenic (Tewoldemedhin *et al.,* 2011a, b). Mazzola (1997) found that only AG-G, -Q, and –I had low virulence toward apple seedlings, whereas AG-A and AG-J were non-pathogenic. Furthermore, not all isolates of AG-G, -Q and –I were pathogenic. In contrast to the pathogen isolates, two binuelceate isolates of unknown AG were even found to significantly enhance seedling growth (Mazzola, 1997).

***'Cylindrocarpon '*- like fungi**

Initially, taxonomic identification of the genus *Cylindrocarpon* was through the use of traditional morphological characters such as colony pigmentation, mycelial growth rate, chlamydospores production and microconidial shape and size (Booth, 1966; Halleen *et al*.,

2006). In this first grouping of *Cylindrocarpon* spp., Booth (1966) first classified *Cylindrocarpon* into four groups. He further segregated the four groups into anamorphs and teleomorphs. Although morphological characters have been used extensively in these studies for *Cylindrocarpon* identification, it was unsatisfactory for ascertaining species differences (Taylor, 2000). Therefore, with the advent of molecular techniques, the use of sequence data of the mitochondrial small subunit (mtSSU) rDNA region and beta-tubulin were first pursued for the identification of *Cylindrocarpon* spp. (Mantiri *et al*., 2001, Alaniz *et al*., 2007). Mitochondrial small subunit (mtSSU) rDNA sequences were used to separate *Cylindrocarpon* spp. into three clades (Mantiri *et al*., 2001). These clades correlated well with the groups of Booth (1966), but it indicated that the fourth group should be eliminated from the genus *Cylindrocarpon,* which was subsequently named as the genus *Campylocarpon* (Halleen *et al*., 2004, 2006). In addition to the mtSSU, many researchers have proposed the application of the internal transcribed spacer (ITS)- and β tubulin regions for *Cylindrocarpon* spp. identification and phylogenetic associations amongst species that are closely related (Halleen *et al*., 2004, 2006; Schroers *et al*., 2008).

The most recent taxonomic changes based on multigene phylogenies has resulted in the introduction of four new genera that have replaced the genus *Cylindrocarpon.* The four genera include *Campylocarpon, Thelonectria, Ilyonectria* and *Rugeronectria* (Halleen *et al*., 2004, Chaverri *et al*., 2011). Subsequently, Lombard *et al*., (2014) reported that *Ilyonectria* is represented by more than one genus. *Dactylonectria* was reported to contain 10 new genera (Lombard *et al*., 2014). Due to the many taxonomic changes occurring in this group of fungi, in the subsequent section this group of fungi will only be referred to as '*Cylindrocarpon'*-like spp.

'Cylindrocarpon'-like spp. include soilborne fungi that have a worldwide distribution (Anon, 1998; Halleen *et al.,* 2006; Alaniz *et al*., 2011). These fungi are pathogenic to a diverse range of hosts and an extensive host range has been reported (Kernaghan *et al.,* 2007). The hosts often include herbaceous and woody plants (Brayford and Samuels, 1993), including forest trees and agricultural hosts such as grapevines, apple, ginseng and conifer seedlings (Brayford and Samuels, 1993). '*Cylindrocarpon'*-like spp. are often considered as weakly virulent pathogens and often function as saprobes on organic matter and the bark of newly killed woody plants (Chaverri *et al*., 2011). As pathogens, '*Cylindrocarpon'*-like species such as *C. destructans* (currently placed in the genus *Ilyonectria* and thought to be comprised of more than 12 different species) has been found to attack immature, wounded as well as senescent roots (Garrett, 1956). '*Cylindrocarpon'*-like spp frequently attack tap and lateral roots (Barbetti, 2005). The pathogenicity of several '*Cylindrocarpon'*-like spp. can be due to the production of phytotoxins (Andolfi *et al*., 2011). These phytotoxins hinder

mitosis in root tips (and incite stunted growth of seedlings (Evans *et al*., 1967; Sweetingham, 1983). On some hosts, damping-off symptoms occur that is most likely due to the production of pectic enzymes (Sweetingham, 1983). Attack of host plants by '*Cylindrocarpon'*-like spp. also results in below and above ground symptoms. Like any other root pathogen, the above ground symptoms occur after the below ground symptoms are at an advanced stage (Reeleder and Brammall, 1994).

The success of '*Cylindrocarpon'*-like spp. as pathogens are promoted by their rapid spore production (conidiospores and chlamydospores) mycelial growth, excellent competitive ability and their capacity to utilize organic as well as inorganic nitrogen. These characteristics allow '*Cylindrocarpon'*-like spp. to act as pioneers in attacking young root tips (Booth, 1966; Kowalski, 1982, Marek *et al*., 2013). The survival structures of '*Cylindrocarpon'*-like spp. can include chlamydospores or ascospores. Chlamydospores generally form within macroconidia in the plant tissue following nutrient depletion (Marek *et al*., 2013). In the soil, carbohydrate exudates from host roots can induce chlamydospores germination (Marek *et al*., 2013). Mycelia from germinated chlamydospores invade root cortical and vascular tissues, inducing root tissue decay (Marek *et al*., 2013) root death or root necrosis (Sweetingham, 1983). Chlamydospores formed at this stage, often become secondary inoculum intended for reinfection (Marek *et al*., 2013).

Role of '*Cylindrocarpon'*-like spp. in ARD

Due to major taxonomic revisions that have occurred in the '*Cylindrocarpon'*-like, the species of these fungi that have been associated with ARD are not well defined. This is due to the fact that several of the studies were done prior to 2011, prior to the implementation of major taxonomic changes. Isolations of '*Cylindrocarpon* destructans' (Zinnsm.), '*Cylindrocarpon lucidum'* Booth, *'Cylindrocarpon macrodidymum'* Schroers, Halleen and Crous, '*Cylindrocarpon leriodendri'* Halleen, Schroers, Groenewald, Rego, Oliveira and Crous from seedlings grown in ARD soils, suggested a role of these '*Cylindrocarpon'*-like spp. in ARD (Jaffee *et al*.,1982b, Braun, 1991, 1995, Dullahide *et al*., 1994; Mazzola, 1998; Manici, 2001,2003, Tewoldemedhin *et al.,* 2011a).

Under glasshouse conditions, the virulence of ARD associated '*Cylindrocarpon'*-like spp. was found to vary from highly virulent to some isolates having only low virulence towards apple seedlings (Dullahide *et al.*, 1994; Mazzola, 1998; Manici, 2001). Symptoms incited in glasshouse trials included root rot and a reduction in plant biomass (Braun, 1991, 1995). Several of the isolates were also found to be non-pathogenic, not causing any symptoms in apple seedling assays (Tewoldemedhin *et al.,* 2011c; Kelderer *et al.,* 2012). The low virulence of '*Cylindrocarpon'*-like fungi and the occurrence of isolates non-

pathogenic to apple in ARD soils, suggest that these fungi are less important in ARD. However, the role of '*Cylindrocarpon'*-like spp. in ARD is more likely due to the important synergistic interaction with *Pythium* spp. It has been shown that co-inoculation of '*Cylindrocarpon'*-like spp. with *P. irregulare* results in an increase in disease severity on apple seedlings, in comparison to the independent inoculation of each species (Braun, 1991, 1995, Tewoldemedhin *et al.,* 2011c). Manici *et al.,* (2013) concluded that '*Cylindrocarpon'*-like spp. are the most important ARD pathogens in central Europe (Germany, Austria and Italy) based on the negative correlation between their root infection frequency in apple seedling roots grown in ARD soils and seedling growth. Furthermore, in gamma irradiated ARD soil, the incidence of '*Cylindrocarpon'*-like fungi were low and correlated with improved seedling growth. The '*Cylindrocarpon'*-like fungi that were identified mainly included *Ilyonectira torresensis* (61% of all '*Cylindrocarpon'*-like fungi), followed by *Ilyonectria europaea* (12%), *Illyonectria robusta* (8%) and *Thelonectria veuillotiana* (Manici *et al.*, 2013).

Pythium **species**

The genus *Pythium* falls under the family Pythiaceaey, order Pythiales, phylum Oomycota and kingdom Chromista, and is characterized by coenocytic hyphae devoid of septations (De Cock and Lévesque, 2004). A wide range of morphological characteristics are associated with *Pythium* spp.,including spherical, filamentous as well as lobulated sporangia, smooth or even ornamented oogonial walls and plerotic and aplerotic oospores. The genus includes homothallic and heterothallic species, with the latter requiring two opposite mating types to produce the sexual oospores (Matsumoto *et al*., 1999; De Cock and Lévesque, 2004; Manici et al., 2003).

Pythium identification can be conducted using morphological criteria and molecular sequence data. Morphological identifications require a great deal of expertise and can furthermore be influenced by the type of media and culture conditions used (Thaher, 2008). Therefore, accurate identification requires traditional morphological characterization along with a molecular approach using sequence data. The barcoding genes for *Pythium* include the internal transcribed spacer (ITS) regions of the ribosomal (rRNA) genes and the cytochrome c oxidase subunit 1 (COI) gene region (De Cock and Lévesque, 2004, Robidue *et al*., 2011). A few *Pythium* species cannot be differentiated using either one of these gene regions, and thus require morphological identification. Furthermore, within the genus, several species complexes reside that are not well defined, sometimes leading to inconclusive results when attempting to identify some isolates to the species level (Robidue *et al*, 2011).

Pythium spp. have a worldwide distribution and cause epidemics of economic importance in many crops including cucurbits, solanaceous as well as in perennial crops (De

Cock and Lévesque, 2004; Taylor, 2008). On annual crops, *Pythium* mostly affects seeds prior to germination, as well as germinating seedlings and young plants causing damping-off (Thaher, 2008). *Pythium* spp. attack root tips as well as feeder roots. Feeder roots are very susceptible to *Pythium* spp. since they do not have secondary wall thickenings like primary root (Cook *et al*., 1987; Olsen and Young, 1998). Plants attacked by *Pythium* thus becomes weakened due to extensive root pruning (De Cock and Lévesque, 2004; Salman and Abuamsha, 2012).

Depending on the host and *Pythium* spp., the interaction of *Pythium* spp. with host plants can range from being saprophytic facultative parasites with an extensive host range to highly pathogenic species with a limited host range (Chen, 1992b). Furthermore, not all *Pythium* spp. are pathogenic to plants. Several *Pythium* species are beneficial, acting as pathogens of other pathogenic fungi and even as pathogens of various other *Pythium* species (Kucharek and Mitchell, 2000), with some also promoting plant growth (Mazzola, 1998). The pathogenic ability of *Pythium* spp. is largely driven by available enzymes produced by these pathogens. These enzymes bring about the invasion of various hosts by *Pythium* species, while other species are generally restricted to one host species (Thaher, 2008). The interaction between the host plant and *Pythium* pathogens involves breaking the host plant biochemical barriers. *Pythium* spp. furthermore secrete enzyme inhibitors to counter act host plant hydrolytic enzymes such as chitinases, glucanases as well as proteases. These hydrolytic enzymes are secreted by the host plant as a defence mechanism to *Pythium* species attack (Stassen and Van den Ackerveken, 2011).

During host plant attack by *Pythium* spp., hyphae absorb nourishment from living and non-living substrates. In the host plant *Pythium* produces effectors and several pectic enzymes. *Pythium* spp. effectors break down host plant cells as well as the host plant middle lamella of the cell wall membrane. This results in the softening of host plant tissues for easy nutrient acquisition by *Pythium* spp. Since *Pythium* spp. are heterotrophic, they can use a broad range of nutrients obtained from the host plant for its growth, development and reproduction (Levesque *et al*., 2010). Since the host plant's defence system will try and defend itself against the attack launched by *Pythium,* the plant will secrete hydrolytic enzymes such as chitinase, glucanases and proteases. In this case, *Pythium* species will often secrete enzyme inhibitors to counter act the host plant hydrolytic enzymes (Stassen and Van den Ackerveken, 2011).

Pythium spp. abundance and diversity are influenced by intensifications in land use, inappropriate crop rotations and reduced fallowing intervals. These factors can contribute to *Pythium* spp. diversity being shifted from saprophytic species to a build-up and accumulation of pathogenic and parasitic species. In a study by Hendrix and Campbell (1971), *Pythium*

was observed to be abundant in cultivated soil in comparison to uncultivated arable soils. This was due to the fact that with cultivation, organic matter is incorporated into the soil where *Pythium* species can then persist saprophytically. In these cultivated soils high in organic matter, *Pythium* spp. can survive as saprophytes by decomposing fresh organic matter and acquiring nutrients for their growth and development (Hendrix and Campbell, 1971). *Pythium* spp. can attack plants as individual spp. or as species complexes (Tedla and Stanghellini, 1992).

Pythium diseases are favoured by specific environmental conditions. In general, excessive soil moisture, moderate to high temperature favour disease development and specific pH levels. The specific optimal temperatures will differ for different species (De Cock and Lévesque, 2004.). For example, *Pythium aphanidermatum* has been shown to have a higher virulence at a pH range between 4.8 and 6.9, whereas at a pH of 7.6 infection severity decreases (Owen-Going, 2008). Although *Pythium* spp. have been recovered in soils with a pH range of 3.6 to 7.2, populations sizes are lower at pH ranges of 6.8 - 7.2, than at a pH range of 3.6 to 5.5. This is likely due to the fact that soil pH affects oospore and sporangia formation (Martin, and Luper, 1999).

Sources of inoculum for *Pythium* spp. can include contaminated irrigation water, alternative hosts like weeds (Wakeham *et al*., 1997). *Pythium* survives in the soil as oospores, and as hypha on old crop debris and on contaminated implements. Oospores often germinate rapidly in response to plant host fatty acids, especially those released by germinating seeds (Ruttledge and Nelson, 1997). The survival mechanisms are important since *Pythium* spp. have poor competitiveness in the absence of fresh organic matter (Stanghellini and Hencock, 1971; Stanghellini, 1974).

In the presence of conducive environmental conditions, such as water logging and in the presence of a susceptible host, *Pythium* oospores germinate and produce sporangia, or hypha that can produce sporangia in which zoospores are formed (Olsen and Young, 1998). Zoospores released from sporangia swim freely in water towards host plant root tips and feeder roots against the force of gravity (Pankhurst *et al.,* 1995). The zoospores are attracted to root tips of the host plant by root exudates released into the rhizosphere. Sporangia can also germinate directly by producing hyphae, depending on the prevailing temperature and species involved (Hendrix and Campbell, 1973). For a few *Pythium* species it is not known that they can produce sporangia and zoospores (Nelson and Craft, 1989).

Role of *Pythium* spp. in ARD

A wide range of *Pythium* spp. have been associated with ARD; often more than one species is associated with a specific orchard, but one species usually dominates (Mazzola, 2002).

Mazzola (2002) identified 17 *Pythium* spp. (*P. aphanidermatum, P. debaryanum, P. dissotocum, P. flevoense, P. heterothallicum, P. intermedium, P. irregulare, P. iwayamae, P. macrosporum, P. sylvaticum, P. torulosum, P. ultimum* and five putative new species) associated with six ARD orchards in Washington state, USA. The species that were widely distributed included *P. intermedium, P. irregulare, P. heterothallicum, P. sylvaticum* and *Pythium* spp. MM1 (*aff. macrosporum*). In South Africa, *P. irregulare* was also the most widely distributed species based on isolation studies. In a New York ARD orchard, *P. irregulare* and *P. sylvaticum* was also among the dominant *Pythium* spp. (Emmett *et al.*, 2014). *Pythium dissotocum, P. heterothallicum* and *P. sylvaticum* also occurred in at least fifty present of the South African ARD orchards based on isolation studies, whereas *P. vexans, P. litorale, P. attrantheridium, P. folliculosum* and *P. minus* were seldom identified (Tewoldemedhin *et al*., 2011b). However, subsequent DNA quantification studies in these orchards showed that *P. vexans* and *P. ultimum* were also widespread in South African ARD orchards (Tewoldemedhin *et al*., 2011c). Manici *et al.* (2013) found that *Pythium* spp. (not identified to the species level) were only associated at a high frequency with ARD orchards in Germany, but not in Italy and Austria. This, however, might be due to the stringent conditions used for surface sterilizing the roots (1 min in 1% sodium hypochlorite), since *Pythium* spp. in feeder roots are sensitive to sterilization on apple.

Several studies have been conducted to investigate the effect of *Pythium* species towards apple seedling growth, which showed that *Pythium* spp. can either promote or suppress apple growth, or have no effect (Mazzola *et al*.,2002; Tewoldemedhin *et al*., 2011c). Symptoms induced by pathogenic species on apple seedlings include root rot, and shoot and/or root growth reductions (Mazzola *et al*., 2002, Tewoldemedhin, 2011a,b). Mazzola *et al*. (2002) reported that all isolates of *P. heterothallicum, P. intermedium, P. irregulare,* and *P. ultimum* from Washington State consistently incited apple seedling stunting and a decrease in plant biomass. In Italy, *P. intermedium* has also been reported as a pathogenic species with isolates varying in virulence from low to being highly virulent (Manici *et al*., 2003). Mazzola *et al*., (2002) also reported variation in the pathogenicity of *Pythium* spp. for *P. sylvaticum,* since only three of four isolates were pathogenic (Mazzola *et al*., 2002). Tewoldemedhin *et al*., (2011c) further also reported that only some isolates of *P. attrantheridium* were pathogenic. *Pythium ultimum*, *P. sylvaticum* and *P. irregulare* have been reported as being highly virulent species toward apple (Mazzola *et al*., 2002; Tewoldemedhin *et al*., 2011c). Other pathogenic species that can significantly reduce the growth of apple seedlings include *P. debaryanum, P. macrosporum, P. dissotocum* and *P. folliculosum* (Mazzola *et al*., 2002; Tewoldemedhin *et al*., 2011c). Some *Pythium* spp. including *P. litorale* are only mildly virulent, causing only root rot and not growth reductions of

apple seedlings (Tewoldemedhin *et al*., 2011c). *Pythium* spp. that have been reported as not having an influence on apple seedling growth include *P. minus, P. aphanidermatum* and a putative new species (Mazzola *et al*., 2002; Tewoldemedhin *et al*., 2011c). A few putative new *Pythium* spp. isolated from ARD orchards have been reported to promote plant growth, and serve as biocontrol agents of apple seedling root rot incited by *P. sylvaticum* and *P. ultimum* (Mazzola, 2002).

Phytophthora as soilborne pathogens

The genus name *Phytophthora* is derived from a Greek term that means plant destroyer (Anton de Bary, 1976). As its name implies, this genus is well known for its devastating nature towards plants (Erwin and Ribeiro, 1996; Agrios, 2005; Cock and Lévesque, 2004). *Phytophthora* has a world-wide distribution and is classified in the order Peronosporales, class oomycetes and kingdom Chromista (Hawksworth *et al*., 1995)

Phytophthora species, like other oomycetes, characteristically contain coenocytic hyphae and cell walls consisting of cellulose (Rossman and Palm, 2006). Although *Phytophthora* spp. can be identified using morphological keys, where characteristics such as sporangial size and shape are important (Ribeiro, 1978), sequence data is required to differentiate some species that are morphologically indistinguishable (Robideau *et al*., 2011). Initially, the genus *Phytophthora* was divided into six morphological groups based on sporangium, antheridium and reproductive features (Stamps, 1990). However, more groups were identified subsequently using multi-gene phylogenies. Various gene regions have been used to investigate the phylogenetic relationship of species including the internal transcribed spacer (ITS) regions of the ribosomal (rRNA) genes and the cytochrome c oxidase subunit 1 (COI), ras-related protein, elicitin, mitochondrial genes (*cox2*, *nad9*, *rps10* and *secY*) as well as B- tubulin genes (Cooke *et al*., 2000; Forster *et al*., 2000; Grunwald *et al*., 2009, Martin *et al*, 2014). The cox1 and ITS regions have been proposed as barcoding regions for the genus *Phytophthora* (Robidue *et al*., 2011). Using molecular phylogenies, the more than 120 described *Phytophthora* species (Hyun and Choi, 2014), have been grouped into eight to 10 phylogenetic clades, i.e. clades 1 to 10 (Cooke *et al*., 2000; Martin *et al.*, 2014).

The genus *Phytophthora* consists of species that vary in their interaction with plants, ranging from highly virulent species to those that are opportunistic pathogens. Only a few species are most likely only important as saprophytes, since they break down plant litter in aquatic systems, especially clade 6 species (Brasier *et al*., 2003; Burgess, 2015). Some *Phytophthora* species target many hosts, for example *P. cinnamomi* affects about 2000 host plants species (Hardham, 2005), and while *P. sojae* has a narrow host range (Tyler, 2007). Yet some other *Phytophthora* species are intermediate in their host range between these

two species. *Phytophthora* continues to be a threat to tree species all over the world, causing massive deforestation (Kroon, 2012; Scott, 2013). Disease symptoms caused by *Phytophthora* spp. on trees include root rot, collar rot, trunk cankers, stem lesions, bud rot, leaf blight, fruit rot, tuber- and corm rot (Agrios, 2005).

Phytophthora spp. reproduce through sexual and asexual structures (Zentmyer and Thorn 1967). For short-term survival and spread, *Phytophthora* species depend on sporangia and zoospores (Zentmyer and Thorn, 1967; Cock and Lévesque, 2004). Chlamydospores and oospores are used for long term survival (Crone *et al*., 2013). Following discharge of zoospores from sporangia, motile biflagellate zoospores swim actively in water to potential infection sites (Duniway, 1976). Many *Phytophthora* spp. generate zoospores at low temperatures of more than 2°C (Granke and Hausbeck, 2010). Zoospores are chemotactically lured to root elongation areas including feeder or secondary roots. At these root elongation regions zoospores settle and encyst (Zentmeyer, 1961, Ho and Zentmeyer, 1977; Day *et al*, 2001). The germ tube subsequently develops and penetrates the host plant root epidermal cells directly or through plant surface wounds (Agrios, 2005). Germ tubes can also develop directly from sporangia, usually when temperatures prevail that are higher than 24˚C (Von Broembsen and Charlton, 2001). Upon entrance into plant epidermal cells the penetrated germ tube develops intra- and inter-cellularly within fine roots. As colonization progresses, *Phytophthora* produces haustoria, which ramifies within root cortical cells drawing nutrients from the plant. In the late stages of root infection, the pathogen experiences nutrient exhaustion and competition from secondary antagonistic fungi, which promotes the formations of resting spores (chlamydospores or oospores) in the root cortical cell tissue. Sporangia can also be formed on the root surface (Day *et al*, 2001). The resting spores are released from roots when the root is completely decomposed by saprophytes (Ribeiro, 1978; Erwin and Ribeiro, 1996). The importance of oospores as a resting structure is dependent on whether the species is homothallic or heterothallic. Homothallic species do not require the presence of two different mating types for sexual reproduction, whereas heterothallic species do. In the latter case, the formation of oospores will be dependent on the presence of both mating types within root cells (De Cock and Lévesque, 2004).

The resting spores, can be dispersed passively by the movement of plant growth media or organic matter, irrigation water, soil on footwear, animals, vehicle tires, tools and equipment (Scott *et al*., 2013). Additionally, slugs, snails, insects and birds have been documented as vectors of *Phytophthora* spp. (Konam and Guest, 2004). Resting spores act as an inoculum supply for subsequent disease cycles. In the presence of favourable

conditions, *Phytophthora* populations can increase from virtually low undetectable levels to high levels within a short period (Agrios, 2005; Erwin and Ribeiro, 1996).

Role of *Phytophthora* spp. in ARD

In several major apple production regions, including South Africa, USA, Italy and Australia, *Phytophthora* spp. have been reported as having a destructive effect on apples (Mircetich and Browne, 1987; Aldwinckle *et al*.,1986; Metheron *et al*., 1988; Tidball and Linderman, 1990; Harris, 1991; Utkhede *et al*., 2001; Zondo *et al*., 2007; Van Schoor *et al*., 2009). Disease symptoms include root- and crown rot. The extent to which *Phytophthora* root and crown rot have affected apple, has also promoted the search for better rootstocks in breeding trials (Borecki and Czynczyk, 1978; Utkhede and Quamme, 1988, Utkhede *et al*., 2001, Browne and Mircetich, 1993, Carisse and Khanizadeh, 2006).

A number of *Phytophthora* spp. have been found associated with ARD in South Africa, North America and Australia. These include *Phytophthora cryptogea* Pethb. and Laff., *Phytophthora cambivora* (Petri) Buisman, *Phytophthora cactorum* (Lebert and Cohn) Schroet., *Phytophthora drechsleri* Tucker (McIntosh, 1975; Dubin and McCrum, 1975; Julis *et al*., 1979, Helton *et al*., 1984; Jeffers and Aldwinckle, 1986, Isutsa and Merwin, 2014), *Phytophthora parasitica* as well as an undescribed *Phytophthora* spp. (Sitepu and Wallace, 1974; Utkhede *et al*., 1992, Isutsa and Merwin, 2014). In central Europe (Germany, Austria and Italy), *Phytophthora* spp. have not been found associated with ARD orchards (Manici *et al.*, 2003, 2013).

Other organisms potentially involved with ARD

Fusarium species have often been associated with ARD, and in some instances some isolates and species were shown to be pathogenic. *Fusarium oxysporum,* followed by *F. solani* are usually the dominant *Fusarium* spp. associated with ARD (Tewoldemedhin *et al*., 2011a; Manici *et al*., 2013). In South Africa, other species that were occasionally associated with ARD include *F. solani, F. equiseti, F. scirpi, F. avenaceum, F. cerealis and F. reticulatum* (Tewoldemedhin *et al*., 2011a). In central Europe, *F. equiseti* was also identified, in addition to *F. acumunatum, F. avenaceum, F. culmorum, F. compactum, F. emitectum* and *F. venenatum* (Manici *et al*, 2013). *Fusarium solani* (Mart.) Sacc. was found to have very low virulence towards apple trees (Utkhede *et al*., 1992; Mazzola, 1998; Manici., 2003; Tewoldemedhin *et al*., 2011c). In Queensland Australia, *Fusarium tricinctum* (Corda) Sacc. is regarded as an important ARD pathogen. This *Fusarium* spp. was observed to substantially reduce the root weight of apple seedlings (Dullahide *et al*., 1994). Manici *et al.* (2013) concluded that *Fusarium* spp. were unlikely to contribute to ARD development, since

their isolation frequency did not correlate with apple seedling growth reductions when seedlings were grown in ARD soils.

Two plant parasitic nematode genera, other than *Pratylenchus,* including *Xiphinema* and *Paratrichodorus,* have also been associated with ARD (Sultan and Ferris, 1991; Lana *et al*., 1983; Tewoldemedhin *et al*., 2011). *Xiphinema,* known as the dagger nematode, has been associated with ARD in New York (Xiphinema americanum (Cobb)) and in South Africa (unidentified *Xhiphinema* spp.) (Tewoldemedhin *et al*., 2011; Isutsa and Merwin, 2014). The damage threshold for *X. americanum* was established at 1 per $10cm^3$ of soil (Isutsa and Merwin, 2014). In South Africa, a *Paratrichodorus* sp. was identified in only one ARD orchard (Tewoldemedhin *et al*., 2011).

MANAGEMENT OF APPLE REPLANT DISEASE

The nature of ARD requires the utilization of a holistic management approach, due to the multiplicity of the causal organisms recognised as causing the disease so far. The use of a single control approach is highly likely to yield ineffective control (Leinfelder and Merwin, 2006). The exception is when broad-spectrum fumigants are used, an approach that has mainly been used for managing ARD. Fumigants, however, are costly and environmentally damaging. In South Africa, failure in disease control has sometimes been observed when using fumigants. This could be due to incorrect application of the fumigants, i.e. suboptimal dosages, too low temperatures at the time of application, sub-optimal soil moisture and too few shanks being used for injecting the fumigant (J.P.B. Wessels, ProCrop, Wellington, South Africa). Alternatively, external inoculum sources such as irrigation water and planting material may re-introduce ARD pathogens into fumigated soil. In South Africa, apple nursery trees have been found to contain several ARD pathogens including *Pratylenchus* spp., *P. irregulare, P. ultimum, P. sylvaticum and Cylindrocarpon*-like spp. (Moein *et al*., 2016). Since, broad-spectrum biocides eliminate most antagonistic organisms from the soil, this biological vacuum can be rapidly re-infested by introduced pathogens.

More recently, sustainable ARD management options have been discovered that rely on changes in the resident microbial community for disease suppression, and other modes of action. These approaches not only have good potential for targeting the range of known ARD pathogens, but also provide a buffering capacity against reinfestation of treated soil by ARD pathogens (Mazzola *et al*., 2015; Hewavitharana and Mazzola, 2016). The latter is a problem with standard fumigation practices, since only the tree row is fumigated resulting in pathogen re-colonization of trees within the first 2-years (Mazzola *et al*., 2015). A sustainable management practice for ARD is the use of mixtures of Brassicaceae seed meals, which have been shown to be effective under orchard conditions, even outperforming standard

fumigation treatments (Mazzola *et al*., 2015). Another promising approach is anaerobic soil disinfestation. This approach relies on the incorporation of an effective carbon source followed by the application of irrigation and subsequent tarping of the soil with an oxygen-impermeable plastic cover. Currently, mainly glasshouse studies have been conducted for the management of ARD using anaerobic disinfestation. However, the approach has also been shown effective for the control of apple nursery replant disease (Hewavitharana and Mazzola, 2016 a, b).

Pre-plant chemical fumigation

Pre-plant chemical fumigation has historically been used as a very successful way of managing ARD (McKenry *et al*., 1994). Methyl bromide is well known and was once a widely used chemical. However, this product has since been phased out by the Montreal protocol of 1995, mainly due to environmental concerns (Ristaino and Thomas, 1997. The post MeBr era has been followed by several studies that have tried finding an equally effective yet safe and inexpensive fumigant. Most of the fumigants that have been evaluated, however, showed variable efficacy across multiple sites, including iodomethane or methyl iodide (MI), chloropicrin (trichloronitromethane) (Pic), 1, 3-dichloropropene (1, 3-D), dimethyl disulfide (DMDS), dazomet and metham sodium or metham potassium among others. These fumigants are are not equally effective against all soilborne pests, differing in spectra of pest effectiveness. For instance, 1,3-D and DMDS offered selective nematode control, butminimal control of bacteria and fungi (Ajwa *et al*., 2003). Pic effectively controls soilborne fungi and insects, but demonstrated limited activity towards weeds and nematodes (Duniway, 2002). Some of the fumigants including MI, although very effective, have negative effects such as pro-longed plant-back times (Browne *et al*., 2006; Fennimore *et al*., 2008; Gao *et al*., 2008).

The limited spectrum of biological agents controlled by most fumigants has resulted in fumigants being combined to obtain improved control. A combination such as that of metham sodium and 1, 3-D or Pic led to rapid loss of the Pic fumigant, thus reducing mixture effectiveness (Zheng *et al*., 2004). A mixture of 1,3-D and Pic was found to be an effective combination of fumigants, since it resulted in consistent yield increases comparable to MB in tomato and strawberry fields (Ajwa *et al*., 2002, 2004; Minuto *et al*., 2006; Porter *et al*., 2006). The 1,3-D/Pic combination is, however, costly and poses a threat to non-target organisms (Di-Primo *et al*., 2003)

Brassicaceae seed meals.

Biofumigation involves the use of volatile chemical compounds or allelochemicals or secondary metabolites of plant origin (Morra *et al*., 2002). Brassicaceae species are most

successful as biofumigation crops due to their biocidal chemistry and modifications they cause in soils. Brassica crops can be incorporated as green material into soil to achieve suppression of soilborne pathogens (Kirkegaard and Sarwa., 1998; Gouws, 2004; Kumar, 2005). This approach, however, often leads to inconsistent control, which has also been found for ARD (Mazzola and Mullinix, 2005). Therefore, the use of Brassica crops in ARD management, has been focused on the use of seed meals that yield more consistent results.

Seed meals (SMs) are waste products of the biodiesel extraction process. SMs contain a wealth of isothiocyanates (ITCs), other chemical compounds including thiocyanates, nitriles as well as oxazolidinethiones and organic side chains such as aliphatic, or aromatic or indole compounds (Clark, 2010). Most of these compounds contain a biocidal action towards pathogenic and parasitic species (Vaughn and Berhow, 1998). For some SMs it has been shown that disease suppression by ITCs is only important during the first 24hr post-plant application period, and thereafter the control is through a change in resident soil biology that suppresses pathogens (Cohen and Mazzola, 2006; Mazzola, 2007). For example soil pasteurization was shown to abolish the suppression of R. *solani* (Cohen & Mazzola, 2006). Similarly, Weerakoon (2011) demonstrated a loss in *B. juncea* SM suppressiveness towards *Pythium abappressorium* when soil was pasteurized prior to SM application. Manici *et al*., (2000) also found that the suppression of R. *solani* by *B. juncea* SMs, which is known to contain active volatile allylisothiocyanate (AITC), was not only due to isothiocyanates, but also through alteration of the resident microbiota (Manici *et al*., 2000). The importance of mechanisms other than biofumigant release, is also evident from the fact that disease suppression is obtained irrespective of glucosinolate content, i.e. often no correlation exists between glucosinolate concentration and disease suppression. For example, *B. napus* SMs, which do not to generate active chemistries against *Rhizoctonia* root rot, provided control of this pathogen (Manici *et al*., 1997). It has further been noted that soil suppressiveness is maintained long after the biofumigation treatment. This was shown by inoculating pathogens into the soil at a stage when ITCs were no longer present in the soil (Mazzola, 2001; Cohen and Mazzola, 2006; Weerakoon *et al*., 2012). Antagonistic *Streptomyces* spp. and host defence induction have been reported as being involved in disease suppression by SMs (Cohen and Mazzola, 2006

Resident soil biology has also been indicated as being important in the control of the lesion nematode using brassica SMs, in addition to compound availability. In a study by Potter *et al*, (1998), no correlation could be established between compound availability and *Pratylenchus neglectus* control. Mazzola *et al* (2001) reported that due to the short-lived compound activity of *B. napus* SM towards nematodes, resident soil biology was more likely involved in control (Mazzola *et al*., 2001). It was indeed found later that beneficial

nematophagous fungi including *Arthrobotrys conoides, A. yunnanensis* and *Dactylella oviparasitica* were exclusively associated with specific SMs (*Brassica juncea–Sinapis alba* or *B. juncea–B. napus*) treated soil, suggesting their involvement in nematode suppression (Mazzola *et al*., 2015). Additionally, SMs applications were shown to promote the predatory nematode *Aporcelaimellus* spp. (Mazzola *et al*., 2010). Some SMs for example *B. juncea* contain glucosinolates active against *Pratylenchus* spp., which are likely to also play a role in nematode control (Mazzola *et al*., 2009).

SMs may further improve plant growth due to the fact that the soil carbon content of the soil is increased, which may improve pedological soil properties such as soil aeration, water infiltration, and water holding capacity (Bellostas *et al*., 2007). The increase in soil carbon through SM applications is important for antagonistic organisms such as predacious nematodes (Reardon and Mazzola, 2010) and protozoa (Cohen and Mazzola, 2006).

The first investigations into SMs for managing ARD, indicated that the use of SMs containing only one *Brassica* spp. was ineffective in controlling the broad spectrum of ARD pathogens. In some instances the SMs even promoted these pathogens, for example *B.napus* and *S. alba* SMs promoted *P. irregulare* and *P. ultimum* respectively (Mazzola *et al*., 2007; 2009; Hoagland *et al*., 2008). *Phytophthora* spp. including *P. cambivora* and *P. mergasperma* were also shown to be stimulated by *B. juncea* SMs (Mazzola unpublished data in Mazzola *et al*., 2007). Consequently, the use of single species SMs resulted in inconsistent control of ARD across different orchards. Since the inconsistent control was mainly due to an increase in oomycete pathogens, the application of a post-plant mefenoxam soil drench following SM application was able to improve yields and tree growth to levels similar to those obtained with fumigants (Mazzola & Brown, 2010).

The use of mixtures of *Brassica* spp. SMs were shown to be effective in ARD control, even outperforming standard fumigation treatments. It was first shown that a mixture of *B. juncea* and *B. napus* SMs was able to improve the vegetative growth of young trees in an organic nursery to levels similar than those obtained with fumigants (Mazzola & Brown, 2010). Subsequently, SM formulations containing a mixture of *B. juncea-S. alba* or *B. juncea-B. napus* were shown to improve tree growth similar to that obtained with standard fumigants. Furthermore, the *B. juncea-S. alba* SM resulted in tree growth and yields at the end of the fourth growing season, which were in general superior to that of fumigants. The SM amended soils, in comparison to the fumigated soil, were shown to be resistant to reinfestation with *P. penetrans* and *Pythium* spp. Rhizosphere microbiome analyses showed that this could be due to a unique microbiome associated with SM treatments, which was distinct from the fumigation treatment microbiome. The SM microbiome contained microbes that are known to be involved in the suppression of ARD pathogens including *Arthrobotrys*

spp. and *Dactylella oviparasitica* active against parasitic nematodes; *Burkholderia* spp. antagonistic to several soilborne fungi including *R. solani;* and *Oidiodendron* spp., which is a known biocontrol agent of *Phytophthora* (Mazzola *et al*., 2015).

Induction of soil suppressiveness by compost and organic amendments.

Soil suppressiveness by compost and organic matter has been described by many workers, and involve physiochemical and biological mechanisms (Hoitink, 1997; De Cueuster and Hoitink, 1999; Boulter *et al*., 2002; Noble and Coventry, 2005). Most of the literature point to the importance of an increase in resident microbial activity in disease suppression (Ristaino and Thomas, 1997; Crecchio and Stotzky, 2001; Bernard *et al.,* 2012). The particular soil biological activity is brought about as a result of carbon source addition by compost (Campbell, 1989). A few studies have evaluated the efficacy of organic matter and compost amendments on the suppression of ARD. A few studies have evaluated the efficacy of organic matter and compost amendments on the suppression of ARD. Van Schoor *et al* (2009) reported improved shoot growth, but not trunk circumference, over three seasons, when compost applied as a soil dressing in combination with a straw mulch was used under replant conditions.

Induction of soil suppressiveness by cover crops.

The use of cover crops has been investigated for the management of ARD. The cover crops can be planted in the driving lanes or orchard lanes (Tedders, 1983; Bugg and Waddington, 1994). Cover crops likely suppress ARD through root exudates that stimulates soil suppressiveness (Mazzola, 1999). Mazzola (1999) showed that there was a substantial decrease in infection by three ARD pathogens namely Cylindrocarpon - like spp destructans, *Phytophthora*, *Pythium Rhizoctonia solani,* following a wheat cover cropping. The decreases in pathogen populations were likely due to the sharp increase of bacterial antagonistic populations (Mazzola, 1999).

USING PHOSPHONATES FUNGICIDES FOR THE MANAGEMENT OF SOILBORNE OOMYCETE PATHOGENS IN AGRICULTURAL CROPS

GENERAL INTRODUCTION

The fungicidal properties of phosphonates have been reported for soilborne pathogens belonging to the oomycetes including *Pythium* spp. and *Phytophthora* spp. (Cook *et al*., 2009, Jackson *et al*., 2000). Phosphonates are used against these pathogens on a very wide range of crops including tree crops, especially in avocado (Bezuidenhout *et al*., 1987, Crane and Shearer, 2014). Other tree crops include apples (Long, 1989), almond and cherry

(Wicks and Hall, 1998; 1990), apricot and peach (Lim, 1990) and cocoa (Holderness, 1990). Phosphonates (H^2P0^3; Phi) are the reduced form of phosphate (H^2PO^4; Pi), and is formulated as fungicides as various alkali salts as well as esters of phosphoric acid (Fenn and Coffey, 1984). The addition of potassium hydroxide to phosphoric acid results in the formation of phosphorous acid with mono or di-potassium, referred to as potassium phosphonate. Potassium phosphonate is the most commonly used formulation for phosphonate based fungicides (McDonald *et al.,* 2001). Ethyl phosphonate is formed when phosphoric acid is combined with ethanol. Aluminium ions may be included within this solution to neutralize ethyl phosphonate ions, resulting in the formation of fosetyl- Al, an aluminium tris-O- ethyl phosphonate (MacDonald, 2001).

Upon its application, phosphonates are taken up by the plant and is eventually hydrolysed to phosphite. Even when diluted in water the phosphonates are hydrolysed into phosphite (Fenn and Coffey, 1985). In this form phosphite (H^2 $P0^3$; Phi) possesses fungicidal properties. Phosphite is quite stable in plants and is not oxidized or even metabolized in plant tissues. Loss of phosphite in plants is most likely through root exudates and due to leaf fall and removal of fruit (Quimette and Coffey, 1990; Carswell, 1996, 1997; Guest and Grant, 1991). Phosphite is in general not toxic to plants. However, in some instances it can cause phytotoxicity at high application rates. Phytotoxic symptoms include leaf burn, foliar necrosis, defoliation, chlorosis, diminished root growth and plant death (Guest., 1995; Komorek. 1997; Aberton. 1999; Ali and Guest, 1998; Pilbeam, 2000; Barrett 2001, Hardy, 2001; Singh, 2003; Shearer, 2006).

Aside from being marketed as systemic fungicides, phosphonates are often also marketed as fertilizers (Guest and Grant, 1991; Lovatt and Mikkelsen, 2006; Thao and Yamakawa, 2009). This can be confusing to growers. However, the labeling of phosphonates as fertilizers is incorrect, since it cannot be used as a source of phosphate by plants (MacDonald, 2001). Although several soil bacteria are known to metabolize phosphite to phosphate, for instance *Escherichia coli, Pseudomonas stutzeri, Alcaligenes faecalis* as well as *Xanthobacter flavus* (White and Metcalf, 2007), the metabolic rates in general is very slow and would hardly have any reasonable relevance for plant growth (McDonald, 2001a). Furthermore, microbes oxidizing phosphite, preferentially utilize phosphate rather than phosphite as a source of phosphate (Adams and Conrad, 1953). Generally, the half-life of phosphite oxidation to phosphate in soil has been reported to be around 12–16 weeks (Adams and Conrad, 1953).

Translocation and persistence of phosphonates and the effect of time of application.

The optimal timing of phosphonate applications requires a good understanding of plant physiology during the time of application (Barrett *et al.,* 2003; Garbelotto *et al.,* 2007a, b; Shearer and Fairman, 2007). Phenological growth stages, greatly impact the efficacy of phosphonate applications (Giblin *et al.,* 2007). This is due to the fact that phosphite is translocated passively or through diffusion equally well upwards (xylem) and downwards (phloem) along with sugar and nutrient streams. Therefore, phosphite will move along the sugar demand in crops in a source/sink relationship (Whiley and Whiley, 2005; Giblin *et al.,* 2007a; Giblin *et al.,* 2007b; Barrette *et al.,* 2001). Since phosphite moves in the transpiration stream the translocation can be influenced by temperature, since temperature affects plant transpiration rates. In avocado, injected phosphonates were detected within 16 -32 days after application in avocado roots (Whiley *et al.,* 1995).

The efficacy of phosphite in controlling soilborne diseases are likely dependent on the accumulation of high root phosphite concentrations, and it is therefore important to know which factors can reduce root phosphite concentrations. Crop load and sink strength have an important influence on root phosphite content. Crop load is important since a negative correlation has been reported in avocado between crop load and root phosphite concentrations, i.e. high yielding orchards have lower root phosphite concentrations (Whiley and Whiley, 2005; Giblin *et al.,* 2007a; Giblin *et al.,* 2007b). The time of year of application has also been proven to influence phosphonate distribution. In avocado the best time for phosphonate foliar sprays and injections was late summer or autumn. This is due to the fact that in late summer and autumn, avocado roots are the key metabolic sink of the trees (Whiley, 1986; Estate, 1994; Hardy *et al*., 2008; Thomas, 2008). Consequently, there is a strong connection between phosphite tissue concentration and metabolic sink strength at the time of application (Whiley *et al*., 1995). Therefore, sink strength must be considered in order to optimize the time of application.

The concentration and longevity of phosphite in different plant species and their protection against pathogens can vary substantially. For instance, in cherry trees, 1g/l and 100g/L phosphonate sprays protected the trees from *P. cambivora* for 17 weeks (Wicks and Hall, 1988). In avocado, it has been demonstrated that a 3-6 month window is required before reapplication (Pegg, 1987). In contrast, in some Australian native tree species such as banksia and eucalyptus, one phosphonate injection can last between 2- to 4-years (Shearer, 2007). This could be due to differences in tree architecture of vascular connections including xylem vessels, xylem cross connections, and three-dimensional xylem arrangements inside the trunk area, which influence the translocation and distribution of phosphite. For example, avocado trees must receive numerous phosphonate injections

spaced at equal distances around the trunk to ensure phosphite distribution to all roots. In contrast, only one phosphonate injection seemed to be adequate for homogeneous phosphite distribution in cocoa trees (Darakis *et al.,* 1985; Pegg *et al.,* 1990; Whiley, 1990; Whiley *et al.,* 1992; Whiley and Schaffer, 1993). Therefore, caution should be taken in making extrapolations on the translocation and longevity of phosphite in different tree species.

The time of phosphonate application is also important for limiting fruit residues, since maximum phosphite residue levels ranging from 2 ppm to 75 ppm are enforced for most fruit crops. Phosphonate application soon after fruit set, can substantially increase phosphite residues in the fruit. These residues continue to persist in high quantities until harvest, which poses risks for exceedances in maximum residue levels (Malusa and Tosi, 2005). Since mature fruit is no longer a strong sink for photosynthates and consequently phosphite, phosphonate applications near harvest were shown to have minimal effects on fruit phosphite residue content in avocados (Whiley, 1995, 2001).

Phosphonate application methods

The systemic nature of phosphite permits the use of various application methods targeting different plant organs. These include soil drenching for root uptake, trunk injection, trunk paints or foliar sprays (Funt, 1985; Hardy *et al*., 2001; Marucchini and Zadra, 2002., Gisi, 2002; Chaluvaraju, 2004; Kennelly, 2005., Godoy and Canteri, 2004; Ishii, 2004; Brown, 2004; Benigni and Bompeix, 2004).

Trunk injections

In avocado, trunk injections have been used extensively for managing *P. cinnamomi* root rot, based on the work done by Darvas (1984). It was shown that trunk injections were superior to foliar sprays when diseased trees were treated. The method was reported to significantly lower fungicide usage and enabled excellent fungicide persistence. The application of only two injections annually during the two root flush window (after summer and spring shoot flush hardened off) were able to result in the recovery of severely declining trees. With phosphonate trunk injections, phosphite first moves with the transpiration stream acropetally through the exterior xylem, into the foliage. Subsequently, phosphite is translocated basipetally through the trunk into the root cambium (Whiley, 1995).

Soil drenches

In the USA, Coffey *et al.* (1984) found that soil applications of fosetyl-Al (8.5 g a.i./m^2) were effective under field conditions if applied two to four times a year on 20-year-old avocado

trees through irrigation on a ground area of about 16 m^2. They investigated soil applications further under glasshouse conditions. It was found that although phosphonates only persisted in soil for 2 weeks, phosphite concentrations remained high for the 8 week period that roots were evaluated (Ouimette & Coffey, 1989a).

Early research in Australia showed that in soils with high microbial activity, phosphonate soil drenches were not effective and only provided protection for 11 weeks (Pegg & Whiley, 1987). Subsequently, it was also concluded that the application of phosphonate fungicides to the soil through fertigation was not cost-effective in avocado. The soil application recommendation that seems to have some potential consisted of a minimum initial application of 20g/l, with subsequent applications of 10g/l every 4 weeks. This equates to 110g of phosphorous acid/ m^2 of canopy area initially, followed by 55g/m^2 every 4 weeks, which yield a total of 770 g/m^2 per annum of canopy surface. A trunk injection program uses 1.25 g/m^2 per annum of canopy surface (Whiley, 2001).

Bark sprays containing penetrants

Recent work in the USA and Australia on native forest trees (Garbelotto *et al*., 2007; Dunstan & Hardy, 2005) and on avocado in Australia (Giblin *et al*., 2007), has shown that organosilicone bark penetrants (Pentra-bark® or Pulse®) greatly assists in the absorption of phosphonates through bark. A further benefit of bark spray applications in avocado was that it provided lower, but more consistent phosphite concentrations in the roots. Furthermore, little or no phosphite ended up in the canopy, thus reducing the risk of fruit residues, when compared to trunk injections (Giblin *et al*., 2007). Giblin *et al.* (2007) found that avocado trunk sprays combined with Pulse® provided sufficient protection and root phosphite concentrations when applied at the same dosage as trunk injections, but two applications, i.e. one every 6 months were required. Dunstan & Hardy (2005) reported that in Australian native species the application of trunk bark phosphonate sprays combined with an organosilicone bark penetrant was as effective as trunk injections and foliar sprays, yielding higher root phosphite levels. An advantage of the barks spray was that the applications took approximately one-third of the time of that required for injection (Dunstan & Hardy, 2005).

Foliar sprays

Coffey *et al.* (1984) were the first to report that fosetyl-Al foliar sprays were effective on tree crops, when it was shown that *P. cinnamomi* could be suppressed on avocado. Foliar sprays applied at 3 g a.i./L and 20L per tree were effective in managing avocado root rot when applied three to five times a year to mature orchard trees. Whiley *et al.* (2001) found that three foliar phosphonate applications at 0.25%, 0.5% or 1% (9L/tree) applied at 6- week

intervals, gave similar results than two trunk injections. Therefore, in Australia, potassium phosphonate was registered as a 0.1 a.i. % foliar application for bearing avocado trees. However, subsequently, an emergency registration was obtained for an increase in dosage to 500g a.i./100L (0.5%), since the 100 g a.i./100L solution did not result in protection of avocado trees against *Phytophthora* (Whiley *et al.*, 2001; Thomas, 2008). In South Africa, McLeod *et al.* (2018) reported that five 0.5% a.i. sprays were comparable to two trunk injections. The variable number of foliar sprays required on avocado is likely is due to the fact that the translocation to roots of foliar applied phosphonates is influenced by various factors and these include crop load, tree phenology and spray volume (Thomas, unpublished report; Whiley, 2001).

In general, spray volume can significantly influence the efficacy of phosphonate foliar sprays. In native Australian vegetation, only high volume aerial sprays are effective, with low volume sprays having low efficacy (Crane & Shrearer, 2014). In Australia, low volume sprays were not effective on avocado (Whiley *et al*, 2001). On the other hand, McLeod *et al*. (2018) did not find a difference in full volume and three-quarter volume foliar sprays based on root phosphite concentrations.

PHOSPHONATES MODE OF ACTION

The mode of action associated with phosphonates is still to be fully elucidated. However, it is likely to involve a direct and/or indirect mode of action. The indirect mode of action involves the plant's defence system, whereas a direct mode of action involves a direct toxic effect against the pathogen (Smillie, 1989). The difficulty in elucidating the specific mode of action involved in each oomycete host pathogen system is likely due to the fact that it is influenced by (i) the time interval between phosphite treatment and inoculation; (ii) the concentration of phosphite applied and its translocation to the target plant organ, (iii) the tolerance of the pathogen to phosphite and (iv) the ability of the host to launch an effective host defence response following phosphite application (Afek and Sztejnberg, 1989, Smillie, 1989; Jackson *et al*., 2000; Massoud *et al*., 2012).

The mode of action of phosphonates is further complicated by the fact that it is likely dependent on by phosphite plant tissue concentrations. Two studies using *Arabidopsis* and *Eucalyptus* have provided evidence that at low phosphite plant tissue concentrations or application rates, an indirect host defence response is involved. This was evidenced by the upregulation of defence genes or compounds. In contrast, when high phosphite tissue concentrations or application dosages were involved, a lack of these host defence responses was seen in the host plant. Furthermore, *Arabidopsis* plants mutated in defence genes had less disease only when high phosphite application dosages were applied; at low

phosphite dosages the mutant plant could not defend itself against the pathogen (Jackson *et al*., 2000; Massoud *et al*., 2012).

Direct mode of action on oomycetes

In vitro studies have provided strong evidence of phosphite having a direct mode of action. High phosphite concentrations minimize the growth and sporulation of oomycete pathogens (Wilkinson, 2001a, b; Garbelotto, 2009). One of the most prominent direct modes of action was shown to be the interruption of biochemical processes. For instance, key phosphorylating enzymes and phosphorous metabolic processes can be suppressed. These specific interruptions largely impact the synthesis of various phosphorous containing compounds (e.g. nicotinamide adenine dinucleotide and adenosin triphosphate) essential for oomycete growth and development (Olaya and Köller, 1999; Taiz and Zeiger, 2002). Phosphite has also been demonstrated to interfere with gene expression at the transcription level (Varadarajan et. al., 2002). In *P. cinnamomi* mycelia, genes coding for annexin and cellulose synthase were down-regulated, whereas genes for adenosine ribosylation factors were upregulated (King, 2010). Most of these genes initiate cell wall activity as well as membrane functionality, important for the survival of the pathogen (Konopka-Postupolska, 2007). Other genes that were shown to be differentially regulated included cellulose synthase enzymes, which are essential for pathogen virulence (Grenville-Briggs, 2008). The biochemical and gene expression alternations caused by phosphite ultimately also affects the morphology of oomycete pathogens. In *Phytophthora* spp. phosphite resulted in hyphal distortion (Dercks and Buchenauer, 1987; Griffith, 1993; King, 2010; Wong, 2010).

The concentration at which phosphite is toxic to *Phytophthora* is species specific, and also developmental stage-specific. In *P. cinnamomi, in vitro* studies showed that phosphite at 40 mg/mL caused hyphal lysis (King, 2010). A very low concentration of 10 µg/mL phosphite inhibited *P. parasitica* as well as *P. citrophthora* sporangium production (Farih, 1981). McCarren (2006) observed that phosphonate application stimulated *P. cinnamomi* chlamydospores dormancy. Horner and Hough (2013) reported EC_{50} values for hyphal *P. cinnamomi* inhibition of 2 µg/mL and EC_{50} of 9 µg/mL for *P. cactorum*. Ouimette & Coffey (1989) found that among nine evaluated *Phytophthora* spp., *P. cactorum* was among the least sensitive species. The EC_{50} values for mycelial inhibition of four *P. cactorum* isolates ranged from 0.25 to 0.30 µg/mL (HPO_3^{-2} per millilitre) when corn meal agar was used.

Even though considerable *in vitro* studies have been conducted for *Phytophthora* spp., limited information is available for *Pythium* spp. (Phillip, 2009). The *in vitro* studies on *Pythium* spp. also showed that species differ in their sensitivity. Early work by Sanders, (1983), reported that potato dextrose agar amended with fosetyl-Al at 1, 10 and 100µg/ml

did not cause any inhibition of 25 isolates representing eight *Pythium* spp. (*Pythium apharnidermatum*). This is likely due to the fact that fosetyl-Al is approximately 2.4 times less fungitoxic to oomycetes than potassium phosphonate *in vitro* (Ouimette & Cofffey, 1989). Fenn and Coffey (1984) reported on the mycelial growth inhibition of four *Pythium* spp. (*P. myriotylum, P. polymorphon, P. aphanidermatum* and *P. ultimum*) when grown on corn meal agar that was amended with 60 to 552 µg/ml phosphorous acid. *Pythium ultimum* was the most sensitive and was completed inhibited by 276 µg/ml, whereas *P. myriotylum* was the least sensitive with only 30% inhibition at 552 µg/ml (Fenn & Coffey, 1984).

The sensitivity of most, but not all *Phytophthora* spp. to phosphite in *in vitro* studies is influenced by the phosphate content of artificial media. Consequently, it is not always straightforward to compare results from different *in vitro* studies since these studies did not always use the same phosphate concentrations in media (Guest & Grant, 1991). Most studies have used full- or half strength corn meal agar that has a low phosphate concentration (0.38mM) or modified Ribeiro's medium, which has an even lower phosphate content (0.084 mM). If media with higher phosphate concentrations are used (0.1-1 mM) this can alter the phosphite sensitivity of isolates, with isolates becoming less sensitive to phosphite with increasing phosphate concentrations in media. For *P. palmivora,* phosphate levels in media did not affect the sensitivity of phosphite sensitive isolates. However, phosphite resistant isolates were inhibited by phosphite only when the phosphate concentrations were low in the medium. For example resistant isolates had a phosphite ED_{50} value of 30 mM phosphite at 7 mM phosphate, but the phosphite ED_{50} was only 1 mM phosphite at 0.1 mM phosphate (Griffith *et al*., 1993). Extrapolations from the sensitivity of *Phytophthora* spp. in phosphate-limiting artificial media relative to their sensitivity in plant tissues, where phosphate concentrations are usually 5-20 mM, can sometimes be unsubstantiated (References within Guest & Grant, 1991).

Indirect host induced mode of action

The first studies on the involvement of an indirect mode of action of phosphonates indicated that phosphonate treated plants generate defence compounds such as phytoalexins or defence enzymes, and alter host plant structural defence responses. The application of fosetyl-Al has been shown to induce the production of the phytoalexin capsidiol. Capsidiol provides good control against *P. nicotianae* in capsicum fruit. The capsidiol activity against *P. nicotianae* was shown to be produced within 18-24 hours following application. Fosetyl-Al was also shown to elicit the hypersensitive response on tobacco foliage (Guest, 1984). Phosphonates can also improve the structural defence response of plants against pathogens including lignification, increased cell wall thickness and plant secondary metabolite

production. Many of the secondary metabolites synthesized by the plant during defence induction possess antimicrobial properties (Guest and Grant, 1991). Phosphonate application in *Banksia brownii*, inhibited *P. cinnamomi* attack through tissue compartmentalization and walling off (Smith, 1997).

In the advent of molecular biology studies on the mode of action of phosphite, the focus has moved towards investigations on the involvement of the hypersensitive response, a primed response and specific defence signalling pathways. The hypersensitive response was shown to be involved in the *Arabidopsis thaliana* interactions with *P. cinnamomi* and *P. palmivora* (Robinson and Cahill, 2003; Daniel and Guest, 2006). A primed host defence was shown to be involved in the *Arabidopsis-Hyaloperonospora* spp. interactions, i.e. defence gene induction only occurs when the host plant is challenged with the pathogen (Massoud *et al*., 2012). The salicylic acid (SA) or jasmonic acid/ethylene (JA/ET) defence response pathways have been reported as being involved in phosphite induced host defence responses. The SA response was involved in the *P. cinnamomi-Eucalyptus, Hyaloperonospora arabidopsidis-Arabidopsis* and *P.* cinnamomi-lupin systems (Jackson *et al*., 2000; Molina *et al*., 1998; Massoud *et al*., 2012; Groves *et al*., 2015). In contrast the JA/ET pathways were shown to be involved in the *P. cinnamomi-Arabidopsis* system (Rookes *et al*., 2008). Dalio *et al*. (2014) furthermore reported that both JA/ET and SA marker genes were upregulated upon phosphite application in *Fagus sylvatica* (European beach) seedlings inoculated with *Phytophthora plurivora.*

MANAGING *PHYTOPHTHORA CACTORUM* ON APPLES USING PHOSPHONATES

The first studies that evaluated phosphonates for managing *P. cactorum* on apple, were done using pot trials or bioassays on orchard trees. Orlikowski *et al*. (1986) evaluated Aliette (80% a.i.) applied as one foliar spray (0.5%) on potted apple trees that were artificially inoculated with *P cactorum*. The effect on the reduction in lesion lengths (40 to 56%) was only seen the following year, and not shortly after application when the product was used curatively. It was further found that phosphonates had a protective effect that lasted for at least 15-months in the trunk of trees (Orlikowski *et al*., 1986). Orlikowski *et al.* (1986) also evaluated Aliette as a 0.25% foliar spray, and 5% and 10% trunk paints, which yielded lesion inhibition lengths that were comparable to the 0.5% foliar spray. Long *et al*. (1989), evaluated the efficacy of fosetyl-Al on 10-year-old orchard trees, which were trunk injected with 4 g a.i. fosetyl-Al. A bioassay, which was used to evaluate the efficacy, showed that the treatment provided at least 15-months of control when *P. cactorum* was inoculated on the shoots and crowns of trees. No correlation was found between the inhibition of lesion length and phosphorous acid concentrations in the shoots (Long *et al*., 1989).

Only a limited number of studies have investigated the efficacy of phosphonates against Phytophthora root- and crown rot on apple under orchard conditions. Most studies were done on *P. cactorum* in the late 1980s and early 1990s by the research group of Utkhede. Utkhede & Smith (1991) evaluated the efficacy of fosetyl-Al foliar sprays and drenches on naturally infected *P. cactorum* apple trees, which showed crown and root rot symptoms. Three trials were conducted in orchards containing trees that were 25, 10 or 7 years old. Fosetyl-Al was applied as a drench/trunk treatment or foliar spray. Application of the drench/trunk application consisted of the application of the product as a 5L trunk spray (60cm of trunk) and soil drench, at a rate of 10 g a.i./tree. The drench/trunk applications were made in autumn and spring in the first 2-years, and spring in the 3rd year. Foliar sprays were applied in spring and autumn, which included two sprays in year one, three sprays in the 2nd year and two sprays in the 3rd year, at a rate of 5 g a.i./tree.

All autumn applications were made after harvest. A significant increase in trunk diameter and yield was only obtained with the foliar spray in the one 7 year orchard, but not in the other orchards (Utkhede & Smith, 1991). Subsequently, Utkhede & Smith (1993) reported on the effect of fosetyl-Al against Phytophthora crown rot in one trial that contained newly planted apple trees that were artificially inoculated annually with *P. cactorum* at the crown region. Fosetyl-Al was applied from planting onwards for the first 4-years in autumn and spring, and in the subsequent 3-years only in spring. The product was applied as a trunk spray (60 cm high) and soil drench at a volume of 4.5 L/tree, and at a final rate of 5g a.i./tree. Over the 7 year trial period, no significant increases in trunk diameter were seen for individual years, but the cumulative increase in trunk diameter over the 7 years was significantly better than the untreated control. The same observation was made for yield, with only the cumulative yield for three years being significantly better than the untreated controls (Utkhede & Smith, 1993). Utkhede and Smith (1995) reported that two annual forsetyl-Al 2g a.i. /l foliar sprays controlled *P. cactorum* in newly planted trees, resulting in enhanced tree growth and yield.

Managing *Pythium* spp. with phosphonates

Although it is well known that *Phytophthora* diseases can be managed by phosphonates, limited reports have been published on the management of *Pythium* species in agricultural or floricultural crops. *Pythium aphanidermatum* and other *Pythium* spp. that incite *Pythium* blight of turfgrasses were preventatively managed using phosphonates (Cook *et al*., 2009). Abbasi, and Lazarovits, (2006) found that the efficacy of phosphonates varied with rate and timing of application. Seed treatment of AG3 phosphonate formulation suppressed *Pythium* damping-off of cucumber (*Pythium aphanidermatum* or *P. ultimum* inoculum or into muck soil

naturally infested with *P. irregulare*, *P. ultimum*, and other *Pythium* spp) provided 80% control of damping-off in all infested substrates tested under growth room conditions (Abbasi and Lazarovits, 2006). Under field conditions, Abbasi and Lazarovits (2006) found that seed treatment of phosphonates resulted in a 63% control of damping-off in a *Pythium*-infested muck soil. A post-plant drench yielded 53% control in comparison to the control (Abbassi and Lazarovits, 2006). Weiland *et al*., (2014) reported the control of damping-off in Douglas-fir (*Pseudotsuga menziesii*) incited by *P. dissotocum, P. irregulare, and P. 'vipa'* when fosetyl-Al and phosphorous acid was applied as a soil drench.

Managing ARD with phosphonates

Only one study evaluated phosphonates for the management of ARD. Autio (1991) evaluated fosetyl-Al in Massachusetts, in the northeastern United States. Apple trees in the orchard trial were planted at a 1 to 3 m offset from the previous tree row to reduce the impact of ARD. Fosetyl-AL foliar sprays, applied as three sprays at bi-monthly intervals in the first year of planting, were evaluated. Two different dosages, a 2.4 g Aliette 80% WP/liter and 4.8 g Aliette 805 WP/liter, were evaluated. There was a significant linear effect of Aliette dosage, with both treatments resulting in a significant increase in trunk diameter and shoot growth in the first year, but not in the subsequent two seasons (Autio, 1991). Fumigation was not included as a treatment in the trials, and therefore the performance of the Aliette treatment to this standard practice is unknown. Furthermore, the causative ARD pathogens were not investigated.

THE USE OF PHENYLAMIDES IN MANAGING OOMYCETES

Products, translocation and mode of action

Phenylamides are systemic compounds that include several compounds such as metalaxyl, furalaxyl, oxadixyl, benalaxyl and ofurace. This specific group of fungicides has a protective, curative and eradicative action against oomycetes (Metalaxyl used to be the most widely used phenylamide, but has been replaced by mefemoxam (methyl N-(2, 6-dimethylphenyl)-N-(methoxyacetyl)-D-alaninate) in many regions of the world. Metalaxyl contains the active racemic mixture of R- and S-enantiomers, whereas mefenoxam only contains the R-enantiomer. Phenylamides are effective over a wide range of ecological soil parameters containing a range of pH values and temperatures (Singh and Tripathi, 1982). Therefore, phenylamides are used in many crops around the world, including temperate, sub-tropical and tropical regions (Sukul and Spiteller, 2000a).

The translocation of phenylamides is generally through the xylem, having an acropetal mobility through the transpiration stream. Some small quantities have been

reported to move basipetal (Tri-parthi and Singh, 1983; Timmer and Castle, 1985). For example in citrus, trunk paints have been found effective in controlling *Phytophthora nicotianae* (Timmer and Castle, 1985). However, drenching of phenylamide is the most frequently used method for the management of soilborne diseases since it results in good root persistence (Sm and Leonian., 1993).

Phenylamides fungicides target the nucleic acid synthesis (Sukul and Spiteller, 2000) and ribosomal RNA polymerases in oomycete pathogens (Davidse, 1988). Nucleic acid synthesis inhibition is due to the inhibition of RNA polymerase I system (Sukul and Spiteller, 2000). Phenylamides have also been shown to cause morphological changes in oomycetes. Jing and Grossmann (1991) observed thickening and degeneration of *Phytophthora infestans* hyphal cells. Hwang (1990) noted excessive cell shrinking in addition to pathogen cell wall membrane separation in *Phytophthora capsici*.

Phenylamide reports of resistance

Problems associated with the use of phenylamides include resistance development and microbial break down in soils. Oomycete pathogens commonly develop resistance against phenylamides when used excessively (Erwin and Ribeiro, 1996; Muller and Gisi, 2007). Therefore, for soilborne pathogens, phenylamides are recommended to be used as a single application each growing season. Drenching applications expose the fungicide to rapid microbial degradation within the soil (Monkiedje and Spiteller, 2002).

The first resistance in oomycetes against phenylamides was reported in 1979, 24-months after metalaxyl was launched as a single use fungicide in some countries. The first phenylamide-resistant isolates were reported for *Pseudoperonospora cubensis* in greenhouse cucumbers (Reuveni, 1980). Subsequently, resistance was also reported for *Plasmopara viticola* on French grapes 1983 (Moreau, 1987), *Bremia lactucae* on lettuce (Crute, 1987; Morton, 1988), *Pythium* spp. in turf grass (Sanders, 1984), and *Peronospora tabacina* in tobacco (Bruck and Apple, 1982). Subsequently, phenylamide resistance has been reported in many more *Pythium* and *Phytophthora* species, including some ARD pathogens such as *P. irregulare* (Hwang and Benson, 2005).

P. sylvaticum isolates in some, but not all ARD orchards were found to be insensitive to metalaxyl in *in vitro* tests, in comparison to *P. heterothallicum* that was sensitive (Mazzola, 2002). The insensitivity of *P. sylvaticum* in some orchards was attributed to previous metalaxyl applications in the orchards. *Pythium dissotocum* isolates were also relatively insensitive to metalaxyl in ARD orchards (Mazzola *et al*., 2002).

The use of phenylamides for the management of *P. cactorum* on apple

Several studies have evaluated the efficacy of metalaxyl against Phytophthora crown and root rot of apple under orchard conditions. Ferree and Ellis (1984) applied metalaxyl (Ridomil ®) at a rate of 500 ppm as a 1 liter soil drench to newly planted apple trees that were inoculated with *P. cactorum.* Applications were made in spring and autumn in two consecutive years. The treatment reduced tree losses and resulted in a significant increase in trunk dia. after 4-years, whereas yield was not significantly increased (Ferree and Ellis, 1984). Utkhede *et al*., (1987) showed that soil drenching with metalaxyl could control *Phytophthora* root and crown rot on apples. Metalaxyl treated trees remained alive and productive in contrast to the non-treated control trees that died in just 3-years (Utkhede & Li, 1987). Utkhede & Smith (1992) evaluated the effect of long-term application of metalaxyl to control crown and root rot of apple trees. Metalaxyl (1 g a.i./tree) was applied for the first 3-years following planting, as a 4.5 liter soil drench and bark spray (60 cm area above the soil line) in spring and autumn, followed by one application in spring for the next 4-years. The treatment did not result in a significant increase in trunk diameter over the 7-years. However, the cumulative yield of three harvests was significantly higher than the untreated control (Utkhede & Smith, 1992). Utkhede & Smith (1991) also evaluated metalaxyl soil granular applications and a soil drench combined with a trunk spray for the control of crown and root rot on apple trees (7 to 25-years old) naturally infected with *P. cactorum* in three orchards. The metalaxyl treatments were applied twice in spring and autumn in the first two years, and in spring in the 3rd year at a rate of 1 g a.i./tree in a 5L volume. Both treatments were effective only in one of the orchards (7-year old), where it resulted in a significant increase in trunk diameter and yield (Utkhede & Smith, 1991). Orlikowski *et al*. (1996) reported that in potted apple trees, metalaxyl applied as a trunk paint (25% a.i. applied at 30-50ml per tree) resulted in at least a 15-month residual activity against *P. cactorum* in trunks and twigs, and completely inhibited the pathogen 1.5 to 4.5 months post-application.

The use of phenylamides for managing ARD

Only one study evaluated metalaxyl for the management of ARD in Massachusetts. Ridomil® was applied once to trees at 2.4 ml/L as a 1 liter soil drench. The treatment resulted in a significant increase in trunk and shoot growth in the 1st year, but not in the subsequent two growing seasons (Autio *et al.,* 1991). The causative ARD pathogens were not investigated at the trial site.

USE OF FENAMIPHOS FOR MANAGING *PRATYLENCHUS* SPP.

Fenamiphos (ethyl3-methyl-4-(methylthio) phenyl (1-methylethyl) phosphoramidate), is an organophosphorous insecticide and nematicide. It is widely used in agriculture as a non-fumigant option, and is flexible for use as a pre- or post-plant application. This nematicide possesses systemic and contact properties towards nematodes. Fenamiphos is generally marketed as Nemacur ® (Loser and Kimmerle, 1971). Fenamiphos is mainly applied to the soil as a drench or granular application, since the formulation may be wettable granules or an emulsifiable concentrate (Tomlin, 2000).

Fenamiphos is active against a wide range of nematodes including *Meloidogyne, Heterodera* and *Pratylenchus.* In apples, fenamiphos has been shown to improve trunk diameter and shoot length when applied at 20.2kg a.i /ha in autumn and spring time (Santo and Wilson, 1990). The product is furthermore registered and used on a wide range of crops (Muchena and Bird, 1987; Santo and Wilson, 1990; Greco and Thomason, 1980).

Upon application, fenamiphos assumes several translocation pathways, depending on application method. Fenamiphos, may be assimilated rapidly through plant foliage or roots and can be translocated via the phloem sieve elements (Zeck, 1971). Within the soil, fenamiphos dissipates slowly and can persist in the soil for 12-weeks. It is effective within 25-60 cm of the soil surface (Homeyer and Wagner, 1981). Fenamiphos has minimal water solubility within the range of 0.04-0.07%. These specific properties reduce its rapid loss in sandy soils during heavy irrigation. Due to its reduced mobility, it is one of several contact and systemic nematicides that is least likely to reach groundwater

The mode of action of fenamiphos is dose dependant. At lower concentrations, it interferes with nematode chemoreception. This results in a reduced ability of nematodes to locate their host root and establish a successful infection. At greater concentrations, fenamiphos interrupts the nematode's hatching process as well as nematode motility (Marban-Mendoza and Viglierchio, 1980b; Pree, 1990). Within the soil matrix and plant tissue, fenamiphos is converted into several metabolites with activity against nematodes. In the soil matrix the thiooxidation process degrades fenamiphos to a sulfoxide and sulfone. The sulfoxide is the toxophore to nematodes. Sulfoxide is usually associated with acetylcholinesterase (Marban-Mendoza and Viglierchio, 1980b; Pree, 1990). Sulfoxide and sulfone compounds possess robust nematicidal properties (Waggoner, 1972; Krause, 1986). For this mechanism of action to occur, the nematode cuticle absorbs the nematicidal compounds. The compounds then interrupt functioning of the nervous system by binding to the enzyme acetyl-cholinesterase (AChE) (Haydock, 2006). Disruption associated with neuroenzyme activity brings about excessive transmission of impulses from the nervous

system. This process impacts both nematode physiological in addition to behavioural activities (Homeyer and Wagner, 1981; Pree *et al.,* 1990).

CONCLUSION

ARD management practices world-wide usually consist of the application of soil fumigation using chloropicrin/1,3 di-chloropropene based fumigants. However, in South Africa, reports have been made on the occurrence of ARD symptoms for soils that have been fumigated. The occurrence of apple replant disease symptoms can be attributed to incorrect fumigant applications. Alternatively, external inoculum sources such as irrigation water and planting material have been found to re-introduce ARD pathogens into fumigated soil. In South Africa, apple nursery trees have been found to contain several ARD pathogens including *Pratylenchus* spp. *P. irregulare, P. ultimum, P. sylvaticum* and *'Cylindrocarpon*'-like spp. (Moein *et al*., 2016).

Efforts should be made to secure cost effective and ecologically friendly integrated ARD management approaches. Alternative approaches to fumigation for managing these agents could be semi-selective chemicals or brassica seed meals. However, the mixture of brassica species seed meals that are known to suppress ARD (Mazzola *et al.,* 2015) is not available in South Africa. Semi-selective chemicals that have potential for managing ARD include phenylamides and fenamiphos. These chemicals have shown potential for suppressing ARD when used independently under glasshouse conditions (Tewoldemedhin *et al.*, 2011c). In addition to phenylamides and fenamiphos, phosphonates may also contribute towards the management of ARD, since phosphonates are known to suppress *P. cactorum* (Utkhede & Smith, 1993), which is an important component of ARD in South Africa. Autio (1991) furthermore reported that phosphonates have potential for managing ARD, although only for the first year of growth (Autio, 1991). Co-application of phenylamides, fenamiphos and phosphonates may thus have potential for suppressing a wide range of ARD pathogens, when applied over an extended 3-year period. Prolonged application of semi-selectives are important since apple trees are most susceptible to ARD during their first 3-years of growth (Mazzola, 1998).

Phytophthora root rot is another soilborne disease of apples, which is economically important. This disease causes tree death and stunted growth, with symptoms becoming evident, one to 2-years after orchard establishment. Phosphonates have been used to effectively manage Phytophthora root- and crown rot on apple (Utkhede and Smith, 1995). Very limited work has been done on the optimisation of phosphonate application methods on Phytophthora crown and root rot on apple. In order to optimize phosphonate application methods for managing Phytophthora root rot, quantification of root phosphite will be

important. The mode of action of phosphonates in the apple *P. cactorum* pathogen system is unknown. Root phosphite concentrations could help to elucidate the mode of action of phosphonates. Limited information is available on the *in vitro* phosphite sensitivity of oomycete ARD pathogens, which can also contribute towards our understanding of the mode of action of phosphonates.

This thesis endeavours to manage ARD and Phytophthora root rot, with specific emphasis on oomycetes and phosphonates. The study evaluated the efficacy of co-applying phosphonates, phenylamides and fenamiphos for managing ARD pathogens on fumigated and non-fumigated orchard soils. The orchard trials also evaluated the efficacy of two fumigants that differed in chloropicrin/dichloropropene content. In a second set of orchard trials, different phosphonate application methods were evaluated on asymptomatic apple trees by monitoring the temporal nature of root phosphite concentrations in the trials. The suppression of the ARD pathogens *P. irregulare* and *P. cactorum* in the roots of the asymptomatic trees were also investigated. In an attempt to better understand the mode of action of phosphite, the *in vitro* phosphite sensitivity of the aforementioned ARD pathogens along with *P. vexans* were also investigated. Subsequently, optimized phosphonate application methods were evaluated in a third set of orchard trials for the management of Phytophthora root rot in young apple orchards. The efficacy of treatments was evaluated by measuring tree growth and *P. cactorum* concentrations in roots.

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CHAPTER 2

Management of apple replant disease using semi-selective chemicals

ABSTRACT

Apple replant disease (ARD) occurs when old apple orchards are replanted, resulting in a reduction in tree growth. Specific groups of fungi, oomycetes and nematodes cause ARD. In South Africa, oomycetes that include a few *Pythium* spp. and *Phytophthora cactorum* were previously shown to play an important role in ARD, with nematodes (*Pratylenchus* spp.) occasionally being involved. The aim of the current research was to evaluate in three orchard trials whether semi-selective chemicals (fenamiphos, metalaxyl, imidacloprid and phosphonates) targeting these groups of pathogens could suppress ARD. Two fumigant formulations differing in chloropicrin and 1,3-dichloropropene ratios were also evaluated. In all orchards, tree growth (increase in trunk diameter and shoot length), 3 or 4- years post-plant, was significantly higher for all treatments relative to the untreated control. This included the independent use of semi-selective chemicals, which furthermore performed in a manner similar to the two fumigation treatments. The two fumigant formulations did not differ significantly in tree growth responses. The effect of treatments on yield data was variable. The independent use of semi-selective chemicals resulted in yields that were significantly lower than the control treatment in all three orchards. In two orchards, fumigant treatments, with the exception of one low rate chloropicrin fumigant, resulted in yields that were significantly higher than the untreated control. In the third orchard, only the semi-selective treatment combined with fumigation resulted in a significant increase in yield. Quantitative real-time PCR analyses of marker ARD pathogens in tree roots 20 months after planting indicated that *Phytophthora cactorum* contributed to disease development at all three orchard sites. This was evident from significant reductions in *P. cactorum* concentrations in treatments that significantly improved tree growth in two orchards. Furthermore, a significant negative correlation existed between *P. cactorum* quantities detected in tree roots and increase in trunk diameter, shoot length and less often yield, in all orchards. *Pratylenchus* spp. densities in two of the orchrds showed the same trends as *P. cactorum* quantities in relation to tree growth response. There was furthermore a significant positive correlation between *P. cactorum* quantities and *Pratylenchus* spp. root densities.

INTRODUCTION

Apple replant disease (ARD) is a root disease of apples that occurs when apple is replanted on soil previously planted to apple or related species. It is a soilborne disease that causes tree stunting and delayed apple fruit-bearing (Mazzola and Manici, 2012). Up to a fifty percent loss in orchard lifespan productivity and profitability can be incited by ARD (Van Schoor *et al*., 2009). The biological agents involved in ARD consist of a wide spectrum of organisms including fungi, nematodes and oomycetes. The occurrence of these pathogens can vary across ARD sites (Mazzola, 1998; Manici *et al*., 2003; Tewoldemedhin *et al*., 2011b). *Pratylenchus penetrans* is known as a causal agent of ARD world-wide, although in South Africa *Pratylenchus* spp. other than *P. penetrans* have been associated with disease development (Tewoldemedhin *et al.,* 2011b). The fungal causative agents include multi- and binucleate *Rhizoctonia* spp., with the multinucleate *R. solani* AG-5 and AG-6 being identified as highly virulent pathogens in the USA (Mazzola 1997).

However, in South Africa, multinucleate *R. solani* AG-5 and AG-6 have not been identified, and only a few bi-nucleate *Rhizoctonia* species have been isolated from apple. Most *Rhizoctonia* bi-nucleate isolates are non-pathogenic, with only a few having low virulence (Mazzola 1997; Manici *et al.,* 2003; Tewoldemedhin *et al*., 2011a). Manici *et al.* (2013) recently hypothesized that bi-nucleate *Rhizoctonia* spp. and *Fusarium* spp. rather have a commensal or mutual symbiotic relationship with apple trees, than a pathogenic relationship. Another group of fungi involved in ARD is the '*Cylindrocarpon'*-like fungi. Isolates within specific species of this group are known to range from non-pathogenic to mildly virulent (Jaffee *et al*., 1982b, Braun, 1991; 1995; Dullahide *et al*., 1994; Mazzola, 1998; Manici *et al*., 2003; Tewoldemedhin *et al*., 2011c). The taxonomy of the '*Cylindrocarpon*'-like fungi has gone through major revisions with some species previously associated with ARD now located within the genera *Neonectria, Thelonectria, Ilyonectria* and *Dactylonectria*. Several species have furthermore been split into multiple species (Chaverri *et al*., 2011; Cabral *et al.*, 2012; Lombard *et al*., 2014). For the purpose of this study, reference will only be made to '*Cylindrocarpon*'-like fungi, and not to the new genera names. In South Africa, oomycetes seem to play a major role in ARD based on their wide-spread occurrence and high virulence. These oomycetes include *Phytophthora cactorum,* several *Pythium* spp. (*P. ultimum, P. irregulare, P.. sylvaticum*) and *Phytopythium vexans* (Tewoldemedhin *et al*., 2011a, b). These pathogens have also been reported as causal agents of ARD in the USA and Italy (Mazzola, 1998; Manici *et al*., 2003).

Due to the complexity of the ARD causal pathogen complex, pre-plant soil application of broad-spectrum fumigants is the primary measure employed to manage the disease. Chloropicrin combined with 1,3-dichloropropene is the most commonly used fumigation

treatment for this purpose (Yao *et al*., 2006., Cabrera *et al*.,2015., Mazzola and Manici, 2012; Mazzola *et al*., 2015). The ratio of these two products in formulations can differ, depending on whether the main target organisms are fungi or nematodes. Chloropicrin primarily has fungicidal activity, whereas 1,3-dichloropropene controls nematodes (Lamberti *et al.*, 2000; Csnos *et al.,* 2000, Gilreath and Santos, 2004; Gilreath *et al*., 2005, Graber *et al*.,2011b). Due to the high cost of fumigation, only the old tree row is fumigated prior to replanting orchard ground. The fumigation effect is relatively short-lived based on growth response (Auvil *et al*., 2011), likely as a result of rapid recolonization of fumigated soils (Mazzola *et al*., 2015). Nonetheless, fumigation is effective in managing ARD since as apple trees age, the trees become less susceptible to the causative ARD agents.

The use of systemic chemicals including phosphonates, metalaxyl and fenamiphos, may hold potential for managing ARD in South Africa since they target oomycetes and nematodes. These chemicals have been used independently on apple trees and other tree species to control either oomycetes or nematodes (Thomidis and Michailidis, 2002, Vawdrey and Westerhuis, 2007, McMahon *et al*., 2010, Akinsanmi, and Drenth, 2013). Phosphonates and phenylamides, specifically metalaxyl and mefenoxam, are well known for controlling *P. cactorum* on apple under orchard conditions (Utkhede and Smith, 1993; Utkhede and Smith, 1995, Boughalleb *et al*., 2010; Sharma *et al*, 2014). Most countries furthermore, have a registration for metalaxyl or mefenoxam (contains only the active R-enantiomer of metalaxyl) on apple. Similarly, fenamiphos is a well-known product registered for managing *Pratylenchus* spp. on apple. However, in several countries, excluding South Africa, it is no longer available due to environmental concerns (Wesseling *et al*., 2005).

The main aims of the study were to evaluate in three ARD orchard trials whether (i) two fumigants differing in their ratio of chloropicrin/1,3-dichoropropene differed in efficacy and (ii) if semi-selective chemicals (fenamiphos, metalaxyl, phosphite and imidacloprid) applied independently or on fumigated soil can improve ARD control. To better understand the performance of treatments, the importance of a few ARD marker pathogens and parasitic nematodes were investigated in orchards. Their quantities were determined, as well as their correlation with tree growth and with each other. The ARD marker microbial pathogens included '*Cylindrocarpon*'-like fungi, *R. solani* AG-5, *P. cactorum*, *P. ultimum, P. irregulare, P. sylvaticum and P. vexans,* and the parasitic nematode *Pratylenchus.* A glasshouse apple seedling bioassay was used to evaluate the potential severity of ARD for each orchard soil, and the relative occurrence of causative ARD pathogens, with an emphasis on *Pratylenchus* spp. and oomycetes.

MATERIALS AND METHODS

Orchard sites

Trials were conducted at three orchard sites (Paardekloof, Glenfruin and Remhoogte), situated in the Western Cape Province in South Africa (Table 1). The region is characterised by a Mediterranean climate with cool, wet winters and warm, dry summers. Two orchards (Remhoogte and Paardekloof) were located in Ceres (34°16'60"S,20°36'00"E,33°00'00"S,19°18'00"E) and one (Glenfruin) in Grabouw (34°10'60"S,19°03'00"E). Two of the orchards were planted in 2013 and the other orchard in 2014. The orchards contained either M7 or MM109 rootstocks (Table 1). The soil types varied from sandy loams to clay soils (Table 2).

Orchard soil evaluations using an apple seedling bioassay under glasshouse conditions

An apple seedling bioassay was conducted for each of the three orchard soils. A bulk soil sample was collected to a depth of 30 cm in all three orchards prior to the application of treatments. The bioassay was used to characterize potential replant disease severity in the orchards under optimal environmental conditions for oomycetes, i.e. frequent irrigation. Additionally, the bioassay was used to determine the presence of nematodes and oomycetes through root isolation studies. qPCR was used to quantify the density of oomycetes (*P. ultimum, P. cactorum, P. sylvaticum, P. irregulare* and *P. vexans*), *'Cylindrocarpon'*-like fungi and *R. solani* AG-5 in seedling roots.

Bioassay establishment and treatments

Four-week-old Golden delicious apple seedlings were produced from germinated seed as previously described (Tewoldemedhin *et al.,* 2011c). The 4-week old seedlings were planted into soil that received the following treatments: (i) untreated control, (ii) pasteurized soil and (iii) pasteurized soil + 15% untreated soil. The soil was steam pasteurized for two hours at 80˚C on two consecutive days, using a Systec Pasteurizer (VE150, Wettenberg, Germany). The pasteurized soil was ventilated for at least 2-days prior to the planting of seedlings. Each treatment was replicated six times, in a completely randomized block design. Each replicate consisted of a 1 L planting bag, containing three apple seedlings. The length and weight of seedlings were recorded just after and prior to planting respectively. Seedlings were grown for 3 months under glasshouse conditions of 26± 2 ˚C and a humidity range of 60 % to 70 %.

Irrigation was applied twice a day for 5 min using drip irrigation. Fertilizer was applied every 10-days by applying 100 ml per planting bag of water soluble classic Multifeed

(Plaaskem, Pvt Ltd, Witfield, South Africa) with the active ingredients of Nitrogen -90g/kg, Phosphorous- 82g/kg, Pottasium-158g/kg, Manganese-900mg/kg, Zinc-350mg/kg, Boron-1000mg/kg, Molybdum-70mg/kg, Iron-750mg/kg, Manganese-300mg/kg, Copper-75mg/kg. Foliar pathogens and pests were managed by applying Agromectin EC (abamectin 18g a.i./l Arysta LifeScience Pvty Ltd), Arcastin Flo (cyhexatin 600g a.i./L, Sipcam Southern Africa Pty Ltd), Topaz 200 EW (penconazole 200g a.i./L, Syngenta Pvty Ltd), Nimrod EC (bupirimate 250g a.i./L, Makhteshim- Agan SA Pvty, Limited) and Mospilan 20 SL (acetamiprid 222g a.i./L, Plaaskem Pty Ltd).

Evaluation of seedling growth parameters

After 3 months, the seedlings were evaluated for the increase in length and fresh weight. The length was measured prior to removal of seedlings from the planting bags. The soil was washed from seeding roots, and the total fresh weight (roots plus shoots) was determined. The increase in weight and height data from the untreated control and the pasteurized treatment were used to calculate the relative percentage increase in height or weight for each orchard, as described by Hoestra (1968). The relative percentage increases were used to determine the ARD severity according to Hoestra (1968).

Isolation and identification of oomycetes

Oomycetes were isolated from the washed seedling roots. Twenty feeder roots from each replicate bag of the untreated control seedlings were plated onto PARPH medium (Jeffers and Martin, 1986) to which 0.8ml/L Benomyl (500g benlate/kg, Villa Crop Protection, Kempton Park, South Africa) was added. Plates were incubated at room temperature for 2 to 3-days. Hyphal tips emerging from roots were transferred to potato dextrose agar (PDA) (Biolab Diagnostics, Midrand, South Africa) amended with 0.04g/L streptomycin, and plates were incubated for 3-days to 7-days at 25°C. Mycelia were scraped from the plates and DNA extracted using a slightly modified CTAB method (Lee and Taylor, 1990).

Oomycete isolates were first grouped into PCR restriction fragment length polymorphism (RFLP) groups (Mazzola *et al*., 2009). PCR-RFLP analyses were conducted by first amplifying the internal transcribed spacer (ITS) region using primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990) and ITS6 (5'-GAAGGTGAAGTCGTAACAAGG-3') (Cooke and Duncan, 1997). The PCR reaction consisted 0.2 µM of each forward and reverse primer, 200 µM of each dNTP (ThermoFisher Scientific, Randburg, South Africa), 1x PCR buffer (Bioline, Inc., Taunton, MA), 0.65 U BIOTAQ™ DNA polymerase (Bioline), 0.2 mg bovine serum albumin (BSA) Fraction V (Roche Diagnostics Randburg, South Africa), 5 µL DNA (5-10 ng) and 2 mM $MgCl_2$ in a final

volume of 40 µL. Amplifications were conducted in a 2700 Applied Biosystems machine (Foster City, CA), starting with an initial denaturation of 5 min at 94°C, followed by 32 cycles of 30 s at 94°C, 30 s at 55°C, 90 s at 72°C and a final extension of 7 min. at 72°C. PCR products were electrophoresed through 1.0% agarose gels containing ethidium bromide in 1x Tris-acetate EDTA buffer (Sambrook, Fritsch and Maniatis, 1989). DNA fragments were visualized under UV illumination. Successful amplifications were restriction digested in a reaction containing the enzymes HinfI (1.25 µl) and HhaI (2.5 µl) (Thermo Scientific, Glen Burnie, Maryland, USA), 1 x tango buffer and 8 µl PCR product in a final volume of 25 µl. The reaction was incubated at 37˚C for 12 to 18 hours in a water bath. DNA fragments were separated on 3 % agarose gels. Isolates that yielded similar PCR-RFLP patterns were grouped into the same PCR-RFLP group.

The PCR products of isolates representing the different PCR-RFLP groups were cleaned using the MSB® Spin PCRapace (Invitek, Berlin, Germany) kit according to manufacturer's instructions. PCR products were sequenced by the Central Analytical facility at Stellenbosch University using a BigDye® terminator V3.1 cycle sequencing kit (Applied Biosystems, CA,USA), followed by capillary electrophoresis on a 3130x1 Genetic analyser (Applied Biosystems). The identity of the sequences was determined by BLAST analyses in GenBank (National Center) (http://www.ncbi.nlm.nih.gov/Genbank/). For *Pythium* species identification, only reference sequences submitted by Levesque and de Cock (2004) were used, or published sequences of recently described new species. *Phytophthora* sequences were submitted to *Phytophthora*-ID database for comparative analysis (version 2.0) (Grunwald *et al.,* 2011; http://phytophthora-id.org/index.html).

Nematode extraction and quantification

Approximately 5 g of washed seedling roots of each replicate of the untreated control seedlings were sent for parasitic nematode analyses at Nemlab (Durbanville, South Africa). The samples, after being taken, were stored at 4 ˚C for one or two days before being delivered immediately to Nemlab. Nematodes were extracted using the centrifugal sugar flotation method (Jenkins, 1964).

qPCR quantification of ARD marker microbial pathogens from roots

DNA extraction from roots. The washed apple seedling feeder roots (approximately 70 mg) from all the replicates of the untreated control seedlings were lyophilized and stored at -80°C. The lyophilized roots from each replicate were analysed separately. The roots were fragmented using a sterile plastic pestle, and DNA was extracted from a 20 mg root sub-sample. The roots were further powdered by adding 0.5 g glass beads (2 mm) and shaking for 10 min in a Retsch MM301 mixer mill (GmbH and Co, Haan, Germany). DNA was

extracted using the NucleoSpin PLANT II kit (Macherey-Nagel GmbH and Ko, Duren, Germany) according to manufacturer's instructions. Extraction buffer PL1 was used, and in the last step, DNA was eluted by applying two 50 µl aliquots of elution buffer.

qPCR standard curves. Standard curves were constructed for each of the investigated ARD marker microbial pathogens including *P. cactorum, P. vexans, P. irregulare, P. ultimum, P. sylvaticum, P. vexans,* 'Cylindrocarpon *macrodidymum'* and *Rhizoctonia* AG-5. The oomycete and '*Cylindrocarpon macrodidymum'* standard curves were generated using genomic DNA which was extracted from pure cultures grown in pea broth (Goodwin and Fry, 1994). Mycelia from cultures were harvested, lyophilized, powdered with a spatula, and 20 mg was used for DNA extraction using the NucleoSpin plant II kit according to manufacturer's instructions. The genomic DNA was quantified using a Nanodrop spectrophotometer (ND1000, Nanodrop technologies, USA). For *R. solani* AG-5, a gBlock (Integrated DNA technologies (IDT), Cape Town, South Africa) was used as DNA template for constructing the standard curve. The gBlock contained the DNA fragment amplified by the qPCR primers. The gBlock fragment was dissolved to a final concentration of 1.568 x 10^{12} copies and was serially diluted for use in the standard curve. Standard curves consisted of a total of eight concentrations obtained through fivefold serial dilutions. Each standard curve concentration was assayed in triplicate. All assays contained a non-template control that used water instead of DNA template.

With the exception of *P. vexans* and *P. ultimum,* all pathogens were quantified using Syber Green ® assays. *Pythium vexans* and *P. ultimum* assays were probe-based assays. The Kapa Syber fast qPCR master mix (Sigma-Aldrich, SA, Cape Town, South Africa) was used for SYBR® Green assays, while the Kapa probe master mix (Sigma-Aldrich, SA, Cape Town, South Africa) was used for the probe-based assays. Assays were conducted in a total volume of 20 µl. The qPCR reactions contained the primers and probes (Integrated DNA technologies [IDT]) targeting the different pathogens at concentrations that are indicated in Table 3. All probes were labelled with 6-FAM and an Iowa Black ® dark quencher. Syber green amplifications consisted of an initial denaturation at 95°C for 5 min followed by 40 cycles of denaturing at 95°C for 10 s, annealing temperature and times as indicated in Table 3, and extension at 72˚C for 20 s. The exception was the *P. cactorum* assay that used a 27 s extension time. The probe-based assays consisted of denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 s, and annealing/extension temperature and times as indicated in Table 3. The standard curve of all pathogens were linear (R^2 = 0.98-0.99). Efficiency and M-slope values are shown in Table 3 for all assays.

qPCR quantification from roots. The ARD pathogens were amplified from root DNA samples using the same reaction and amplification conditions that were employed for

constructing the standard curves. Each reaction contained 2 ul root DNA , which was diluted fivefold. The roots from each replicate bag were analysed in duplicate. Two standard curve control (calibrators) samples were included in all runs, which allowed the importation of standard curves for pathogen quantification.

Orchard trials evaluating different management approaches

Trial design and treatments

Three orchard trials were established two in 2013 (Paardekloof and Glenfruin) and one in 2014 (Remhoogte). The cultivars and rootstocks used in each trial, and the soil type of the orchards are shown in Table 1 and Table 2 repectively.

The following treatments were applied in the two orchards that were planted in 2013 (Paardekloof and Glenfruin):

(i) untreated control
(ii) pre-plant fumigation with 33.3% chloropicrin and 60.8% 1,3-dichloropropene [Low chl/dichl]
(iii) pre-plant fumigation with 57.0% chloropicrin and 38.0% 1,3-dichloropropene [High chl/dichl]
(iv) semi-selective chemicals [fenamiphos, imidacloprid, metalaxyl and potassium phosphonate) [Independent semi-selectives]
(v) pre-plant fumigation with 33.3% chloropicrin and 60.8% 1,3-dichloropropene + semi-selective chemicals [Low chl/dichl + semi-selectives]
(vi) methyl bromide fumigation [Mbr].

The Remhoogte trial planted in 2014 contained treatments i to v, but the vi treatment (Mbr fumigation) was replaced by a treatment consisting of pre-plant fumigation with the high chl/dichl fumigant + semi-selective chemicals (High chl/dichl + semi-selective) (Table 5).

In all three orchards, treatments were replicated six times in a completely randomized design. Each replicate consisted of ten trees.

The semi-selective treatment applications consisted of a soil drench at planting, followed by a 2-year phosphonate application program. The soil drench was applied shortly after planting, when trees started budding and showed clear signs of growth initiation. The soil drench was applied as a 2 L drench per tree, which contained 1.05 g imidacloprid, 1 g fenamiphos, 2 g metalaxyl and 12 g mancozeb. The 12 g mancozeb formed part of a cost-effective metalaxyl formulation Metazeb 700 WP (Villa Crop Protection, Aston Manner, South Africa) that was used in the trials. Metazeb contains metalaxyl at 100 g/kg and

mancozeb at 600 g/kg mancozeb. The fenamiphos product used was Nemacur 400 EC (Villa Crop Protection, 400g fenamiphos/L), and the imidacloprid product Confidor 70 WG (Bayer, Isando, South Africa, 700 g/kg). The first phosphonate application was made in the next season (2nd year of growth) in spring (September), when trees started to show the first signs of budding. Potassium phosphonate was applied as a trunk paint using a 200 g a.i./L solution (Phosguard 400 SL, Witfield, South Africa; 400 g phosphorous acid/L), approximately 50 ml per tree. The trunk paint was applied from the soil surface upwards onto stems (approximately 30 cm) using a paint brush. The spring trunk paint phosphonate applications were followed up by a second phosphonate application in December of the same year. The December applications consisted of three weekly foliar sprays using Phosguard at 2 g a.i./L solution, approximately 50 ml per tree. Foliar applications were made using a motorised mistblower backpack sprayer (SR 400, STIHL, Virginia, USA). The phosphonate trunk paint in September and foliar spray applications in December were repeated in the 3rd year of growth.

The fumigation treatments were applied only to the planting rows (12-20m long x 80cm-100cm width plots) through shank injection, followed by immediate tarping with impermeable black plastic. Fumigants were applied by a registered pesticide applicator (BioScience Research CC, Durbanville, South Africa). Methylbromide (1000g/kg, methylbromide 980/20g chloropicrin/kg, Mebrom Chemicals, SA) was applied at 50 g/m^2. The different chloropicrin/1,3-dichloropropene formulations containing the low 33.3% chloropicrin concentration (Low chl/dichl) and the formulation containing the high 57.0% chloropicrin concentration (High chl/dichl) consisted of the products Agrocelone NE (Agroquimicos de levanter [AQL], S.A., Valencia, Spain) and Agrocelone FE (AQL) respectively. Both Agrocelone fumigants were applied at a dosage of 52 g/m^2.

Orchard trial evaluations

Tree growth measurements and yield. All tree growth measurements were made on the centre eight trees of each replicate. Tree growth was evaluated by determining the increase in trunk diameter and shoot length. Trunk diameter was determined at planting, and subsequently on an annual basis to calculate the increase in trunk diameter. In the first year, the total shoot length was determined at the start of the trials, and again in the 1st year of growth. This allowed the calculation of an increase in total shoot length. Growers did not conduct shoot pruning in the first year of growth, only the leader was pruned. From the 2nd year onwards, only the shoot length of 1-year old shoots was measured using one shoot per tree from each of the eight replicate trees per treatment.

The ARD severity of each orchard was calculated based on the relative increase in stem diameter in comparison to the no treatment control with the high rate fumigation treatment. Only the increase in stem diameter in the 3rd year of growth was used, since this was available for all three orchards. The percentage increase data was used to estimate severity using the classification of Hoestra (1986), where severe is an increase in > 200%, moderate is between 150 % and 200%, and low is less than 150 %.

The Paardekloof and Remhoogte orchards had their first yields in the 3rd year after planting, whereas the Glenfruin orchard only had its first measurable yield in the 4th year after planting. For the Paardekloof trial, cumulative yield was determined by summating the yield from the 3rd and 4th year after planting. At Glenfruin and Remhoogte, yield was only taken in the 4th and 3rd year respectively, i.e. the year in which the first measurable yield was obtained.

Root sample collection and analyses. Root sampling was conducted 20 months after planting, in a 20 to 40 cm tree radius, on opposite sides of the tree row at a depth of 30cm. Roots were sampled from three trees in the middle of the 10 trees within each replicate. Each root sample was divided into two groups for (i) ARD marker microbial pathogen qPCR quantifications and (ii) nematode analysis. DNA extraction from roots, qPCR of the ARD marker pathogens and nematode analyses were also done as described in the apple seedling bioassay section. The identity of the *Phytophthora* spp. amplified in qPCR reactions were determined through sequence analyses of the PCR products as described in Chapter 4.

Statistical analyses

Tree growth data (increase in shoot length, increase in stem diameter), yield and pathogen DNA quantities were subjected to an analysis of variance (ANOVA) using the GLM (General Linear Models) Procedure of SAS statistical software (Version 9.4; SAS Institute Inc, Cary, USA). The Shapiro-Wilk test was used to test for deviation from normality (Shapiro and Francia, 1972). The pathogen DNA concentration data deviated significantly from normality, and therefore the data were Ln (x+1) transformed, resulting in the data being normally distributed. Fisher's least significant difference (LSD) test was calculated at the 5 % level to separate means for significant effects. A probability of 5 % was considered significant. Levene's variance ratio test was used to calculate variation within replications (Levene 1960).

Pearson correlation analyses were conducted on (i) tree growth data and yield, (ii) *Pratylenchus* spp. and ARD marker microbial pathogen DNA concentration and tree growth

and yield. The association of different ARD marker microbes with each other and with *Pratylenchus* spp. were also investigated using correlation analyses. Correlation analyses and significance tests were also conducted using SAS statistical software.

RESULTS

Orchard soil evaluations using an apple seedling bioassay under glasshouse conditions

Bioassay growth results

The two approaches used, relative percentage increase in height or weight, for determining the ARD severity of the three orchard soils, in general yielded the same ARD severity level for the orchard soils. Using the relative percentage increase in height or weight of apple seedlings in pasteurized and untreated soil, the Paardekloof soil was identified as having a moderate ARD severity, whereas the Remhoogte soil had a severe ARD status. For the Glenfruin soil, the two approaches yielded different results; moderately and severe ARD status was indicated based upon apple seedling height and weight responses, respectively (Table 4).

For all three orchard soil bioassay trials there were significant differences between treatments for height ($P < 0.0001$-0.0095) and weight ($P < 0.0001$-0.00195). The pasteurised soil significantly enhanced seedling height and weight relative to the untreated control soil across all three orchard trial soils. Mixing pasteurized soil with 15% (v/v) untreated control soil, resulted in a significantly lower height and weight, relative to the untreated control (data not shown).

Isolation and identification of oomycetes and nematodes from roots

The oomycetes identified through isolation studies included one *Phytophthora* spp. and four *Pythium* spp. *Pythium irregulare* was most widespread, since it was isolated from the roots of seedlings in two orchard soils (Paardekloof and Remhoogte) (Table 4). *Phytophthora cactorum* was only isolated from seedling roots grown in Glenfruin orchard soil and *P.ultimum* only from Paardekloof. The Remhoogte seedling isolations differed from the other orchards in that it also contained the moderately pathogenic *Pythium* sp. complex B2A and *P. heterothallicum* (Tewoldemedhin *et al*., 2011a). *Pythium* sp. complex B2A is a species complex that includes several species (*P. dissotocum, P. coloratum, P. lutarium, P. marinum, P. diclinum, P.* aff. *dictyosporum*, *Pythium* sp. group F and P. sp. '*tumidum*') that cannot be differentiated based on ITS sequence data (Robideau *et al*., 2011). Among these listed species *P. dissotocum* has been identified as a pathogen of apple (Tewoldemedhin *et al.*, 2011a).

Pratylenchus spp. were present in the roots of seedlings grown in all three orchards soils, although at different levels. The root infestations were high in the Paardekloof and Remhoogte orchards but very low in the Glenfruin orchard seedling roots (Table 4).

qPCR quantification of ARD marker microbial pathogens from roots

Almost all of the ARD marker microbial pathogens, including '*Cylindrocarpon'*-like spp*., P.cactorum, P.vexans, P.irregulare* and *P.ultimum* were detected in the roots of seedling for all orchard soils (Table 4). The exceptions were *P. vexans* that was absent from the Remhoogte seedling roots, and *P. sylvaticum* and *R. solani* AG-5, which were not detected at any orchard site. The pathogen quantities varied somewhat in the three orchards. The Paardekloof seedling roots had relatively higher *P. irregulare* and *P. ultimum* quantities in comparison to the other two orchards.

Orchard trials evaluating different management approaches

Tree growth measurements and yield

ARD severity, based on relative percentage increase in stem diameter of the untreated control versus the High chl/dichl treatment, after 3-years of growth differed among the three orchard sites. Under orchard conditions, ARD severity at Glenfruin was moderate (150 % increase in trunk dia.), whereas Paardekloof had a high ARD severity (207 % increase in trunk dia.) as well as the Remhoogte orchard (280 % increase).

In general, there were significant and relatively high correlations among the measured tree growth parameters (increase in stem diameter and shoot length). Increase in leader length was also measured, but the data are not shown since the correlation of this parameter with increase in stem diameter was usually lower than for shoot growth. This likely was the result of growers "topping" the trees in orchards in order to obtain even tree growth. In all three orchards, there were significant and moderate to high correlations between shoot length and increase in stem diameter for a specific year; Glenfruin ($P \leq 0.002$; r = 0.505 to 0.812), Paardekloof ($P \leq 0.011$; r = 0.472 to 0.877) and Remhoogte ($P \leq 0.003$; r = 0.485 to 0.832). The yield correlated mostly, but not always, with shoot length measured from the 2nd year onwards; Glenfruin ($P \leq 0.003$; r = 0.443 to 0.562), Paardekloof ($P \leq 0.432$; r = 0.158 to 0.398), Remhoogte ($P \leq 0.0001$; r = 0.504 to 0.551). In general, there was a higher and always significant correlation between yield and increase in stem diameter; Glenfruin ($P \leq 0.002$; r = 0.506 to 0.717), Paardekloof ($P \leq 0.015$; r = 0.457 to 0.645) and Remhoogte ($P < 0.0001$; r = 0.600 to 0.835), in comparison to shoot length in all three orchards.

The shoot length and increase in stem diameter measured over the 4-year (Paardekloof and Glenfruin) or 3-year (Remhoogte) growth period typically yielded similar results with regards to the performance of treatments in each of the years (data not shown). Therefore, along with the (i) high correlation between shoot length and increase in stem diameter and (ii) increase in stem diameter and yield, the results from the increase in stem diameter will mainly be shown and discussed. Shoot length data is only shown for the final year of growth measurements in each orchard (Table 5).

There were significant differences among treatments for increase in stem diameter for all annual measurements in all three orchards; Glenfruin ($P \leq 0.0027$), Paardekloof ($P \leq 0.0018$) and Remhoogte ($P \leq 0.0045$). In all orchards, the increase in stem diameter in the first year of growth was significantly higher than the untreated control for almost all of the applied treatments, with the exception being the Independent semi-selective treatment (Fig.1). From the second year onwards, nearly all treatments inclusive of the Independent semi-selective treatment, resulted in a significantly higher increase in stem diameter relative to the untreated control across for all orchards. The exception was the Remhoogte orchard, where the Independent semi-selective treatment in the 2nd year of growth was not significantly different from the untreated control (Fig. 1C). In all three orchards, from the 3rd year onwards, all of the treatments resulted in a similar significant increase in stem diameter relative to the untreated control (Table 5, Fig. 1).

Considering only the final year of growth data (increase in stem diameter and shoot length) for all the orchards, all treatments performed significantly better than the untreated control (Table 3). There were, in general, no significant differences between the efficacy of treatments, specifically between the High- and Low-chl/dichl treatments, and where semi-selectives were added to these fumigation treatments. The Independent semi-selective treatment was sometimes less effective in increasing stem diameter and shoot length, since for this treatment (i) a significantly lower increase in stem diameter was recorded relative to the methyl bromide treatment at Glenfruin and (ii) at Paardekloof the treatment had a significantly lower increase in trunk diameter than the Low chl/dichl + semi-selective treatment. At Glenfruin, the Low chl/dichl treatment + Semi-selective treatment had a significantly lower shoot length than the methyl bromide treatment. Although not significantly different, the Independent semi-selective treatment tended to have the lowest shoot length and increase in trunk diameter in all three orchards (Table 5).

Yield

The efficacy of treatments with regards to yield differed in the orchards. At Remhoogte, almost all treatments were equally effective, and resulted in significantly higher yields than

the untreated control. The exception was the Independent semi-selective treatment that did not result in a significant increase in yield relative to the untreated control. The Independent semi-selective treatment also did not result in significantly higher yields in the other two orchards relative to the untreated control. For Glenfruin, the Low chl/dichl was the only fumigant treatment that did not result in a significantly higher yield than the untreated control. At Paardekloof, only one treatment, the Low chl/dichl + semi-selective treatment, had significantly higher yields than the untreated control (Table 5). The two Chl/dichl treatments differing in the ratio of fumigant chemistries did not differ significantly from each other in yield in any of the three orchards (Table 5).

The addition of semi-selective chemicals to the Low chl/dichl fumigation treatment, did not results in significant higher yields compared to the fumigant only treatment in all three trials (Table 5). The exception was at Paardekloof, where the Low chl/dichl + Semi-selective treatment had a significant higher yield than the independent use of this fumigant (Table 5).

qPCR quantification of ARD marker microbial pathogens from roots

qPCR assays successfully detected several of the ARD marker pathogens in the apple roots at the study sites and included '*Cylindrocarpon'*-like spp., *Phytophthora* spp., *P. ultimum* and *P. irregulare*. *P. ultimum* was detected in apple roots from Glenfruin and Paardekloof, but not the Remhoogte orchard. The *P. sylvaticum, P. vexans* and *R. solani* AG-5 were not detected in any of the orchards (Table 6). Sequence analysis of the *Phytophthora* genus specific qPCR assay products from each trial, showed that the species involved was *P. cactorum.*

Although there were significant differences between treatments ($P \leq 0.0338$) in the quantity of '*Cylindrocarpon'*-like spp. DNA detected in apple roots, DNA quantities of '*Cylindrocarpon*'-like spp. were often significantly higher than the control in treatments that exhibited a significant increase in tree growth relative to the untreated control. In two of the orchards (Glenfruin and Paardekloof), none of the treatments resulted in a significant reduction in the amount of '*Cylindrocarpon'*-like spp. DNA detected in roots, relative to the untreated control. Notably, the methyl bromide treatment, although having the best tree performance, had a significantly higher '*Cylindrocarpon'*-like spp. concentration (39.14 pg/g) at the Glenfruin orchard relative to the untreated control (12.43 pg/g). Furthermore, at Paardekloof the Independent semi-selective treatment also had a significantly higher '*Cylindrocarpon'*-like spp. Concentration (4.8 pg/g) in comparison to the untreated control (1.3 pg/g). At the Remhoogte orchard, significant reductions in '*Cylindrocarpon'*-like spp. were observed in response to certain treatments relative to the control. Only the High chl/dichl (2.87 pg/g) and the Low chl/dichl + semi-selective (2.26 pg/g) treatments had

significantly lower quantities of '*Cylindrocarpon*'-like spp. DNA relative to the untreated control (12.45 pg/g) (Table 6).

Pythium irregulare quantities were only significantly different between treatments at the Paardekloof orchard (P = 0.0197) (Table 6). However, the difference was not due to a reduction in pathogen quantity relative to the untreated control. The High chl/dichl treatment had a significantly higher *P. irregulare* concentration (1.17 pg/g) than the untreated control (0.03 pg/g).

Pythium ultimum DNA concentrations detected in apple roots was low (< 0.07 pg/g) at all orchard sites with the pathogen not being detected at the Remhoogte orchard (Table 6). For the Paardekloof and Remhoogte orchards, there were no significant differences (P > 0.1795) between treatments for this pathogen. However, at Paardekloof the pathogen might have been important since all treatments resulted in a substantial reduction in its quantities (< 0.04 pg/g) relative to the untreated control (0.07 pg/g) (Table 6). The importance of *P. ultimum* in the Paardekloof orchards was also supported by correlation analyses between its quantities and tree growth (see below).

Phytophthora cactorum was a significant pathogen at the Glenfruin and Remhoogte orchards since there were significant differences between treatments ($P \leq 0.0090$). These differences were due to some treatments reducing the pathogen concentrations relative to the untreated control. The exceptions were for the Low chl/dichl and the Methylbromide treatments in the Glenfruin orchard, which did not differ significantly from the untreated control. Although there were no significant differences between treatments in *P. cactorum* quantities at Paardekloof, the untreated control had a much higher concentration than the applied treatments (Table 6). The importance *of P. cactorum* at Paardekloof, was also supported by correlation analyses between its quantities and tree growth (see below).

Correlation between ARD marker microbial pathogens, tree growth and yield

For most of the ARD marker pathogens, with the exception of *P. cactorum* and *P. ultimum,* there were no significant correlations between DNA concentrations determined 20 months after planting and tree growth (increase stem diameter and shoot length) and yield (data not shown). The importance of *P. cactorum* in limiting tree performance at all three orchards was evident from several significant negative correlations between *P. cactorum* quantities and some tree growth parameters and yield (Table 7). In all three orchards, there was a significant negative correlation between shoot growth and *P. cactorum* DNA quantities in the final year of orchard growth analyses; 4th year for Paardekloof (r = - 0452; P = 0.016) and Glenfruin (r = - 0.355; P = 0.034) and 3rd year for Remhoogte (r = – 0.632; P < 0.0001). Additionally for all orchards there was also a significant moderate negative correlation

between *P. cactorum* and the increase in stem diameter in the 3rd year of growth (r = -0.333 to -0.410; P < 0.047), but not the 4th year. For Glenfruin there was also a significant moderate negative correlation between yield and *P. cactorum* quantities (r = -0.378; P = 0.034) (Table 7).

Pythium ultimum quantities correlated negatively with tree growth at the Paardekloof and Glenfruin orchards. The importance of *P. ultimum* in the Paardekloof orchard was evident from a significant moderate negative correlation between *P. ultimum* concentrations and increase in stem diameter in the 3rd year of growth (r = -0.458; P = 0.030). At the Glenfruin orchard, there was also a significant moderate negative correlation between *P. ultimum* and yield (r = -0.378; P = 0.023) (Table 7).

Nematode extraction and quantifications

Pratylenchus spp. was the only parasitic nematode genus identified in the orchards. *Pratylenchus* spp. infestation was observed in the Paardekloof and Remhoogte orchards in the 2nd (20 months after planting) year of growth, but not at Glenfruin (Table 6). At Glenfruin, *Pratylenchus* spp. were only detected in the 3rd year of growth (30 months after planting) (data not shown). *Pratylenchus* spp. root densities were significantly different among treatments in the Paardekloof orchard (P = 0.0260), but not the Remhoogte orchard (P = 0.5339). At Paardekloof all treatments significantly reduced *Pratylenchus* spp. root densities (< 47 nematodes/5g roots) relative to the control (548 nematodes). Although no significant differences were observed among treatments at the Remhoogte orchard, a trend similar than that at Paardekloof was evident (Table 6).

Correlation between Pratylenchus spp. root densities, tree growth and yield

Correlation analyses between *Pratylenchus* spp. root densities and tree growth responses (shoot length and increase in stem diameter) and yield, revealed significant negative correlations for the Paardekloof and Remhoogte orchards (Table 7). *Pratylenchus* spp. were important in reducing tree growth at the Paardekloof orchard since there were significant moderate negative correlations between their root densities and shoot length (2nd, 3rd and 4th year) and increase in stem diameter (3rd and 4th year). At Remhoogte, yield and the 2nd year of shoot growth was significantly negatively correlated with *Pratylenchus* spp. root densities (Table 7)

Correlation between the DNA concentrations of different ARD marker microbial pathogens with each other and Pratylenchus spp.

Correlation analyses between the different pathogen (fungal and oomycete) DNA quantities and *Pratylenchus* spp. root densities, yielded interesting associations in two orchards. The associations were between (i) *Pythium* spp. and '*Cylindrocarpon'*-like spp. and (ii) *P. cactorum* and *Pratylenchus* spp. Significantly high to moderate correlations were present between *P. cactorum* and *Pratylenchus* spp. at the Paardekloof (r = 0.942; P < 0.0001) and Remhoogte (r = 0.43; P = 0.009) orchards.

'Cylindrocarpon'-like spp. DNA quantities were significantly correlated with two different *Pythium* spp. in two of the orchards. Significant moderate to high correlations were obtained between '*Cylindrocarpon'*-like spp. and *P. irregulare* (r = 0.714; P < 0.0001) at Paardekloof, and at Glenfruin with *P. ultimum* (r = 0.432; P = 0.009).

DISCUSSION

The study evaluated different strategies for the management of ARD at three orchard sites in South Africa. It was shown that two fumigants differing in chloropicrin/1,3-dichloropropene concentrations were, in general, equally effective in improving tree growth. The combined use of semi-selective chemicals (fenamiphos, metalaxyl, phosphonates and imidacloprid) without a preplant fumigation treatment improved tree growth (shoot length and increase in trunk diameter) to a level similar to that attained with fumigants. However, this was not true for yield. The addition of semi-selective chemicals to fumigated soil in one of the orchards, significantly increased yield relative to a fumigant only treatment. Analyses of marker ARD microbes (*Phytophthora* spp., *P. vexans, P. irregulare, P. sylvaticum* and *P. ultimum*) and nematodes (*Pratylenchus* spp.) suggested that *P. ultimum* likely only contributed to disease development in one of the three study orchards. *Phytophthora cactorum* was important in all three orchards, whereas *Pratylenchus* spp. were important in two orchards. The latter two groups of organisms likely interacted synergistically based on significant correlations between their quantities. Correlation analyses also suggested synergistic interactions between '*Cylindrocarpon'*-like spp. and *Pythium* spp. The possibility of synergistic interactions between these groups of pathogens will have to be investigated in future studies.

An apple seedling bioassay was useful for predicting the ARD status of the three investigated ARD orchard soils, and the involvement of ARD microbes and nematodes. However, the assay did have some limitations. The seedling assay accurately predicted the high ARD severity of the Remhoogte orchard soil but for the Paardekloof and Glenfruin soils, disease severity predicted by the seedling bioassay was somewhat different than disease

development ultimately observed under orchard conditions. At Paardekloof the seedling assay, both in terms of relative increase in weight and height, under predicted ARD severity. For the Glenfruin soil, the use of the relative increase in height, rather than weight, in the seedling bioassay accurately predicted ARD severity of the orchard. Therefore, although Hoestra (1968) and Sewell *et al.* (1992) suggested the use of weight rather than length for predicting ARD incidence in seedling assays, length was found to be a better approach in one of the orchards. However, since Sewell *et al.* (1992) evaluated a large number of soils (506) in order to come to their conclusion, it shows that there will always be exceptions to the rule. Therefore, the seedling bioassay cannot consistently predict ARD severity accurately and can result in an under-estimation (this study) or over-estimation (Merwin *et al*., 2001).

The seedling bioassay was useful for determining the importance of *Pratylenchus* spp. but not *P. vexans* in all three orchards, *Pratylenchus* spp. populations were accurately predicted as being high or low to absent. Although the seedling bioassay indicated the importance of *P.vexans* in two orchards, *P. vexans* was not detected in roots assayed from any of the orchard trials. This is likely due to seedlings being more susceptible to *P. vexans* than older orchard trees, along with the highly conducive conditions created in the seedling bioassay for pathogen infection (Tao *et al*., 2011). The seedling bioassay also accurately predicted the presence of *P. cactorum*, although only when qPCR was used in the assessment, and not direct isolation from plant roots. It is known that *Phytophthora* spp. can be difficult to isolate due to dormancy (Collins *et al.*, 2012), or the presence of other pathogens in the root tissue, such as *P. vexans* which also grows on the semi-selective isolation medium. The qPCR assay, due to its high sensitivity (5 fg), is likely to also improve detection of *P. cactorum* in comparison to root isolations. The *P. cactorum* DNA root quantities in the bioassay were low for all soils, whereas in two of the orchard trials, tree roots contained much higher pathogen DNA concentrations. Factors that might contribute to this is that nursery planting material might be contaminated with *P. cactorum.* It is known that in South Africa, nursery trees contain several of the ARD pathogens, although *P. cactorum* was not specifically identified (Moein, 2016).

The study demonstrated that the cause of ARD in the three orchard soils was biological, based on several factors including that (i) seedling growth in bioassays was significantly greater in the pasteurized soil, (ii) the dilution of pasteurized soil with 15% untreated soil resulted in growth reductions similar to that of the untreated control, (iii) all fumigation treatments (High chl/dichl or MetB) significantly enhanced apple tree growth and in most cases yield, and (iv) the presence of significant negative correlations between tree growth parameters and DNA quantities of apple replant marker microbes (*Phytophthora*

cactorum and *P. ultimum*) and nematodes (*Pratylenchus* spp). The biological nature of ARD is well known and has been reported by several other studies (Mazzola, 1998, Yao *et al.*, 2006., Yao *et al*., 2006., Tewoldemedhin *et al*., 2011c).

The occurrence and incidence of ARD pathogens can be somewhat site specific (Manici *et al.*, 2003; Mazzola, 1998), which was also evident in the current study. In all three orchards, *P. cactorum* contributed significantly to disease development. This was evident from *P. cactorum* quantities having a significantly low to moderate negative correlation with tree growth (shoot length and increase in stem diameter) or yield. Furthermore, fumigation treatments resulted in a significant reduction of the pathogen in some orchards. *Pratylenchus* spp. were involved in ARD in only two orchards. The site-specific importance of *Pratylenchus* spp. has been reported (Mazzola, 1998; Mazzola and Manici, 2012). *Pratylenchus* spp. root densities had a negative moderate correlation with yield or tree growth in the orchards. The possible synergistic interaction between *P. cactorum* and *Pratylenchus* spp. in increasing disease severity was evident from significant high to moderate correlations between *P*. cactorum quantities and *Pratylenchus* spp. root densities. *Pythium ultimum* was important in two orchards based on low to moderate negative correlation of its DNA quantities with yield or tree growth in two orchards. Other ARD pathogens that might have been involved include the moderately virulent *Pythium* spp., *P. dissotocum* and *P. heterothallicum* (Tewoldemedhin *et al.,* 2011a) that were detected in the bioassay study at Remhoogte. Since the quantities of the two species were not investigated under orchard trial conditions, it is unclear what their role was.

The ARD marker pathogens *P. sylvaticum, Rhizoctonia solani* AG-5 and *P. vexans* were not detected in any of the orchards. The lack of detection of *P. vexans* is likely, as previously discussed, due to its low virulence or lack of pathogenicity towards older trees. The absence of *P. sylvaticum* in orchard trial roots was also supported by the seedling bioassay results. The absence of *R. solani* AG-5 in the orchards, agrees with previous studies conducted in South African orchards (Tewoldemedhin *et al.*, 2011a, b).

At the Paardekloof orchard, some ARD pathogens might have been introduced by the planting material and residual soil innoculum. First of all, there was high *P. cactorum* DNA quantities in orchard roots, compared to the seedling bioassay where high irrigation was applied to promote the pathogen. Furthermore, most fumigation treatments, including methylbromide, were ineffective in significantly reducing *P. cactorum* quantities. The introduction of pathogens introduced on planting material would also explain why the only treatment that significantly increased yield at this orchard was the Low chl/cl + semi-selective treatment. The performance of the aforementioned treatment could also have been due to the fact that old trees were removed and the orchard was replanted within the same

year, without care being taken to remove large root pieces. The latter would have continued to be an inoculum source after fumigation.

The role of '*Cylindrocarpon'*–like spp. and *P. irregulare* in ARD in the three orchard trials is difficult to determine based on root quantification data and correlation analyses. For *P. irregulare,* the root quantities were mostly not significantly different between fumigant treatments and the untreated control. No significant correlations were furthermore found with *P. irregulare* quantities and tree growth. Yet, this pathogen is a well-known highly virulent ARD pathogen (Tewoldemedhin *et al*., 2011a, Souli *et al*., 2014.). The '*Cylindrocarpon'*-like spp. quantities, although showing significant differences between orchard treatments, did not correspond to the efficacy of treatments. For example significantly higher quantities were present in the methylbromide treatment at Glenfruin. A similar phenomenon was found at Paardekloof for *P. irregulare* in the High cl/chl treatment. For '*Cylindrocarpon'*-like fungi, this is most likely due to the fact that the primers used to target this group of pathogens amplified pathogenic and non-pathogenic isolates. It is known that pathogenic and non-pathogenic spp. occur within this group (Tewoldemedhin *et al.*, 2011c; Manici *et al.,* 2015). The significant correlation between '*Cylindrocarpon'*-like spp. and *Pythium* spp. in two of the orchards is interesting. It is known that '*Cylindrocarpon'*-like spp*.* act synergistically with *P. irregulare* (Braun, 1991 1995; Tewoldemedhin *et al.,* 2011b). There was indeed a significant and high correlation between these two pathogens at Paardekloof. Furthermore, at Glenfruin there was also a significantly moderate correlation between '*Cylindrocarpon'*-like spp. and *P. ultimum.*

Some abiotic factors such as soil properties, orchard soil management and differences in rootstocks, may have affected pathogen activity in the orchards and ultimately ARD severity. The importance of oomycetes at Glenfruin might be due to the clay loam soil type, as opposed to the other two orchards having a sandy loam. The latter soil type would also be more conducive to *Pratylenchus* spp. (Zasada *et al*., 2015). Furthermore, aside from affecting pathogen activity, different soil textures are known to support different microbial communities (Grandy *et al*., 2009), which can act synergistically or antagonistic with pathogens in orchard soils. Different rootstocks were used in the trials, which could have favoured certain pathogens. For example, the MM109 rootstock used at Paardekloof is known to be more susceptible to *Phytophthora* spp. than M7 used in the other trials (McIntosch, 1975).

The independent use of semi-selective chemicals may have potential for managing ARD based on an improvement in tree growth, and suppression of ARD marker microbes. In all seasons and across all trials, the independent use of semi-selective chemicals enhanced tree growth (shoot length and increase in stem diameter) to levels attained by fumigants in

the the 3rd and 4th year of growth. However, this was not true for yield. The yields were furthermore not comparable to the fumigant treatments. Since, the Remhoogte orchard site had a high disease potential, the independent use of semi-selectives only resulted in improved tree growth responses similar to soil fumigation in the 3rd year, not the 2nd year. This is most likely due to the high inoculum pressure in this orchard at planting. The semi-selective chemical mixture significantly suppressed prominent ARD pathogens such as *P.cactorum* (Glenfruin and Remhoogte orchards) and *Pratylenchus* spp. (Paardekloof). There was also a trend towards reductions, although not significant, for *Pratylenchus* spp. (Remhoogte) and *P. cactorum* (Paardekloof). The fenamiphos in the semi-selective mixture would have contributed towards suppression of *Pratylenchus* spp. (LaMondia, 1999) whereas metalaxyl and phosphonates will suppress *P. cactorum* (Utkhede, 1987). The semi-selective chemicals also included imidaclorpid, which is known as a resistance inducer on citrus against bacterial diseases (Francis *et al*., 2009). Phosphonates are also increasingly being seen as plant resistance inducers (Massoud *et al.,* 2012; Jackson *et al*., 2000). Therefore, it might be possible that these products functioned together in suppressing pathogens and in improving tree growth. The suppression of pathogenic '*Cylindrocarpon*'-like spp. could not be deduced because the primers used for quantification do not discriminate between non-pathogenic and aggressive species. However, it was clear that the semi-selectives and putative plant resistance inducing chemicals in the mix were unable to suppress this group of organisms significantly.

This is the first study to show that the independent use of semi-selective chemicals can improve tree growth in ARD orchards to levels attained with fumigants under orchard conditions for a period of 3 to 4-years. A few studies have shown that the independent use of metalaxyl, mefenoxam or phosphonates or combined with other management practices can help to manage ARD. Only one study evaluated metalaxyl for the management of ARD under orchard conditions in Massachusetts, USA (Autio *et al*., 1991). Metalaxyl was applied as a soil drench at planting, and resulted in a significant increase in trunk and shoot growth in the first year, but not in the subsequent two growing seasons. Autio *et al.* (1991) furthermore also evaluated fosetyl-Al (alkyl phosphonate) foliar spray applications in the first year of planting and obtained a significant increase in trunk diameter and shoot growth, but also only in the first year of growth. The causative ARD pathogens were not investigated in the trial site, and a standard fumigation treatment was also not included. The application of a mefenoxam soil drench to a *Brassicae napus* seed meal treatment, improved apple tree growth and yield in ARD orchards; however, this treatment was only effective in ARD orchards where parasitic nematodes were absent (Mazzola and Mullinix, 2005; Mazzola and Brown, 2010).

In South Africa, growers currently mainly use the Low chl/dichl treatment, since this was the first fumigant registered following the banning of methyl bromide. It was therefore important to determine if the High chl/dichl treatment, which is now also registered in South Africa, would be a better option for growers. There were no significant differences between these treatments for tree growth and yield. However, there was a trend across all orchards for a slightly higher increase in trunk diameter and yield obtained with the High chl/dichl fumigant. Furthermore, in the Glenfruin orchard where oomycetes were the primary disease causing agents, the Low chl/dichl treatment did not significantly increase yields relative to the untreated control, whereas the High chl/dichl did. Thus, the use of higher chloropicrin dosages could be very important in orchards where oomycetes prevail. It will be important to obtain another year of yield data for all the orchards to confirm the superior nature for the High chl/dichl fumigant. This formulation, due to the higher chloropicrin content that suppresses microbes, would be expected to improve the suppression of oomycetes. Although there were no significant differences in pathogen quantities between the two treatments, there was a trend for the improved suppression of *P. cactorum* at Glenfruin. There was also a trend at Paardekloof and Remhoogte for a reduction in *Pratylenchus* spp. root densities by the High chl/dichl treatment compared to the Low chl/dichl treatment. This is unexpected, since the Low chl/dichl product would be expected to have a reduced activity against parasitic nematodes, compared to the High chl/dichl product, due to its lower 1,3-dichloropropene content. Perhaps this phenomenon is due to the importance of *P. cactorum* in both orchards and the synergistic interaction with *Pratylenchus* spp., i.e. the reduction of *P. cactorum* by the higher chlorocropin concentration reduced the ability of *Pratylenchus* spp. to infect and multiply. Another factor that could have contributed to a better performance of the High chl/dichl treatment is that the co-formulation of chloropicrin with 1,3-dichloropropene can result in increased degradation rates of chloropicrin by approximately 15 % (Zheng *et al*., 2003). Ashworth *et al.* (2015) also found with a meta data analyses of a large number of published field studies that total chloropicrin emissions were ~4.5 times lower when it was co-formulated with 1,3-dichloropropene. However, when evaluating this under controlled laboratory conditions it was found that co-formulation was not a significant factor in emission losses from columns (Ashworth *et al.*, 2015).

In summary, the study showed that semi-selective chemicals may have potential for managing ARD, and that a higher chloropicrin content fumigant has potential for improving ARD management in South Africa. The independent use of semi-selective chemicals significantly improved tree growth, but not yield. The semi-selective chemicals applied to fumigated soils, also has potential for improving ARD management in orchards where tree roots are not removed adequately prior to fumigation, and/or where nursery material is

contaminated with pathogens. The inclusion of fenamiphos in the mixture is problematic, since this nematicide is likely to also be lost from markets in South Africa in the near future, as has occurred internationally. Therefore, future studies should investigate alternative nematicides that are more environmentally friendly. The study highlighted the biological nature of ARD and the causal agents in three South African ARD orchard. *Phytophthora cactorum* and *Pratylenchus* spp. are prominent role players. The correlation found between the root quantities and densities of these two organisms and their suggested interaction requires further investigation under (i) controlled glasshouse conditions and (ii) larger orchard scale investigations. The role of *Cylindrocarpon*-like spp. and *P. irregulare* and their interaction is not well understood and requires further investigation. A re-evaluation of the '*Cylindrocarpon'*-like spp. associated with ARD and their pathogenicity is required in view of recent taxonomic changes in this group. This could assist in the development of molecular markers that only amplify pathogenic groups within this complex. The involvement of *Rhizoctonia* in the current study was only limited to *R. solani* AG-5 due to a lack in marker availability for the bi-nucleate groups. This aspect also needs to be addressed in future and to determine whether the hypothesis of Manici *et al.* (2013) is correct in that bi-nucleate *Rhizoctonia* spp. rather have a symbiotic or mutualistic interaction with apple. In the current study pathogens and nematodes were only quantified 20 months after planting. In future studies itt will be interesting to also investigate at an earlier time point such as 12 months after planting, whether the same or different groups of organisms are involved in ARD.

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Table 1. Information on apple orchards that were used in apple replant disease management trials.

Production region	Farm name	Fumigation date	Planting date	Rootstock	Variety	Spacing
Witzenberg valley	Paardekloof	10 September 2013	12 October 2013	MM109	Early redone	3.75m x1.25m
Kouebokkeveld	Remhoogte	13 September 2014	06 October 2014	M7	Gale gala	4m x1.5m
Grabouw	Glenfruin	16 September 2013	03 October 2013	M7	Royal beauty	4.5m x 2m

`**Table 2. Orchard soil properties** of three orchard soils

Orchard	pH	Classification	Resistance (Ohm) [a]	CEC (Cmol (+)/kg [b]	Clay %	Silt %	Sand %	Water holding Capacity (mm/m) [c]
Glenfruin	5.2	Clay loam	200	10.09	39	38	23	59.55
Paardekloof	5.4	Sandy loam	1400	17.46	13	20	67	109.20
Remhoogte	4.8	Sandy loam	790	8.68	19	14	67	94.92

Soil sampling was conducted in the top 30cm depth, ten random soils were collected and thoroughly mixed to make a representative sample. Representative samples were then send for soil analysis at Bemlab (Somerset, South Africa).

[a] Resistance (Ohm) A low soil resistance indicates the presence of large quantities of salts in the soil, i.e. the soil is saline.

[b] CEC (Cmol (+)/kg are the total exchangeable cations, a measure of the soil's ability to retain and supply nutrients, specifically the positively charged nutrient ions called cations. These include the cations calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^{1+}), ammonium (NH_4^+), and many of the micronutrients**.** The higher the CEC the harder it becomes to change factors as pH and less leaching of cations and anions.

[c] Water holding Capacity (mm/m) is the depth of water held between field capacity and permanent wilting point per metre depth of soil.

Table 3. Quantitative real-time PCR (qPCR) primers, probes and amplification conditions used for quantifying apple replant disease marker pathogens (*Pythium irregulare, Pytopythium vexans, Pythium ultimum,* the genus *Phytophthora, Pythium sylvaticum* and '*Cylindrocarpon'*-like fungi) from apple roots.

Target species	Primers (nM)		Probe (nM)	Annealing[a]		$MgCl_2$ [b]	Efficiency	M-slope	Limit of detection	Reference
				Temp.	Time					
P. sylvaticum	Syl1F (200)	Syl1R (200)	_	65	15* [c]	500 nM*	0.93	- 3.3	18.8 fg	Schroeder *et al*., 2006
P. irregulare	PirF1 (300)	PirR1 (900	_	60	5	100 nM*	0.96	- 3.4	0.54 fg	Spies *et al*., 2011
Phytophthora genus	Yph1F (250) *	Yph2R (250) *	_	62	20*	_	1	- 3.10	5.80 fg	Schena *et al*., 2008
P. ultimum	PulF2 (300)	PulR2 (300)	PulP2 (150)	60	30	_	0.98	- 3.5	57.0 fg	Spies *et al*., 2011
P. vexans	PV390P (300)	PV455P (300)	PV412P (200)	60	30	_	0.95	- 3.4	12.6 fg	Moein, 2016
'Cylindrocarpon'-like fungi	YT1F (300)	CylR (300)				300 nM	0.92	- 3.2	6.90 fg	Tewoldemedhin *et al*., 2011c
Rhizoctonia AG5	RSAG5F (900)	RSAG5R (300)		60	15		0.98	-3.4	2.56 copies	Mazzola and Zhao, 2010

[a] Annealing temperatures and extension times used in assays. All assays were Syber Green based, with the exception of the *P. ultimum* and *P. vexans* assays, which were probe based.

[b] The qPCR master mix used in all assays contained a final concentration of 2.5 mM $MgCl_2$, and therefore most assays did not require additional $MgCl_{2.}$ except for the *P. sylvaticum* and *P. irregulare* assays.

[c] Values followed by * were modified from the published assay.

Table 4. Apple replant disease (ARD) severity of three orchard soils determined using an apple seedling bioassay conducted under glasshouse conditions, and the associated diagnostic DNA concentrations of ARD pathogens.

Orchard soil	Growth response pasteurized and un-treated soil [a]		ARD severity [b]		Oomycete spp. isolated (% of oomycete isolates) [c]	*Pratylen-chus* spp. (5 g roots)[c]	Quantification of pathogen DNA in roots (pg / g roots) [c]				
	% RIH	% RIW	Height	Weight			*Cylindrocar-pon*-like spp.	*P. cactorum*	*P. vexans*	*P. irregulare*	*P. ultimum*
Glenfruin	155	207	Moderate	Severe	*P. cactorum* (100 %)	10	0.184	0.009	4.02	0.006	0.0006
Paardekloof	165	162	Moderate	Moderate	*P. ultimum* (57 %) *P. irregulare* (43 %)	250	0.700	0.004	0.484	0.120	0.016
Remhoogte	280	378	Severe	Severe	*Pythium* sp. complex B2A (39 %) *P.heterothallicum* (8 %) *P. irregulare* (3 %)	590	0.3435	0.002	0	0.084	0.007

[a] Golden delicious apple seedlings (4-weeks old) were grown in untreated and pasteurized soils for 3 months. The increase in seedling fresh weight (shoot and roots) or height was determined at the end of the trial for each treatment. Each treatment consisted of six replicates. The relative percentage increase in seedling weight or height was determined in pasteurized versus the un-treated soil. Percentage relative increase in height (%RIH) = (Increase in height in pasteurized soil / increase in height untreated soil) × 100. Percentage relative increase in weight (%RIW) = (increase in weight in pasteurized soil / increase in weight untreated soil) × 100.

[b] The ARD severity status was determined according to the ARD classification of Hoestra (1968). Hoestra (1968) identified ARD soils based on the percentage increase in seedling weight as severe (> 200 % increase), moderate (between 150 and 200 % increase) and low (less than 150 % increase).

[c] All pathogen isolations and quantitative real-time PCR (qPCR) analyses were performed after 3 months on the roots of apple seedlings obtained from untreated control soils.

Table 5. The effect of various soil fumigation and semi-selective chemical treatments on apple tree growth (shoot length and increase in stem diameter) and yield, in three apple replant orchard trials (Glenfruin, Paardekloof and Remhoogte).

Orchards	Treatments [a]	Increase in shoot length (cm) in the 3rd or 4th year of growth [b]	Increase in trunk diameter (mm), 3- or 4-years post-planting [c]	Yield (kg/tree) [d]
Glenfruin	Untreated control	39.05c	30.35c	0.65c
	Low chl/dichl	65.42ab	39.86ab	1.45bc
	High chl/dichl	62.86ab	41.86ab	2.57ab
	Methylbromide	68.18a	42.75a	4.40a
	Independent semi-selectives	61.25ab	37.83b	2.01c
	Low chl/dichl + semi-selectives	60.36b	39.37ab	2.52b
	P value	**< 0.0001**	**< 0.0001**	**0.0054**
Paardekloof	Untreated control	38.32b	14.30c	1.62b
	Low chl/dichl	72.78a	20.54ab	1.53.50b
	High chl/dichl	62.91a	20.83ab	1.79b
	Methylbromide	68.14a	21.62ab	3.47ab
	Independent semi-selectives	63.43a	19.64b	1.87b
	Low chl/dichl + semi-selectives	65.13a	25.10a	4.21a
	P value	< 0.0001	**0.0018**	0.0228
Remhoogte	Untreated control	20.48b	22.16b	3.63b
	Low chl/dichl	38.15a	27.60a	8.85a
	High chl/dichl	38.22a	29.48a	10.17a
	Independent semi-selectives	35.46a	27.37a	5.89b
	Low chl/dichl + semi-selectives	35.78a	28.94a	11.05a
	High chl/dichl + semi-selectives	39.29a	29.07a	9.38a
	P value	< 0.0001	0.0045	< 0.0001

[a] The semi-selective treatments consisted of a soil drench at planting (metalaxyl, fenamiphos and imidacloprid) followed by 2-years of potassium phosphonate applications, twice annually. The fumigant treatments were all applied pre-plant. Two of the fumigants differed in their chloropicrin content; the Low chl/dichl fumigant had a 33.3% and 60.8% chloropicrin and 1,3-dichloropropene content respectively, whereas the High chl/dichl fumigant had a 57% and 38% chloropicrin and 1,3-dichloropropene content respectively.

[b] Shoot length for the Paardekloof and Glenfruin orchards was taken in the 4th year of growth, whereas in the Remhoogte orchard, it was taken in the 3rd year of growth. The Paardekloof and Glenfruin orchards were established in 2013, and Remhoogte in 2014.

[c] Increase in stem diameter values are for a 4-year period (2013 to 2017) for Paardekloof and Glenfruin, and for a 3-year period for Remhoogte (2014 to 2017).

[d] Yield for Paardekloof is the cumulative yield for the 3rd and 4th year of growth (2016 and 2017). The yield for Glenfruin is the yield recorded in the 4th year of growth (2017). For Remhoogte, the yield is that obtained in the 3rd year of growth (2017).

Values in columns are the average of six replicates (eight trees per replicate). For each orchard, values within a column followed by the same letter do not differ significantly ($P > 0.05$) according to Fisher's least significant difference test.

Table 6. The effect of various soil fumigation and semi-selective chemical treatments on the quantities of apple replant disease marker microbes (‘*Cylindrocarpon’*-like fungi, *Pythium irregulare, Pythium ultimum* and *Phytophthora cactorum*) and parasitic nematodes in the roots of apple trees. The roots were obtained from three orchard trials

Orchards	Treatments	*‘Cylindrocarpon’*-like fungi *(pg/g)*	*P. irregulare (pg/g)*	*P. ultimum(pg/g)*	*P. cactorum (pg/g)*	*Pratylenchus* spp. (5 g roots)
Glenfruin orchard	Untreated control	12.430 b	3.960	0.002	40.330 a	ND
	Low chl/dichl	10.140 b	1.180	0.003	21.499 abc	ND
	High chl/dichl	11.450 b	0.005	0.028	0.000 c	ND
	Methylbromide	39.140 a	0.180	0.040	20.28ab	ND
	Independent semi-selectives	10.850 b	0.500	0.003	0.000 c	ND
	Low chl/dichl + semi-selectives	7.390 b	0.440	0.005	10.910 bc	ND
	P value	0.0172	0.1518	0.7445	0.0090	ND
Paardekloof orchard	Untreated control	1.300 abc	0.028 b	0.068	34.000	548b
	Low chl/dichl	3.790 ab	0.030 b	0.019	6.090	47a
	High chl/dichl	10.970 abc	1.178 a	0.015	3.290	42a
	Methylbromide	1.210 bc	0.012 b	0.003	0.730	75a
	Independent semi-selectives	4.800 a	0.030 b	0.007	3.720	30a
	Low chl/dichl + semi-selectives	0.190 c	0.041 b	0.002	2.550	10a
	P value	0.0338	0.0197	0.1795	0.3273	0.0260
Remhoogte orchard	Untreated control	12.450 a	0.040	**ND**	0.370 a	231
	Low chl/dichl	3.650 a	0.010	ND	0.000 b	95
	High chl/dichl	2.870 b	0.030	ND	0.070 b	31
	Independent semi-selectives	21.920 a	0.030	ND	0.000 b	70
	Low chl/dichl + semi-selectives	2.260 b	0.020	ND	0.060 b	21
	High chl/dichl + semi-selectives	13.050 a	0.0300	ND	0.010 b	50
	P value	0.0019	0.4832	ND	0.0008	0.5339

Root samples used for pathogen and *Pratylenchus* spp. quantification were obtained 20 months after planting (i.e., in September 2015) for the Glenfruin and Paardekloof orchards, and 20 months after planting (i.e., in September 2016) for the Remhoogte orchard. Sampling was conducted in a 20 to 40 cm tree radius, on opposite sides of the row at a depth of 30 cm. Values in columns are the average of six replicates. For each replicate, roots sampled from three trees were pooled into one sample. For each orchard, values in columns followed by the same letter do not differ significant according to Fisher’s least significance difference test at the 95 % significance level. **ND** indicates that the organisms were not detected. *Pythium sylvaticum, Pythium vexans* and *Rhizoctonia solani* AG-5 were not detected in any of the orchards.

Table 7. Correlation between apple tree growth parameters (shoot length and increase in stem diameter) and yield, with DNA quantities of apple replant disease (ARD) marker microbes (*Phytophthora cactorum* and *P. ultimum*) and nematodes (*Pratylenchus* spp.) in three apple ARD orchard trials (Glenfruin, Paardekloof and Remhoogte).

	Pearsons' correlation coefficient (*P* value) [a]						
Orchard	Increase stem dia. 2nd year	Shoot length 2nd year	Increase stem dia. 3rd year	Shoot length 3rd year	Increase stem dia. 4th year	Shoot length 4th	Yield
		P. cactorum					
Glenfruin	NS	NS	-0.358 (0.032)	-0.34 (0.042)	NS	-0.355 (0.034)	-0.378 (0.023)
Paardekloof	NS	-0.398 (0.036)	-0.410 (0.030)	-0.413 (0.029)	NS	-0.452 (0.016)	NS
Remhoogte	-0.488 (0.003)	-0.585 (< 0.0001)	-0.333 (0.047)	-0.632 (< 0.0001)	ND	ND	NS
		P. ultimum					
Glenfruin	NS	NS	NS	NS	NS	NS	-0.378 (0.023)
Paardekloof	-0.425; 0.024	NS	-0.458 (0.03)	NS	NS	NS	NS
Remhoogte	NS	NS	NS	NS	ND	ND	NS
		***Pratylenchus* spp.**					
Paardekloof	NS	-0.428 (0.026)	-0.49 (0.009)	-0.49 (0.009)	-0.393 (0.042)	-0.516 (0.006)	NS
Remhoogte	-0.361 (0.031)	NS	NS	NS	NS	NS	-0.371 (0.026)

Pearson's correlation and significance levels were calculated between (i) DNA quantities of ARD microbes or *Pratylenchus* spp. in apple roots and (ii) the apple tree growth responses (increase in stem diameter and shoot length) and yield that were measured over a 3-year (Remhoogte) or 4-year (Glenfruin and Paardekloof) growth period. Yield for the Paardekloof orchard was cumulative yield for the 3rd and 4th year of growth, whereas that of Remhoogte and Glenfruin was for the 3rd and 4th year of growth respectively. Microbial DNA quantities were determined through quantitative real-time PCR, whereas *Pratylenchus* spp. were determined through conventional counting. Microbes and *Pratylenchus* spp. were quantified 20 months after planting.
NS = non-significant; ND = not done

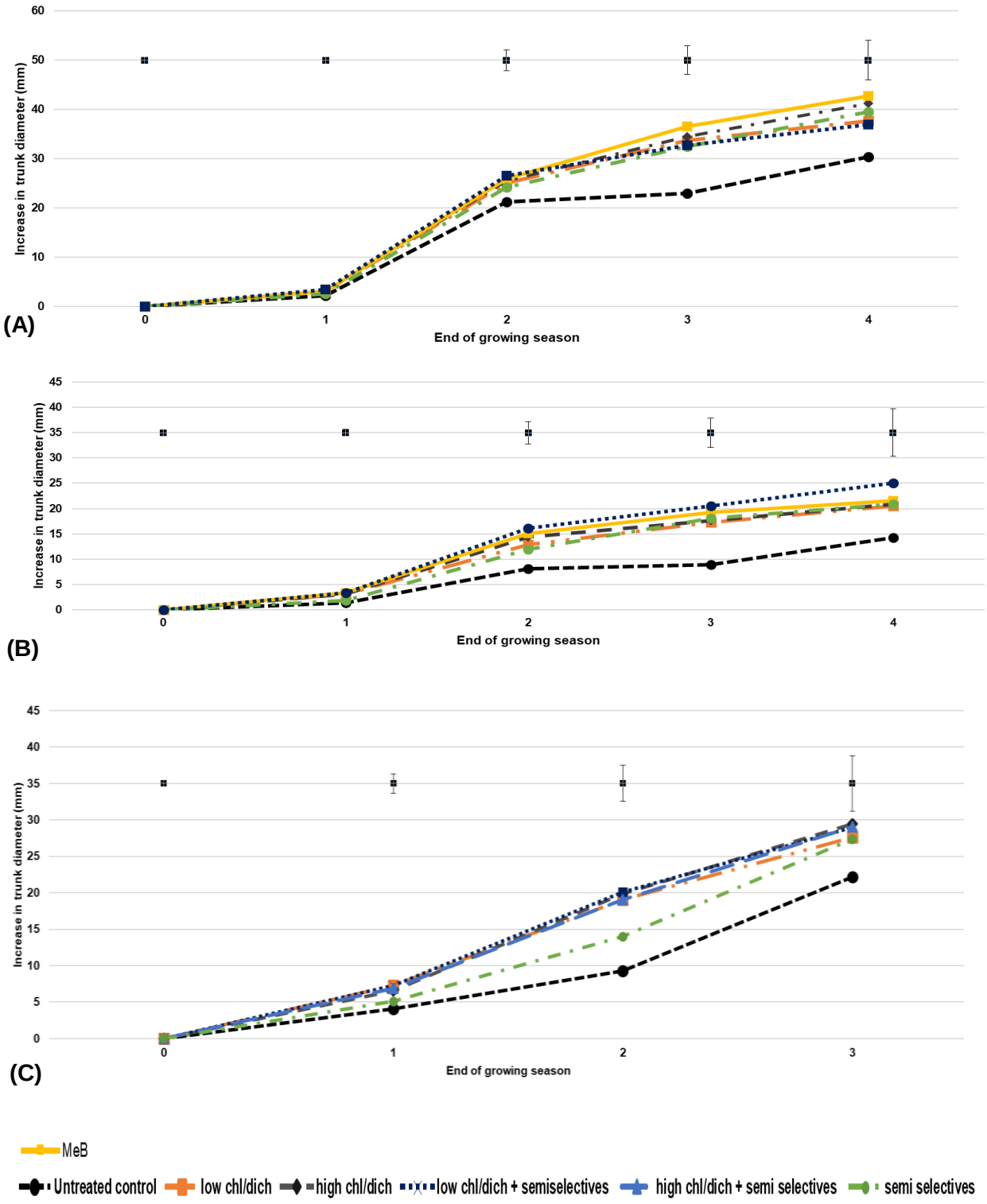

Fig. 1. Increase in the trunk diameter of apple trees planted in three apple replant orchard trials at (A) Glenfruin (B) Paardekloof and (C) Remhoogte, in response to various soil fumigation and semi-selective chemical treatments. Increase in trunk diameter is shown over a 3-year (Remhoogte) or 4-year (Paardekloof and Glenfruin) period. Values are the average of six replicates (eight trees per replicate). Error bars indicate the least significant difference for each time-point. The semi-selective treatments consisted of a soil drench at planting (metalaxyl, fenamiphos and imidacloprid) followed by 2-years of potassium phosphonate applications, twice per year. The fumigant treatments were all applied pre-plant and included methyl bromide (MeB) and two fumigants differing in their chloropicrin content; the Low chl/dichl fumigant had a 33.3 % chloropicrin and 60.8 % 1,3-dichloropropene content, whereas the High chl/dichl fumigant had a 57 % chloropicrin and 38 % 1,3-dichloropropene content. At the Glenfruin and Paardekloof orchards, the high chl/dich + semi selectives treatment was not included. The latter treatment was only included at the Remhoogte trial. At the Remhoogte trial, the methyl bromide treatment was not included.

CHAPTER 3

Phosphite translocation and persistence in apple roots and their spectrum of activity against oomycete root rot pathogens

ABSTRACT

Phosphonate fungicides are widely used for the management of *Phytophthora* diseases, and also show potential for managing apple replant disease. The aims of the study were to better understand (i) the temporal nature of phosphite concentrations in the roots of asymptomatic apple trees (no aboveground symptoms, but oomycete root rot pathogens were present) in response to different phosphonate application methods (foliar sprays, stem sprays, soil drenching and trunk paints) in two orchard trials, and (ii) the phosphite sensitivity of three oomycete root rot pathogens. Root phosphite (active breakdown product of phopshonates in plants) concentrations peaked at 4- to 8-weeks after winter phosphonate applications, and for summer applications at 2- to 4-weeks. For the summer applications, root phosphite decreased significantly over a 12-week period post-application. There were no consistent decreasing trends for the post-winter application period, since trends differed between the two trials. The trunk paint application yielded significantly higher root phosphite concentrations than the other application methods. In the two trials, the root phosphite concentrations in the trunk paint application ranged from 46.26 to 268.33 µg/g $_{FW}$ at 12-weeks after the winter application, 72.42 to 103.36 µg/g $_{FW}$ at 17-weeks after the winter application, and 14.48 to 878.73 µg/g $_{FW}$ at 12 weeks after the summer application. The soil drench application was least effective in accumulation of phosphite in tree roots. It was not possible to meaningfully compare efficacy of the different phosphonate application methods, because they were applied on different schedules; foliar sprays occured in summer, whereas other application methods were also applied in winter. Foliar sprays were nonetheless able to outperform the trunk sprays. *Phytophthora cactorum* and *Pythium irregulare* DNA quantities in the roots of trees receiving phosphonate treatment were significantly lower than the control treatment. *Phytopythium vexans* was more sensitive *in vitro* to phosphite than *P. cactorum* and *P. irregulare.* All three species exhibited variation among isolates in phosphite sensitivity *in vitro*. Inhibitory activity of phosphite towards all the species in liquid medium assays was much lower in comparison to a solid medium. Phosphate, which is known to influence the efficacy of phosphite *in vitro*, had variable effects. *Phytophthora cactorum* was inhibited significantly less by phosphite at 15 mM phosphate than at 1 mM phosphate, whereas *P. vexans* showed an opposite trend. The effect of phosphate on phosphite inhibition of *P. irregulare* was medium dependent (liquid or solid).

INTRODUCTION

Various oomycete root rot pathogens can contribute to growth and yield reductions of apple. *Phytophthora cactorum* is known for causing root rot, as well as crown- and collar rot on apple. Trees of all ages can suffer from the disease under favourable environmental conditions (Matheron *et al*., 1988, Wilkocks, 1993). Several *Pythium* spp. and *Pythopythium vexans* can also cause root rot of apple, but these species are mainly known for attacking newly planted young apple trees. The aforementioned oomycete pathogens form part of the apple replant disease (ARD) complex of pathogens that also include fungal pathogens and parasitic nematodes. ARD occurs when apples are replanted onto soil previously cultivated with apple or closely related tree species, resulting in tree growth and yield reductions (Mazzola & Manici, 2012). In South Africa, *Phytopythium vexans, Pythium irregulare, Pythium ultimum* and *Pythium sylvaticum* are highly virulent oomycete ARD pathogens that are widely distributed among orchard locations (Tewoldemedhin *et al*., 2011b,c). Soilborne oomycete pathogens can be managed using phosphonate fungicides. A wide range of *Phytophthora* spp. attacking a broad range of hosts including vegetables, shrubs and tree crops have been effectively managed using phosphonate fungicides (Cohen and Coffey, 1986). In contrast, only a few reports have been published on the effifcay of phosphonate fungicides against Pythium diseases. Phosphonates have been reported as being effective in managing a few *Pythium* spp. causing Pythium blight of turfgrasses, soybean- and Cape gooseberry daming-off (Cook *et al.*, 2009; Miyake *et al.,* 2015; Carmona *et al.*, 2018). On apple, phosphonates have been used for managing root- and crown rot caused by *P. cactorum* (Utkhede and Smith, 1993). In South Africa, *Phytopythium vexans, Pythium irregulare, Pythium ultimum* and *Pythium sylvaticum* are highly virulent oomycete ARD pathogens that are widely distributed among orchard locations (Tewoldemedhin *et al*., 2011b,c).There are no reports on the efficacy of phosphonates against *Pythium* spp. causing root rot and apple replant disease on apple.

Phosphonate fungicides are alkali metal salts of phosphorous acid (H_3PO_3). Various commercial formulations are available for example potassium phosphonate and calcium phosphonate. In planta, phosphonates dissociate into various ions, including phosphite ions (Mc Donald *et al*., 2001; Martínez, 2016; Scott, *et al*., 2016, Borza *et al*., 2017) that have activity against oomycetes, and to a lesser extend against fungal and bacterial pathogens (Guest and Grant, 1991; Lobato *et al*., 2010). Phosphite is fully systemic in plants and can be translocated in the xylem acropetally and in the phloem basipetally. This high mobility in plants enables the use of various fungicide application methods when treating plants with phosphonates. Application methods include soil drenching, trunk injection, trunk paint, trunk sprays and foliar sprays (Pegg *et al*., 1995; González, Caetano and Sánchez, 2017;

Duvenhage, 1999; Hardy, Barrett and Shearer, 2001; Cooke and Little, 2002; Nartvaranant *et al*, 2004). Following application, phosphite will be translocated in plants in a source-sink manner. Thus, the plant organs that are the strongest sink at the time of application will accumulate the highest phosphite concentrations (Malusa and Tosi, 2005; Whiley *et al*., 1995).

The control of oomycete-induced root rot in apple will likely require application of phosphonates at the time that roots are a sink as in periods of active root development. Limited information is available on the temporal growth of roots in young apple trees (Artikson, 1980, Psarras *et al*., 2000). In South Africa specifically, no studies have been published on the seasonal dynamics of root growth on apples. Recent research conducted by E. Lotze in the Grabouw region showed that young trees show continued root growth, but that root growth peaks during summer and autumn (personal communication, E. Lotze, Stellenbosch University, Department of Horticulture).

Several studies over the past few decades have investigated the mode of action of phosphonates against oomycetes. The mode of action can be a direct toxic (fungistatic) effect towards the pathogen and/or an indirect effect where host resistance is induced (Guest and Grant, 1991; Machinandiarena *et al.*, 2012; Massoud *et al.*, 2012). Recent studies in the *Arabidopsis-Hyaloperonospora* and *Eucalyptus-Phytophthora cinnamomi* host-pathogen systems, have provided evidence that at low *in planta* phosphite concentrations, host resistance induction contributes to disease suppression. However, at higher *in planta* concentrations a direct toxic effect will limit disease development (Jackson *et al.*, 2000; Massoud *et al.,* 2012). The direct toxic effect of phosphite will likely differ in different host-pathogen interactions since suppression will depend on the (i) concentration and persistence of phosphite in the plant organ attacked by the pathogen, and (ii) phosphite sensitivity of the pathogen involved. Plant species are known to vary in the concentration and persistence of phosphite following phosphonate applications (Hardy *et al.*, 2001). The translocation and persistence of phosphite in apple trees have not been investigated previously, aside from a study by Long *et al.* (1989) on trunk injections and Malusa and Tosi (2005) on foliar sprays. However, both of the aforementioned studies focused on translocation to shoots, trunks and fruits, not to roots.

Substantial information is available on the direct toxic effect of phosphite towards *Phytophthora* spp., whereas less is known about *Pythium* and *Phytopythium* spp. The direct toxic effect towards oomycetes has been investigated using *in vitro* studies and artificial growth media. In *Phytophthora,* phosphite is known to affect all life stages *in vitro,* including mycelia and the formation of sporangia, oospores and chlamydospores (Coffey & Joseph, 1985). However, most studies have evaluated the effect of phosphite on mycelial growth

inhibition on solid agar media. Although this is a very practical and easy manner for evaluating the sensitivity of isolates, it can be inaccurate. This is due to the fact that only radial growth and not density is taken into account. The use of liquid media has been suggested as being a more accurate evaluation method and can overcome this potential problem (Guest & Grant, 1991). Another factor that can influence the *in vitro* phosphite sensitivity of isolates is phosphate concentration in the growth medium. This could be due to the fact that phosphite and phosphate are taken up by the same transporters in *Phytophthora*. Consequently, it has been found that at high phosphate concentrations, isolates may demonstrate reduced sensitivity to phosphite relative to lower phosphate concentrations (Griffith *et al*., 1993, 1989).

Phytophthora spp. and isolates within species, vary in their sensitivity towards phosphite *in vitro* (Ouimette and Coffey, 1989; Wilkinson *et al*., 2001a). The *in vitro* sensitivity of *P. cactorum* has only been evaluated for four isolates in one study. The EC_{50} values for *P. cactorum* mycelial growth inhibition were relatively low ranging from 20.3 to 24.3 ug/ml, when a low phosphate-containing (0.14 mM phosphate) 0.5 % corn meal agar was used (Ouimette and Coffey, 1989). A phosphate concentration of 0.14 mM is low considering that phosphate levels in plants can range between 0.5 to 20 mM (Bieleski, 1973). Although only four studies have investigated the sensitivity of *Pythium* spp., it is clear that *Pythium* spp. also vary in their *in vitro* sensitivity to phosphite (Sanders *et al.*, 1983; Fenn & Coffey, 1984; Cook *et al.*, 2009; Weiland *et al*., 2014). Of the ARD oomycete pathogens, the *in vitro* sensitivity of only four to five isolates to potassium phosphonate have been evaluated for *P. irregulare* and *P. ultimum* (Cook *et al*., 2009, Weiland *et al*., 2014), and more than 29 isolates of each of *P. irregulare, P. ultimum* and *P. sylvaticum* against fosetyl-Al (Sanders *et al.,* 1983; Fenn & Coffey, 1984; Weiland *et al.*, 2014).

The overall aim of this study was to learn more about phosphite translocation and persistence in young apple tree roots, and the *in planta* and *in vitro* effect of phosphite on selected oomycete pathogens. Two orchard trials were used to evaluate the effect of different phosphonate application methods (soil drenching, stem sprays, trunk paint and foliar sprays) on root phosphite concentrations and its persistence in asymptomatic apple trees. The asymptomatic trees were known to be infected by some oomycete pathogens, but no aboveground symptoms were evident. The persistence of phosphite after a winter, followed by a summer application was also evaluated. At the end of the trials, the extent of *P. cactorum, P. vexans* and *P. irregulare* colonization in the roots of phosphonate treated and control trees was evaluated using real-time quantitative PCR (qPCR). The *in vitro* toxicity of phosphite to the three aforementioned oomycete species were also investigated. Since medium type (i.e., liquid or solid) and medium phosphate content can influence

sensitivity to phosphite, these factors were also investigated *in vitro*. The degree of inhibition of *P. irregulare* and *P. cactorum in vitro* and in roots was investigated in an attempt to make inferences regarding the mode of action of phosphite.

MATERIALS AND METHODS

Orchard trials with phosphonate to assess phosphite concentrations and oomycete root infection

Orchards and experimental layout

Trials were conducted in two orchards containing asymptomatic apple trees in their second year of growth. The orchard trees were established on apple replant sites that had been fumigated using 1,3-dichloropropene/chloropicrin prior to planting. The orchards were situated on the Paardekloof and Vastrap farms in the Witzenberg valley, South Africa (340 16' 60" S ,200 36' 0" E). Both trials were planted with the cultivar Early Red One grafted onto the MM109 rootstock. The soil type was a sandy loam at Paardekloof, while Vastrap had a sandy clay loam soil.

Seven treatments were evaluated in both orchard trials (Table 1), which included four different application methods; trunk paint, soil drench, foliar sprays and trunk sprays. The phosphonate formulation that was evaluated in all application methods was a potassium phosphonate fungicide (Phosguard 400 SL, 400 g phosphorous acid/L, Nulandis, Witfield, South Africa). Additionally, an ammonium phosphonate formulation (Brilliant SL, 300g phosphorous acid/L, Arysta, South Africa,) was also evaluated as a foliar spray. The pH of the 5g a.i./L foliar sprays was adjusted to a pH of 7.2 using potassium hydroxide pellets in order to prevent leaf burn. Foliar sprays were applied using a mist blower backpack sprayer (SR 400, STIHL, Virginia, USA). For the trunk sprays, the bark penetrant Charge (1000 g a.i./l polyether-polymethylsiloxane-copolymer; Villa Crop, South Africa) was evaluated at two different dosages. The penetrant was added at a concentration of 0.50 ml/L for the full rate application and at 0.25 ml/L for the half rate application. Trunk sprays were applied using a 750 ml laboratory spray bottle. The different phosphonate application treatments were conducted in winter followed by a summer application, with the exception of the foliar sprays that were only applied in summer. The soil drench and trunk paint and –spray applications were applied on 12 June 2015 for the winter applications and on 18 November 2015 for the summer applications. The foliar sprays were only applied in summer, and not in winter. The summer foliar applications consisted of three weekly sprays, with the first application being

on 4 November 2015 and the last application on 18 November 2015 (Table 1). Each treatment was replicated six times with a replicate consisting of six trees.

Root phosphite quantification

Root sampling. Root sampling for phosphite quantification was conducted at 2, 4, 8 and 12-weeks after phosphonates were applied in winter and summer. Additionally, a pre-summer sampling time point, 17-weeks after the winter applications, was included just prior to the application of the summer applications. An approximately 30 g root sample was obtained from the four centred trees within each replicate, which was pooled to yield one composite sample per replicate. Roots were washed under running tap water, and fresh weight was recorded. The roots were placed in brown paper bags and dried for 3 days at 60°C.

Extraction and phosphite quantification using liquid chromatography-mass spectrometry (LC-MS/MS). The dried root samples were first pulverised into a powder using an electric IKA basic analytical mill R (IKAR - Werke GmbH and Co.KG, Staufen, Germany). Five hundred milligram of root powder was added to 10 ml of deionized water. The solution was incubated overnight on a rotary shake incubator at 100 rpm and 25˚C (3082U, Labcon, Midrand, South Africa). Subsequently, the samples were centrifuged (Centrifuge 5810R, Eppendorf, Hamburg Germany) using a swinging bucket rotor head, for 10 min. at 4000 rpm at 20˚C. From the resultant supernatant, a 1000 µl volume was passed through a 0.22 µm PALL acrodisc ® syringe filter containing a Supor ® membrane (Pall Corporation, Midrand, South Africa). Seven hundred microliters of the filtrate were subsequently passed through a 10K Nanosep ® centrifugal device (Pall corporation) by centrifugation at 5000 g for 20 min. The flow through filtrate was used for LC/MS-MS phosphite quantification.

For each LC/MS-MS analyses run, a standard curve was prepared. A stock solution of 200 g/l of phosphorous acid (Sigma-Aldrich-Aldrich, Oakville, ON), adjusted to a pH of 6.5 with potassium hydroxide, was serially diluted to obtain a standard curve with ten points ranging in concentration from 0.05 µg/ml to 30 µg/ml.

All root extracts were analysed by the Central Analytical Facility Mass Spectrophotometry division at Stellenbosch University (Stellenbosch, South Africa). LC/MS-MS analyses were conducted using the European Commission Reference Laboratories for residues of pesticides Single Residue Methods: Quick method for the analyses of numerous highly polar pesticides in foods of plant origin; method 1.3 “Glyphosate and Co. AS 11-(HC (http://www.crl-pesticides.eu/library/docs/srm/meth_QuPPe.pdf). 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). A Thermo Hypercarb (100 x 2.1 mm, 5 μM particle size) (Thermo Fisher Scientific, Waltham, USA) column was used for LC separation. The flow rate, mobile phase and MS parameters were as described in McLeod *et al.* (2018). Quantitative data was processed by Masslynx and Targetlynx software (Ver.4.1).

The recovery rate and matrix effect of the extraction method were determined using roots from the untreated control, which were spiked with phosphite. Roots were spiked with phosphite at the start of the phosphite extraction process and after phosphite was purified through the 10-K Nanosep device. The latter allows evaluating whether a matrix effect exists in samples. The pre-and post-extraction spiking were conducted at phosphite concentrations of 0.5 µg/ml, 1 µg/ml and 2 µg/ml.

Quantification of P. irregulare, P. cactorum and P. vexans DNA from roots.

Fine feeder roots for pathogen quantification were sampled from trees just immediately prior to the phosphonate applications made in June 2015 and 12-months later (June 2016). Roots were sampled at the centre four trees of each replicate to make one composite sample per replicate. The roots were washed free from soil and lyophilized. DNA was extracted from roots using the NucleoSpin PLANT II kit (Macherey-Nagel GmbH and Ko, Duren, Germany) as described in Chapter 2. Quantitative real-time PCR (qPCR) amplifications of *P. irregulare, Phytophthora* spp. and *P. vexans* were conducted using primers and reaction- and amplification conditions described in Chapter 2. qPCR was conducted in duplicate for each replicate of each treatment. Since the *Phytophthora* spp. qPCR does not identify the species involved, a subset of the amplicons generated from positive samples was sequenced to determine the species as described in Chapter 2. The change in pathogen DNA concentration was calculated by subtracting the initial pathogen DNA quantity from that obtained 12-months later.

In vitro phosphite sensitivity of *P. irregulare, P. vexans* and *P. cactorum*

Isolate collection

Eight *P. cactorum* isolates and 10 isolates each of *P. irregulare*, and *P. vexans* were obtained from various sources. All ten *P. irregulare* isolates and four *P. cactorum* isolates (PCD, PCE, PCF, PCG) originated from ARD orchard trials (Chapter 2). Three of the other *P. cactorum* isolates (PCA, PCB, PCC) were obtained through soil baiting in *Phytophthora* root rot control trials (Chapter 4) and one isolate (7204) was from the Stellenbosch culture collection. All ten *P. vexans* isolates were obtained from the Stellenbosch university culture collection. The culture collection isolates were all derived from the ARD study of Tewoldemedhin *et al.* (2011b). Isolates from ARD orchards were obtained by plating root

segments of apple seedlings planted in the ARD soils as described in Chapter 2. Alternatively, isolates were obtained by soil baiting using avocado leaf disks floated on an ARD soil slurry as described in Chapter 4. All isolates were hyphal tipped twice to obtain pure cultures. Species identity of the isolates was determined and confirmed through sequencing of the internal transcribed spacer region, followed by BLAST analyses as described in Chapter 2.

Solid agar medium assay

The sensitivity of *Pythium irregulare* (10 isolates), *P. cactorum* (eight isolates) and *P. vexans* (10 isolates) isolates to phosphite was evaluated in an agar assay. Assessments were conducted using media containing phosphate at 1 mM and 15 mM phosphate and employed three different phosphite concentrations. *Phytopythium vexans* isolates were evaluated at 30,100 and 200 µg/ml phosphite, *P. irregulare* at 250, 500 and 1000 µg/ml and *Phytophthora cactorum* was evaluated at 200, 500 and 1000 µg/ml. Preliminary screening trials showed that *P. vexans* was more sensitive than the other species, and it was therefore evaluated at lower phosphite concentrations. The isolates were first grown on potato dextrose agar plus streptomycin (PDA^{+}) for 5-days at 25˚C in the dark. A mycelial plug (0.5 cm in diameter) from the edge of the colony was used to inoculate a Ribeiro's agar medium plate (90 mm dia.) containing no phosphite (control), and the range of required phosphite concentrations. For each isolate, two plates were inoculated per treatment and the experiment was repeated once.

Ribeiro's medium is a minimal defined salts medium, and was prepared according to Ribeiro *et al*. (1975) as modified by Fenn and Coffey (1984). The exception was that the medium phosphate quantity was adjusted to final concentrations of 1 mM or 15 mM. Bacteriological agar was added at 15 g / L (Merck, Darmstadt, Germany). The medium was autoclaved, and allowed to reach 50°C before the addition of phosphorous acid (Sigma-Aldrich-Aldrich, Oakville, ON), to yield the specified phosphite concentrations. Prior to use, the phosphorous acid was filter sterilised through a 0.22 µm PALL acrodisc ® syringe filter containing a Supor ® membrane (Pall Corporation, Washington, USA).

The agar plates inoculated with the respective test isolates were incubated at 24°C in the dark. Mycelial growth on each plate was determined by measuring the diameter of the colony (in two directions) after 5-days for *P. vexans* and *P. irregulare* isolates, and after 9-days for *Phytophthora cactorum* isolates. The colony plug dia. was subtracted from the colony size prior to calculating the percentage growth inhibition. The percentage growth inhibition was calculated as follows:

$$\% \text{ mycelial growth inhibition} = \left(1 - \frac{\text{growth on amended medium}}{\text{growth on unamended medium}}\right) \times 100$$

Liquid medium assay

A total of seven isolates were evaluated in the liquid medium assay, which included two *P. cactorum* isolates, two *P. irregulare* isolates and three *P. vexans* isolates. These isolates were selected based on the results of the agar assay at 15 mM phosphate and 200 µg/ml (*P. vexans*) or 500 µg/ml (*P. irregulare* and *P. cactorum*) phosphite, and represented isolates that were either among the most phosphite sensitive or tolerant for each species. The liquid assay was conducted in the same manner as the solid agar assay employing the same concentrations of phosphite and phosphate. The exception was that agar was omitted from the Ribeiro's medium and that each 90-mm-plate was inoculated with four agar plugs (6 mm dia.). Two plates were inoculated per treatment for each isolate. Control plates containing phosphate at 1 mM and 15 mM were also included. *Pythium irregulare* and *P. vexans* isolates were incubated at 24°C in the dark for 7-days, while *P. cactorum* isolates were incubated for 14-days. Mycelial growth in each plate was determined by first harvesting the mycelia onto Whatman number 1 filter paper, using vacuum filtration to remove growth medium and rinsing with water. The harvested mycelia on filter papers were dried at 60°C for 2 days in an oven. The weight was recorded for each replicate, and the weight of the filter paper was subtracted. The percentage growth inhibition was calculated as described for the solid agar assay and the experiment was repeated once.

Statistical data analyses

Root phosphite concentrations, the change in pathogen DNA concentrations, *in vitro* pathogen growth inhibition on solid- and liquid media data were subjected to analysis of variance (ANOVA) using the GLM (General Linear Models) Procedure of SAS statistical software (Version 9.4; SAS Institute Inc, Cary, USA). The data of the two repeat experiments from the *in vitro* pathogen growth inhibition was used as blocks in the analyses. The Shapiro-Wilk test was used to test data normality. The *P. irregulare* DNA concentration data and root phosphite concentration deviated significantly from normality. The data were therefore Ln (x+1) transformed to improve normality. For the comparison of the liquid and solid media *in vitro* data, a weighted analysis was conducted, since there was significant variance ($P > 0.05$) for medium based on Leven's test. Differences between means were investigated using Fisher's least significant difference (LSD) test at the 5 % level. All analyses were conducted using SAS statistical software.

RESULTS

Orchard trials with phosphonate to assess phosphite concentrations and oomycete root infection

Root phosphite quantification using LC/MS-MS analyses

The phosphite standard curve was linear (R^2 =0.99876) over the evaluated concentration range (0.01 to 30µg/g). The recovery rate of phosphite spiked control root samples was 42% ± 6 for both the pre- and post-clean up samples. Therefore, the final root phosphite concentrations were calculated by correcting for the recovery rate, i.e. each value was multiplied by 100 ÷ 42.

ANOVA analyses performed on the combined trials showed a significant interaction for trial x treatment ($P < 0.0006$), therefore the trials were analyzed separately. For each of the trials, for each season (winter and summer) separately, there was a significant treatment x time interaction ($P < 0.0001$). Therefore, the data of the sampling time points (2 to 12 weeks post-treatment) are shown separately for each season. The data of the actual root phosphite concentrations (µg/g $_{FW}$) are shown in Table 2 along with the Fisher's least significant test results of the transformed data used for statistical analyses. To better illustrate trends and the temporal nature of root phosphite concentrations over the 12-week periods, the transformed data of Table 2 is also shown as line graphs in Figs. 1 and 2.

Considering all treatments, the root phosphite concentrations detected after the winter applications fluctuated over the 12-week period, but showed similar trends at the two trial locations (Vastrap and Paardekloof) (Table 2; Figs. 1 & 2). At Vastrap, root phosphite concentrations peaked for all treatments after 8-weeks (18.7 – 263.24 µg/g_{FW}) and were significantly higher than at the other winter sampling time points. At Paardekloof, this was also true for the trunk paint and trunk spray + ½ penetrant treatments, which were significantly higher or equal to the other winter time points (35.43 – 268.33 µg/g_{FW}). Root phosphite levels for the remaining treatments at Paardekloof peaked at 4- weeks post-application in winter (16.62 – 120.67 µg/g_{FW}).

There were no clear trends, considering all treatments, for a change in root phosphite concentrations over the 12-week period following winter applications. Root phosphite concentration increased significantly in the trunk paint treatment at both trials between the 2-week and 12-week time points (46.26 – 268. 33 µg/g_{FW}). For the other treatment methods, the root phosphite concentrations for the two time points after the winter applications exhibited either a significant decrease (mainly at Paardekloof) or no significant change (mainly at Vastrap) (Table 2; Figs. 1 & 2).

For the summer applications, considering all treatments, root phosphite concentrations increased and then decreased over the 12-week period following applications (Table 2; Figs. 1 and 2). At both trials, root phosphite concentrations peaked 4-weeks after application for the foliar sprays (21.50 – 32.31 µg/g_{FW}), and for the other treatments after 2-weeks (18.33 – 878.74 µg/g). Subsequently, root phosphite concentrations for the summer applications declined significantly for all treatments to the 12-week time point (3.00 – 32.90 µg/g_{FW}). The exception was for the foliar ammonium phosphonate treatment, which peaked at 4-weeks post-application in both trials (23.60 – 24.24 µg/g_{FW}).

The overall performance of treatments considering all time points for the winter and summer applications were similar in the two trials (Table 2; Figs. 1 & 2). The trunk paint application yielded significantly higher root phosphite concentrations than all other phosphonate application methods considering all time points for winter and summer applications (14.48 – 878.71 µg/g_{FW}). The second highest root phosphite level was detected in response to the trunk spray + penetrant and trunk spray + ½ penetrant treatments, but only for the winter applications (6.08 – 91.05 µg/g_{FW}). Both of these treatments were significantly better than the Soil drench treatment (3.00 -25.86 µg/g_{FW}) for all the winter and summer time points, with the exception of the 12-week summer time point. However, when compared to the foliar spray treatments at the 4- to 12-week time points after summer applications, the two trunk spray treatments were diminished with respect to relative phosphite levels detected in roots among all treatments. The two trunk spray treatments had similar or significantly lower root phosphite concentrations (3.45 – 9.98 µg/g_{FW}) than the two foliar spray treatments in both trials 4- to 12-weeks after the summer applications (5.12 – 32.31 µg/g_{FW}). There was no consistent trend for the trunk spray + penetrant and trunk spray + ½ penetrant to differ in delivering root phosphite concentrations (Table 2; Figs 1 and 2).

The relative efficacy of the two foliar spray treatments when compared to other treatments was difficult to evaluate since they were only applied in summer. The limited number of applications restricted to the summer application resulted in root phosphite concentrations that were significantly lower than that observed for other treatments at 17-weeks (0-week summer application time point) after the winter phosphonate stem applications were conducted (Table 2; Figs. 1 & 2). The two foliar treatments nonetheless outperformed the two trunk spray treatments in terms of root phosphonate concentrations as discussed above. The foliar potassium phosphonate treatment yielded significantly higher root phosphite concentrations than the foliar ammonium phosphonate treatment at the early summer time points (Paardekloof: 2- and 4-weeks; Vastrap: 2-weeks) but not at the later 8- and 12-week time points. The two foliar spray treatments also yielded significantly higher root phosphite concentrations than the soil drench treatment; the exception was the 2-week

summer time point at Vastrap. The range of root phosphite concentrations obtained with the potassium phosphonate foliar sprays at 2- to 4-weeks post-application were relatively low and ranged between 16.45 to 32.31 µg/g_{FW}. Subsequently, concentrations at 8 to 12-weeks after application declined to a range of 5.12 to 11.86 µg/g_{FW} (Table 2).

Quantification of P. irregulare, P. cactorum and P. vexans DNA from roots.

In both trials, *Phytophthora* spp. and *P. irregulare* were detected in apple roots by qPCR for all treatments. Sequencing of a subset of the qPCR products from the *Phytophthora* spp. assay, showed that *P. cactorum* was present in the trials. No positive amplifications were obtained for *P. vexans* in either trial for any of the treatments.

At the two trials there were significant differences between the phosphonate and control treatments ($P \leq 0.0265$) in *P. irregulare* root DNA concentrations over the 12-month trial period. The quantity of *P. irregulare* DNA detected in roots from the control treatment increased over the 12-month period, which was significantly different from the decrease in *P. irregulare* quantities observed for the phosphonate treatments in both trials (Table 3). There was no significant difference in quantity of *P. irregulare* DNA detected in apple roots among the different phosphonate treatments at Vastrap, but at Paardekloof the two stem-spray treatments were less effective in reducing the quantity of pathogen DNA in roots relative to the other phosphonate treatments.

The *P. cactorum* DNA quantities also differed significantly between phosphonate and control treatments in both trials ($P \leq 0.0186$). In both trials, the *P. cactorum* DNA quantities increased in the control treatment, which was significantly higher in comparison to the other phosphonate treatments. The exception was the trunk spray + penetrant treatment at Vastrap that did not differ significantly from the control (Table 3). At Paardekloof, the phosphonate treatments did not differ significantly from each other in their effect on *P. cactorum* DNA quantities. At Vastrap, the phosphonate treatments also did not differ significantly from each other in *P. cactorum* DNA levels; the exception was for the foliar ammonium phosphonate treatment that had a greater reduction in *P. cactorum* quantities than some of the other phosphonate treatments.

In vitro phosphite sensitivity of *P. irregulare*, *P. vexans* and *P. cactorum*

Solid agar medium assay

ANOVA analyses showed that for all three oomycete species, relative growth inhibition when exposed to phosphite in the presence of 15 mM phosphate differed significantly ($P < 0.0001$) among isolates within a species. The *P. cactorum* isolates PCC, PCD and PCG were

significantly less sensitive (52.43 to 58.78 % inhibition) to 500 µg/ml phosphite than the other *P. cactorum* isolates (82.67 to 89.66 % inhibition) (Table 4). The least sensitive *P. irregulare* isolates at 500 µg/ml phosphite were NN11, NN13, NN15 and PSR3.4 (21.64 % to 26.85 % inhibition). These isolates did not differ significantly from each other in sensitivity, but had a significantly lower percentage inhibition than five of the other isolates that were inhibited 44.15% to 80.38%. *Pythium irregulare* isolate PSR3.1 was the most sensitive, showing a significantly higher (80.35%) inhibition than the other isolates. The *P. vexans* isolates, screened at 200 µg/ml phosphite, were all very sensitive showing more than 72.30% inhibition at this concentration. Isolate 6737 was highly sensitive (91.50%) and was inhibited to a significantly greater degree than isolates 6742, 6743, 6748, 6736 and 6718 (72.30 to 81.0%) (Table 4).

Influence of medium (liquid or solid) and phosphate concentration on phosphite sensitivity

ANOVA analyses showed that there was no significant effect of medium or phosphate concentration on phosphite sensitivity of isolates within a given species (Table 5). However, there was a significant interaction between medium x species x phosphite conc. x phosphate conc. (P =0.0190), which was investigated further.

The percentage inhibition by phosphite on the three species was significantly influenced by the use of liquid versus solid agar medium (Table 6). For all three species inhibition was significantly less in liquid medium versus solid medium. This was true for all phosphite and phosphate concentrations, with a more or less 50% lower inhibition observed in liquid medium than on solid medium (Table 6).

The effect of phosphate on percentage inhibition differed for all three species (Table 6). Inhibition of *P. cactorum* was significantly less at 15 mM phosphate than at 1 mM phosphate regardless of medium type, resulting in a 8.31% to 11.76% lower inhibition at 15 mM than at 1 mM phosphate. Phosphate had an opposite effect on *P. vexans* in comparison to *P. cactorum*, since the percentage inhibition was significantly higher in liquid and solid mediums at 15 mM than at 1mM for *P. vexans*. This was more evident for *P. vexans* in the liquid medium where percentage inhibition was significantly higher (7.99 to 12.59% difference) at all three phosphite concentrations in the presence of 15 mM than at 1 mM phosphate. The effect of phosphate on *P. irregulare* inhibition was medium dependent. In the liquid medium at the 500 µg/ml phosphite concentration, percentage inhibition was 9.99% less at 15 mM than at 1 mM phosphate. In contrast, on the solid agar medium the percentage inhibition for *P. irregulare* was significantly higher (9.16 to 17.19% difference) at 15 mM than at 1 mM phosphate for all three phosphite concentrations (Table 6).

For the two isolates of *P. cactorum* and *P. irregulare* that were screened *in vitro* in the liquid and solid media at various phosphate concentrations, these two factors (medium type and phosphate concentration) influenced the inhibitory activity of phosphite significantly (Table 7). The isolates were initially selected for representing the top and lower range of phosphite sensitivities within each species by using an agar assay containing 15 mM phosphate and 500 µg/ml phosphite (Table 4). However, when considering the effect of medium (liquid and solid) and phosphate (1 mM and 15 mM) the ranking of the two evaluated isolates changed for *P. cactorum,* and for *P. irregulare* no significant differences were sometimes evident in the sensitivity of the two isolates. For example, at 15 mM phosphate and 500 µg/ml phosphite on solid medium, *P. cactorum* isolate PCC was significantly more tolerant (58.79% inhibition) compared to isolate PCA (87.29% inhibition). However, in liquid medium at 1 mM phosphate and 200 µg/ml, *P. cactorum* isolate PCA was significantly more tolerant (15.34%) than isolate PCC (27.12% inhibition). In several other phosphite and phosphate concentrations in the liquid and agar assays there were no significant differences in percentage inhibition between the two *P. cactorum* isolates. For *P. irregulare,* isolate E19 was significantly more tolerant (44.15% inhibition) than isolate PS3.1 (80.38) on the initially screened solid medium at 15 mM phosphate and 500 µg/ml. This was also true when the two *P. irregulare* isolates were screened at some of the other phosphate and phosphite concentrations on solid and liquid medium. However, there were some phosphate and phosphite concentrations in the liquid medium where no significant differences in sensitivity were evident between the two isolates, for example at 500 µg/ml phosphite and 15 mM phosphate in liquid medium (14.38 and 20.66% inhibition) (Table 7).

DISCUSSION

The current study revealed the temporal nature of phosphite in apple roots in two orchard trials when phosphonate fungicides were applied using various application methods in winter and summer. Root phosphite concentrations peaked, depending on application method, at 4- to 8-weeks after winter applications, and after 2- to 4-weeks for summer applications. A relative rapid decline in root phosphite over a 12-week period was observed after the summer applications, but this trend was not consistent with the winter applications in both trials. The trunk paint application method yielded the highest root phosphite concentrations, and showed large fluctuations in root phosphite concentrations 12-weeks post application, especially in summer. In contrast, the foliar sprays, which were only applied in summer, although yielding lower root phosphite concentrations, exhibited relatively lower fluctuations in root phosphite concentrations. The fluctuating and varied root phosphite concentrations yielded by the different application methods were all sufficient for suppressing *P. cactorum*

and *P. irregulare* DNA quantities in the roots of phosphonate treated trees. The phosphite concentrations in roots would have in general only resulted in a low level or no suppression of the pathogens, when considering the lowest percentage mycelial growth inhibition *in vitro* by phosphite among all of the evaluated phosphate and media types (agar or liquid). Phosphate and medium type resulted in isolates varying in percentage growth inhibition at the same phosphite concentration.

Only a few studies have investigated the temporal nature of root phosphite in tree crops. This is most likely due to the high cost involved in phosphite quantifications when analysed by commercial laboratories. Furthermore, most phosphite quantification methods are not high throughput, unlike the relatively cost-effective LC/MS-MS method used in the current study (Mcleod *et al*., 2018). One of the most extensive studies on the temporal nature in root phosphite concentrations was done by Whiley *et al*. (1995) on avocado for potassium phosphonate trunk injections. Monitoring root phosphite over a 125-day period, showed that phosphonate applications made when the summer shoot flush was mature (autumn to winter period) or when the spring shoot growth was mature (summer period), resulted in root phosphite reaching a maximum at around 30-days post-application. In the current study, root phosphite concentrations after the summer applications also peaked at around this time period (2- to 4-weeks), but a delayed peak (4- to 8 weeks) was observed following the winter applications. Whiley *et al.* (1995) also noted a decrease in root phosphite concentrations after their summer applications, similar to the current study. However, root phosphite concentrations remained stable for the winter applications (Whiley *et al.,* 1995). In the current study, this trend was evident in only one of the two trials for the winter applications. At the Vastrap trial, the trend was the same since the root phosphite at the 2-week and 12-week time points (85-days) remained stable for most application methods; at Paardekloof a decrease was seen in root phosphite concentrations for most application methods for the post-winter application period; the exception was the trunk paint treatment. In citrus, phosphonates foliar applied in autumn also only resulted in the highest root phosphite concentrations being reached rather later after 65-days, in comparison to the other time points that were evaluated at 25- and 45-days post-application (Graham, 2011).

Differences in the temporal nature in root phosphite concentrations following different application times, methods and in different tree crops, are due to differences in tree phenological stages, which influence source-sink relationships. Phosphite is known to be translocated in a source-sink manner. For example, in avocado it is well known that roots become a sink following the maturation of the summer- and spring shoot flush. Therefore, during shoot growth, root flushing is limited (Whiley *et al.*, 1995). In young apple trees this association would be more difficult to make, since trees produce new shoot flushes

continuously during spring and summer. Furthermore, unlike avocado and citrus, apple trees lose their leaves and go into dormancy in winter. During the winter period carbohydrates are translocated to roots in apples (Heide and Prestrud, 2005; Tartachnyk and Blanke, 2001; Malusa and Tosi, 2005). Along with this carbohydrate translocation in winter, the roots of young trees also show a peak in root flush (personal communication, E. Lotze). Considering the phenological stages of apple trees, the observed temporal nature of root phosphite in the current study after the summer phosphonate applications is as expected. Phosphonates applied to trunks, will be translocated in the phloem directly to the roots, rapidly reaching a peak after 2-weeks. The phosphite from foliar phosphonate applications will first have to be translocated from the foliage through the phloem downwards to the roots, thus only reaching a peak later mainly at 4-weeks. Subsequently, phosphite for all application methods decreased over a 12-week period due to a dilution effect of the enlarging roots system that will flush and most likely re-translocation of root phosphite to the growing shoot and fruit sinks. During the summer application period, small fruits are present on apple trees, which would be a strong sink. In the current study, following winter applications, phosphonates applied to trunks peaked later (4- to 8-weeks after application) in roots. This would suggest that phosphite in winter is translocated from trunks to roots more slowly, and over an extended period than when applications are made in summer. The temporal nature of root phosphite for the winter applications at the Vastrap trial, and the trunk paint application at Paardekloof, was as expected based on tree phenology, i.e. root phosphite remained relatively stable over the 12-week post-application period, due to shoots and fruits not being a sink. The significant decline in root phosphite at the Paardekloof trial between the 2-week and 12-week time points for the other applications methods is difficult to explain. It is likely that phosphite translocation in this trial could have been altered due to nematode infections. Towards the end of the study, the grower reported that *Pratylenchus* spp. infestation was problematic at the Paardekloof orchard. This could have resulted in an altered allocation of carbohydrate resources. Mazzafera *et al.* (2004) reported that in *Coffea arabica, Pratylenchus coffeae* infestations resulted in a decreased partitioning of carbohydrates to roots.

The best method of phosphonate application was difficult to identify, since foliar applications were only made in summer, and not also in winter as for the other application methods. Nonetheless, it was clear that the trunk paint treatment was very effective in translocating phosphite to roots since it consistently yielded the highest root phosphite concentrations in winter and summer among all the evaluated application methods. This is most likely due to the high rate (40 g phosphorous acid in total) used for trunk paint applications. The trunk spray treatments yielded significantly lower root phosphite

concentrations than the trunk paint. This relatively poor performance of the trunk spray treatment in comparison to the trunk paint, could potentially be improved by increasing the trunk spray treatment dosage (20 g phosphorous acid in total) to that of the trunk paint application (40 g phosphorous acid in total). Trunk spray applications will be preferred by growers as opposed to trunk paint applications, due to the labour intensive nature of trunk paint applications. In the current study a laboratory spray bottle was used for trunk spray applications, which was time consuming. However, a knap sack sprayer head could easily be designed for this application method, which will be much less time and labour consuming than trunk paints. Foliar applications will be the preferred application method for growers since these applications will require the least amount of labour, and furthermore have a low product cost due to the low dosage used (3.6 – 6 g phosphorous acid in total). The low dosage foliar applications were very effective in yielding high root phosphite concentrations considering that it outperformed the trunk spray applications that were applied at a much higher dosage (20 g phosphorous acid in total). In apple production systems, trunk spray applications will likely remain relevant for bearing apple trees, since it is likely to have the advantage of yielding lower fruit phosphite residues when phosphonates are applied in summer, due to the fruit sink in trees. Foliar sprays should in future be evaluated in winter to determine if foliar winter applications will be effective in delivering root phosphite. In the current study, the winter phosphonate applications were made only in June, when tree leaves started to colour in autumn. Therefore, foliar applications were not applied in winter in the current study. However, foliar applications will be feasible earlier in April/May just after harvest when leaf quality is better, which will also coincide with the predicted autumn root flush. The soil drench application yielded the lowest root phosphite concentrations, but this could be due to the low total dosage of 7.5 g phosphorous acid used for this application method. Although soil drench applications have been found effective in citrus (Graham, 2011), their continued use could result in the conversion of phosphite to phosphate by soil microbes. It is known that this can occur in soils, although the observed rates have been very slow (McDonald *et al*., 2001; Graham, 2011). Nonetheless, the risk does exist that in some soil types this effect might be higher and that phosphite degrading soil microbes can increase with repeated phosphonate soil applications, especially when high dosages are applied.

Irrespective of the fact that root phosphite concentrations varied significantly across time points, trials and application methods, all phosphonate treatments resulted in a significant reduction in *P. cactorum* and *P. irregulare* DNA quantities in apple tree roots in the two orchard trials over the 12-month period. The only exception was the two trunk spray treatments at the Paardekloof trial, which did not differ significantly from the control

treatment in pathogen DNA concentrations. Based on root phosphite concentrations, there was no reason for these two application methods to not reduce pathogen concentrations. This is evident from the fact that the root phosphite concentrations of the trunk spray treatments were significantly higher than those of the soil applications that did suppress pathogen quantities. It was surprising that the two foliar spray treatments, which were only applied in summer, also significantly suppressed pathogen concentrations, even though they only harboured root phosphite for 6-months or less. Root phosphite concentrations for the foliar spray treatments ranged between 5.12 and 32.31 ug/g_{FW} during the 12-week monitoring period, and would have been even lower in the remaining 16-weeks before pathogen quantifications were conducted. Van der Merwe and Kotze (1994) found that very low root phosphite concentrations were required for suppression of *Phytophthora cinnamomi* in avocado roots, since 9.8 µg/g_{FW} phosphite resulted in 87% inhibition of colonization.

In vitro assays evaluating the phosphite sensitivity of *P. cactorum, P. vexans* and *P. irregulare* isolates revealed that the percentage inhibition of mycelial growth was dependent on whether a liquid or solid medium was used in assays, and on the phosphate concentrations (1 mM or 15 mM) in media. The agar assay method, which is used by most studies, overestimated the percentage inhibition of mycelial growth compared to the liquid medium assay. Davis *et al*., (1994) suggested that the use of colony radial growth does not take into account the dry weight of the colony. This might be the reason for obtaining high inhibition percentage in solid agar than in liquid medium. Darakis *et al*. (1997) reported that the effect of phosphate was more evident in liquid medium than solid medium, since liquid medium is aqueous

The observed outcome is most likely due to the competition between phosphite and phosphate anions for the transport system prominent in the aqueous environment. Furthermore, phosphate was found to inhibit phosphonate transport (Barchietto *et al*., 1989, Griffith *et al*., 1989). The differences in the inhibition of mycelial growth at low and high phosphate observed in this study might be due to different kinetics of transport in the species studied, these are dependent on the internal phosphorus content of the organism (Straker & Mitchel, 1987). However, in the current study this was only found to be true for *P. irregulare.* The *in vitro* effect of phosphate in decreasing the phosphite sensitivity of *P. cactorum* at a high phosphate concentration (15 mM), and for *P. vexans* to increase in the phosphite sensitivity at a high phosphate concentration (15 mM), was evident both in liquid and solid media. The effect of phosphate on phosphite inhibition, however, was found to vary considerably with species (Fenn and Coffey 1989). In the current study, *P. vexans* was the most sensitive species in *in vitro* assay analyses in comparison to *P. irregulare* and *P. cactorum.* For all three species, the isolates within each species varied in their sensitivity to

phosphite. This phenomenon has been reported previously for *Pythium* spp. and *Phytophthora* spp. (Fenn and Coffey, 1984; Guest & Grant, 1991). The *in vitro* phosphite sensitivity of *Phytopythium* spp. has not been reported previously. Furthermore concentrations of less than 100ug/ml were found to have no action against *Pythium* spp. for example, among 25 *Pythium* isolates, including *P. dissotocum* and *P. irregulare*, treated with fosetyl-Al Sanders *et al.* (1983). Similarly the study of Weiland *et al*., (2014) using a total of 117 isolates of *P.irregulare*, *P.syvatticum* and *P.ultimum* obtained EC_{50} ranging from 990 – 1652 µg/ml, in this study V8 medium was used, and phosphate was not reported. For *Phytophthora* spp phosphite concentrations comparable to our study was obtained fosetyl-Al EC_{50} values have been reported for *Phytophthora* spp., including *Phytophthora citrophthora* (24 to 285 µg/ml) Farih *et al*., 1981, *P. infestans* (62 to 1,021 µg/ml) Bashan *et al*., 1990, and *P. parasitica* (1,146 µg/ml) (Metheron *et al*., 2000).

The variable effects of medium type (solid or liquid) and phosphate concentration on the *in vitro* inhibitory activity of phosphite towards *P. irregulare* and *P. cactorum* is problematic in assessing the relative effect of root phosphite concentrations as a determinant of pathogen suppression in orchard tree roots. *In vitro* mycelial growth inhibition was only 11 to 15 % for both species at 200 ug/ml phosphite, for the lowest percentage phosphite inhibition obtained *in vitro* among the evaluated media types, phosphate concentrations and isolates. The lowest root phosphite concentrations where the species were inhibited in orchard trials were for the foliar treatments where root phosphite concentrations were much lower (5.12 and 32.31 ug/g) than the *in vitro* tested 200ug/ml. Furthermore, even the highest root phosphite concentrations achieved with the trunk paint treatment (444.67 and 878.74 ug/g), would have a limited effect on the two species based on *in vitro* results. *In vitro,* at 500 ug/ml phosphite, mycelial growth inhibitions considering the lowest percentage inhibition for all media and phosphate concentrations were 28% for *P. cactorum* and 14% for *P. irregulare.* Increasing the *in vitro* concentration to 1000 ug/ml phosphite, only increased the percentage inhibition to 38% and 27% for *P. cactorum* and *P. irregulare* respectively. It therefore seems unlikely that a direct mode of action would have been involved for phosphite in suppressing *P. cactorum* and *P. irregulare* in tree roots in the Paardekloof and Vastrap orchard trials.

In summary, the study has shown the potential of phosphonates for suppressing *Pythium* and *Phytophthora* root rot preventatively based on pathogen suppression in the roots of orchard trees. Although *Pythium irregulare* was found to be suppressed by root phosphite in roots in the current study, there are few studies that have been performed *in planta* to demonstrate suppression of *P. irregulare* (Abbasi and Lazarovits, 2006) The dynamics in root phosphite seemed to be affected by the time of application, which is likely

due to phenological differences at the time of phosphonate applications. Phosphonate applications using various application methods should be assessed for their effect on phosphite fruit residues, which is set at 75 mg/kg for apple (EFSA, 2014). The current study was only able to provide very limited indications as to whether oomycete pathogen suppression in apple roots could be due to a direct mode of action of phosphite. In future, conducting a time course study on the extent of pathogen root infections that coincide with root phosphite quantification time points will be more informative regarding the direct mode of action. Furthermore, future studies should also focus on evaluating defence gene expression in response to a range of phosphonate dosages. This will determine whether an indirect mode of action involving host resistance induction is involved. In these studies, acquiring data on pathogen suppression in the roots of phosphonate treated plants, and the effect of root phosphite concentrations on defence gene induction would also be important to evaluate. It is known that several plant resistance inducers show a dose dependant induction of host defences as has been shown for phosphite in the *Arabidopsis-Pseudoperonospora* system (Massoud *et al.,* 2012). It would also be important to determine whether defence gene induction will differ when phosphonates are applied using different application methods. Some plant resistance inducers are known to differ in their efficacy based on the method of application, for example soil versus foliar applications in the suppression of citrus cancer (Francis *et al*., 2009)

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Table 1. Phosphonate treatments and date of application for summer and winter applications at two apple orchard trials (Paardekloof and Vastrap) evaluating the of effect phosphonate treatments on root phosphite concentrations and DNA quantities of oomycetes in roots.

Treatment name [a]	Formulation [b]	Phos-phorous acid (g/L)	Volume applied per tree	Winter application dates	Summer application dates	Total amount of phosphorous acid applied per tree (summer + winter applications)
Untreated control	Water	0	0	12 June 2015	18 November 2015	0
Foliar potassium phosphonate	Potassium phosphonate	2	300 – 500 ml	Nd	4, 11 and 18 November 2015	3.6 – 6 g
Foliar ammonium phosphonate	Ammonium phosphonate	2	300 – 500 ml	Nd	4, 11 and 18 November 2015	3.6 – 6 g
Trunk spray + penetrant	Potassium phosphonate + polyether-polymethylsiloxan-copolymer	200	50 ml	12 June 2015	18 November 2015	20 g
Trunk spray+ ½ penetrant	Potassium phosphonate + polyether-polymethylsiloxan-copolymer	200	50 ml	12 June 2015	18 November 2015	20 g
Trunk paint	Potassium phosphonate	200	100 ml	12 June 2015	18 November 2015	40 g
Soil drench	Potassium phosphonate	0.75	5 L	12 June 2015	18 November 2015	7.5 g

[a] Foliar spray applications were applied with a knapsack sprayer. The pH of the 5 g/L foliar sprays were adjusted to 7.2 using potassium hydroxide. Trunk paints were applied using a 100 mm width paint brush. Trunk sprays were applied using a 750 ml laboratory spray bottle. Foliar applications were not applied (Nd) in winter.

[b] The potassium phosphonate formulation was Phosguard (Nulandis, Phosguard 400 SL, Witfield, South Africa; 400 g phosphorous acid/L), and the ammonium phosphonate formulation was Brilliant (Arysta, Brilliant SL, South Africa, 300g phosphorous acid/L). The bark penetrant, was Charge (Villa Crop, South Africa, 1000 g a.i./l polyether-polymethylsiloxane-copolymer). The penetrant was added at a concentration of 0.50 ml/L for the full rate application and 0.25 ml/L for the half rate application.

Table 2. Root phosphite concentrations monitored over a 12-week period in apple tree roots, following summer and winter phosphonate applications to apple trees at two trials (Paardekloof and Vastrap) where different application methods, formulations and dosages were evaluated.

Treatment [a]	Winter				Summer				
	2 w (26 June 2015)	4 w (10 July 2015)	8 w (7 Aug. 2015)	12 w (4 Sept. 2015)	0 w (28 Oct. 2015)	2 w (2 Dec. 2015)	4 w (16 Dec. 2015)	8 w (13 Jan. 2016)	12 w (10 Feb. 2016)
	Paardekloof								
Trunk paint	81.83c	120.67 b	268.33 a	113.43 b	72.24b	444.67 a	69.98 b	14.48 gh	18.17 fg
Trunk spray + penetrant	46.11 de	59.38 d	35.43 f	14.74 g	14.95 gh	48.24 c	16.11 jkl	7.12 lm	3.45 p
Trunk spray + ½ bark penetrant	42.45 ef	34.40 f	49.17 de	6.08 g	6.07 lmn	69.14 b	18.48 fg	7.26 kl	4.45 nop
Soil drench	9.24 h	16.62 g	5.71 i	3.62 j	2.05 q	18.33 fg	10.38 ij	3.26 p	4.05 op
Untreated control	0.76 kl	1.43 k	0.62 l	0.74 kl	0.48 st	0.95 r	1.76 q	0.68 t	0.98 rs
Foliar potassium phosphonate	Nd	Nd	Nd	Nd	0.62 rst	29.76 de	32.31 d	5.12 mno	9.67 ijk
Foliar ammonium phosphonate	Nd	Nd	Nd	Nd	0.67 rst	5.93 g	23.60 ef	6.62 lm	11.24 hi
	Vastrap								
Trunk paint	83.81 c	46.26 e	263.24 a	162.05 b	103.36 b	878.74 a	107.19b	42.86 cd	32.90 de
Trunk spray + penetrant	24.93 f	15.48 g	91.05 c	30.14 f	6.00 c	100.26 b	9.98 jk	5.40 lmno	4.60 nop
Trunk spray + ½ bark penetrant	17.29 g	15.52 g	61.29d	30.31 f	58.83 c	130.71 b	12.24 ij	9.98 jk	4.74 lmno
Soil drench	11.55 h	8.48 i	18.17 g	14.24 gh	22.83 fg	25.86 ef	13.19 ij	3.10 pq	3.00 pq
Untreated control	5.24 j	6.00 ji	7.14 ij	7.29 i	6.07 lmn	7.40 mno	4.05 op	1.33 r	2.33 qr
Foliar potassium phosphonate	Nd	Nd	Nd	Nd	7.10 kl	16.45 ef	21.50 fgh	11.67 ij	11.86 ij
Foliar ammonium phosphonate	Nd	Nd	Nd	Nd	6.83 klm	6.91 ghi	24.24 ef	10.64 j	16.38 hi

[a] All treatments consisted of potassium phosphonate formulations that were applied using different application methods, with the exception of the foliar sprays that were evaluated as ammonium- and potassium phosphonate formulations. The dosages and times of application are shown in Table 1.

Root phosphite concentration (µg/g $_{\text{fresh weight}}$) values in columns are the mean of six replicates (four trees per replicate). The 0-week time point is the time point just before the summer applications were made, which was 17-weeks after the first winter applications were made. For each trial separately, and each application time (winter or summer) separately, values followed by the same letter do not differ significantly ($P \geq 0.05$) according to Fisher's least significance difference test. The significance test results are for the Ln (x + 1) 1transformed root phosphite concentration data.

Table 3. Effect of different phosphonate application methods on the change in *Phytophthora cactorum* and *Pythium irregulare* DNA concentrations in apple tree roots at two orchard trials (Paardekloof and Vastrap).

	Pathogen DNA concentrations (pg/g) in roots [a]			
	P. irregulare		*P. cactorum*	
Treatment [b]	Paardekloof	Vastrap	Paardekloof	Vastrap
Trunk paint	-1.732 ab	-0.052 a	0.18 b	0.15 b
Trunk spray + penetrant	-0.018 cd	-0.026 a	-0.80 b	0.66 ab
Trunk spray + ½ bark penetrant	-0.003 bcd	-0.065 a	0.06 b	-1.255 bc
Soil drench	-0.683 ab	-0.196 a	-0.76 b	-0.72 bc
Foliar potassium phosphonate	-0.073 abc	-0.190 a	0.03 b	- 0.01 b
Foliar ammonium phosphonate	-0.601 a	-0.150 a	-0.76 b	-2.56 c
Control	0.043 d	0.436 b	2.07 a	2.37 a
P value	0.0079	0.0265	0.0186	0.003

[a] Pathogen DNA concentrations (pg/g) in roots were determined using quantitative real-time PCR assays. The change in DNA concentration was calculated by subtracting pathogen concentrations obtained at the end of the trials from those obtained at the start of the trials, 12 months earlier. Values are the average of six replicates. Values in columns followed by the same letter do not differ significantly ($P \geq 0.05$) according to Fisher's least significance difference test. For *P. irregulare,* t-test results from Ln (x + 1) transformed data are shown. *Phytophthora cactorum* data were not transformed.

[b] All treatments consisted of potassium phosphonate formulations that were applied using different application methods, with the exception of the foliar sprays that were evaluated as ammonium- and potassium phosphonate formulations. The dosages and times of application are shown in Table 1.

Table 4. Percentage inhibition by phosphite of mycelial radial growth of different isolates of three oomycete pathogens (*Phytophthora cactorum, Pythium irregulare* and *Phytopythium vexans*) in *in vitro* agar assays.

P. cactorum (500 µg/ml phosphite)		*P. irregulare* (500 µg/ml phosphite)		*P. vexans* (200 µg/ml phosphite)	
Isolate	% Inhibition	Isolate	% Inhibition	Isolate	% Inhibition
7204	82.67 bc	E19	44.15 c	6718	72.30 c
PCA	87.19 ab	E20	43.17 c	6728	89.12 ab
PCB	89.66 a	E6	63.46 b	6730	83.56 ab
PCC	58.78 d	NN11	26.39 d	6736	81.00 bc
PCD	55.96 de	NN13	21.64 d	6737	91.50 a
PCE	76.68 c	NN15	26.85 d	6739	87.45 ab
PCF	83.60 ab	PSR3.1	80.38 a	6742	73.03 c
PCG	52.43 e	PSR3.3	65.11 b	6743	72.47 c
		PSR3.4	24.71 d	6745	86.01 ab
		PSR5.5	32.96 cd	6748	73.15 c

Mycelial growth inhibition for the three oomycete species was evaluated on Ribeiro's agar medium containing 500 µg/ml (*P. cactorum* and *P. irregulare*) or 200 µg/ml phosphite (*P. vexans*) and a phosphate concentration of 15 mM. Radial growth was measured after 5-days for *P. vexans* and *P. irregulare* and after 9-days for *P. cactorum.* The percentage inhibition of radial growth was calculated relative to the control plates that only contained 15 mM phosphate and no phosphite. Values are the average of two experiments, where experiment was used as a block for statistical analyses. Values in columns followed by the same letter do not differ significantly ($P \geq 0.05$) according to Fisher's least significance difference test.

Table 5. Analyses of variance for the effect of different culture media (liquid and solid) and phosphate concentrations (1 mM and 15 mM) on the percentage inhibition by phosphite of three oomycete species (*Phytophthora cactorum*, *Pythium irregulare* and *P. vexans*).

Source of variation	df	Type I SS	Mean Square	F Value	Pr > F
Medium	1	70.7851919	70.7851919	1563.00	<.0001
Medium(Rep)	3	0.2242023	0.0747341	1.65	0.1798
Species	2	9.2436951	4.6218475	102.05	<.0001
Species (isolate)	4	4.0646881	1.0161720	22.44	<.0001
Phosphite conc.	6	194.951673	32.4919460	717.45	<.0001
Species x Phosphite conc.	3	14.1292613	4.7097538	104.00	<.0001
Species x Isolate	12	14.1292613	4.7097538	104.00	<.0001
Phosphate conc.	1	0.4106983	0.4106983	9.07	0.0030
Species x phosphate conc	2	4.7639980	2.3819990	52.60	<.0001
Species x phosphate conc (Isolate)	4	1.8828580	0.4707145	110.39	<.0001
Phosphite conc x phosphate conc.	6	2.3118396	0.3853066	8.51	<.0001
Species x phosphite conc. x phosphate conc.	3	0.6682930	0.2227643	4.92	0.0027
Species x oomycete (Isolate)	12	1.7334348	0.1444529	3.19	0.0004
Medium x species	2	0.2204768	0.1102384	2.43	0.0908
Medium x species (Isolate)	4	4.5733272	1.1433318	25.25	<.0001
Medium x Phosphite conc.	6	25.7276410	4.2879402	94.68	<.0001
Medium x species x phosphite conc.	3	1.0416221	0.3472074	17.67	<.0001
Medium x species (Isolate)	12	4.3468434	0.3622369	8.00	<.0001
Medium x phosphate conc.	1	0.0648395	0.0648395	1.43	0.2332
Medium x species x phosphate conc.	2	1.4640386	0.7320193	16.16	<.0001
Medium x species (Isolate)	4	0.3186756	0.0796689	1.76	0.1395
Medium x phosphite conc. x phosphate conc.	6	0.4781564	0.0796927	1.76	0.1104
Medium x species x phosphite conc x phosphate conc	3	0.4629566	0.1543189	3.41	0.0190
Medium x species (Isolate)	12	0.7653095	0.0637758	1.41	0.1666

Table 6. Effect of medium (solid or liquid) and phosphate concentration on the percentage growth inhibition by different phosphite concentrations on *Phytophthora cactorum, Pythium irregulare* and *Pythium vexans*.

		P. cactorum			*P. irregulare*			*P. vexans*		
Phosphate conc.(mM)	Phosphite conc. (µg/ml)	Liquid	Solid	Phosphite conc (µg/ml)	Liquid	Solid	Phosphite conc(µg/ml)	Liquid	Solid
1	200	21.22 nopq	52.94 f	250	13.97 qr	28.92 klm	30	6.47 st	38.26 ij
1	500	35.23 ijk	83.83 b	500	27.51 lmno	48.67 fg	100	20.30 opq	66.98 de
1	1000	50.15 f	93.30 a	1000	35.05 ijk	73.01 cd	200	32.41 jkl	77.97 bc
15	200	22.41 mnop	41.18 hi	250	12.63 rs	46.11 fgh	30	19.06 pqr	47.90 fgh
15	500	35.06 ijk	72.98 cd	500	17.52 pqr	62.26 e	100	28.29 klmn	66.53 de
15	1000	41.84 ghi	92.53 a	1000	29.18 klm	82.17 b	200	41.02 hi	84.03 b

Mycelial growth inhibition for the three species was evaluated on Ribeiro's medium containing various phosphite and phosphate concentrations. The liquid test media did not contain agar, whereas the solid test media did. Percentage inhibition calculations for the solid medium test was done by first measuring radial growth after 5-days of growth for *P. irregulare* and *P. vexans,* and after 9-days for *P. cactorum* on the amended and un-amended media. Percentage inhibition was determined for the solid test by comparing radial growth on phosphite amended media relative to the control containing only the relevant phosphate concentration, but no phosphite. Percentage inhibition for the liquid test was calculated in a similar manner than for the solid test, except that mycelial weight was used for calculations after 7-days of growth for *P. irregulare* and *P. vexans,* and after 14-days of growth for *P. cactorum*. Values are the average of two experiments, and for *P. cactorum* and *P. irregulare* the average of two isolates each, and for *P. vexans* the average of three isolates. Values followed by the same letter do not differ significantly ($P \geq 0.05$) according to Fisher's least significance difference test conducted on the Ln transformed data.

Table 7. Effect of medium (solid or liquid) and phosphate concentration on the percentage growth inhibition by different phosphite concentrations on two isolates each of *Phytophthora cactorum* and *Pythium irregulare* isolates.

		Phytophthora cactorum				*Pythium irregulare*			
		Isolate PCA		Isolate PCC		Isolate PS3.1		Isolate E19	
Phosphate conc. (mM)	Phosphite conc. (µg/ml)	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid
1	200 (*P. cac*) ; 250 (*P. irr.)*	15.34 j	55.90 de	27.12 i	49.99 defg	14.18 j	45.87 e	13.76 j	11.99 j
1	500	31.29 hi	83.03 c	39.18 fghi	84.64 c	33.89 fg	66.46 cd	21.14 j	30.89 fg
1	1000	49.45 defg	97.17 a	50.85 def	89.45 c	40.22 ef	88.10 a	29.89 gh	57.92 d
15	200 (*P. cac*) ; 250 (*P. irr.)*	14.14 j	49.94 defg	30.69 hi	35.43 ghi	11.46 j	70.56 c	13.81 j	21.68 hi
15	500	28.434 hi	87.29 c	41.70 efgh	58.79 d	14.38 i	80.38 b	20.66 i	44.15 e
15	1000	37.54 gfhi	88.48 c	46.15 defg	96.58 b	27.20 ghi	90.97 a	31.18 fg	73.39 bc

Mycelial growth inhibition for the two species was evaluated on Ribeiro's medium containing various phosphite and phosphate concentrations. The results are shown for each of two isolates per species separately, whereas Table 6 contains the averages of the two isolates evaluated for each species. The liquid test media did not contain agar, whereas the solid test media did. Percentage inhibition calculations for the solid medium test was done by first measuring radial growth after 5-days of growth for *P. irregulare* and after 9-days for *P. cactorum* on the amended and un-amended media. Percentage inhibition was determined in the solid test by comparing radial growth on phosphite amended media relative to the control containing only the relevant phosphate concentration, but no phosphite. Percentage inhibition for the liquid test was calculated in a similar manner than for the solid test, except that mycelial weight was used after 7-days of growth for *P. irregulare* and after 14-days of growth for *P. cactorum*. Values are the average percentage inhibition of two experiments. For each species, values followed by the same letter do not differ significantly ($P \geq 0.05$) according to Fisher's least significance difference test conducted on the Ln transformed data.

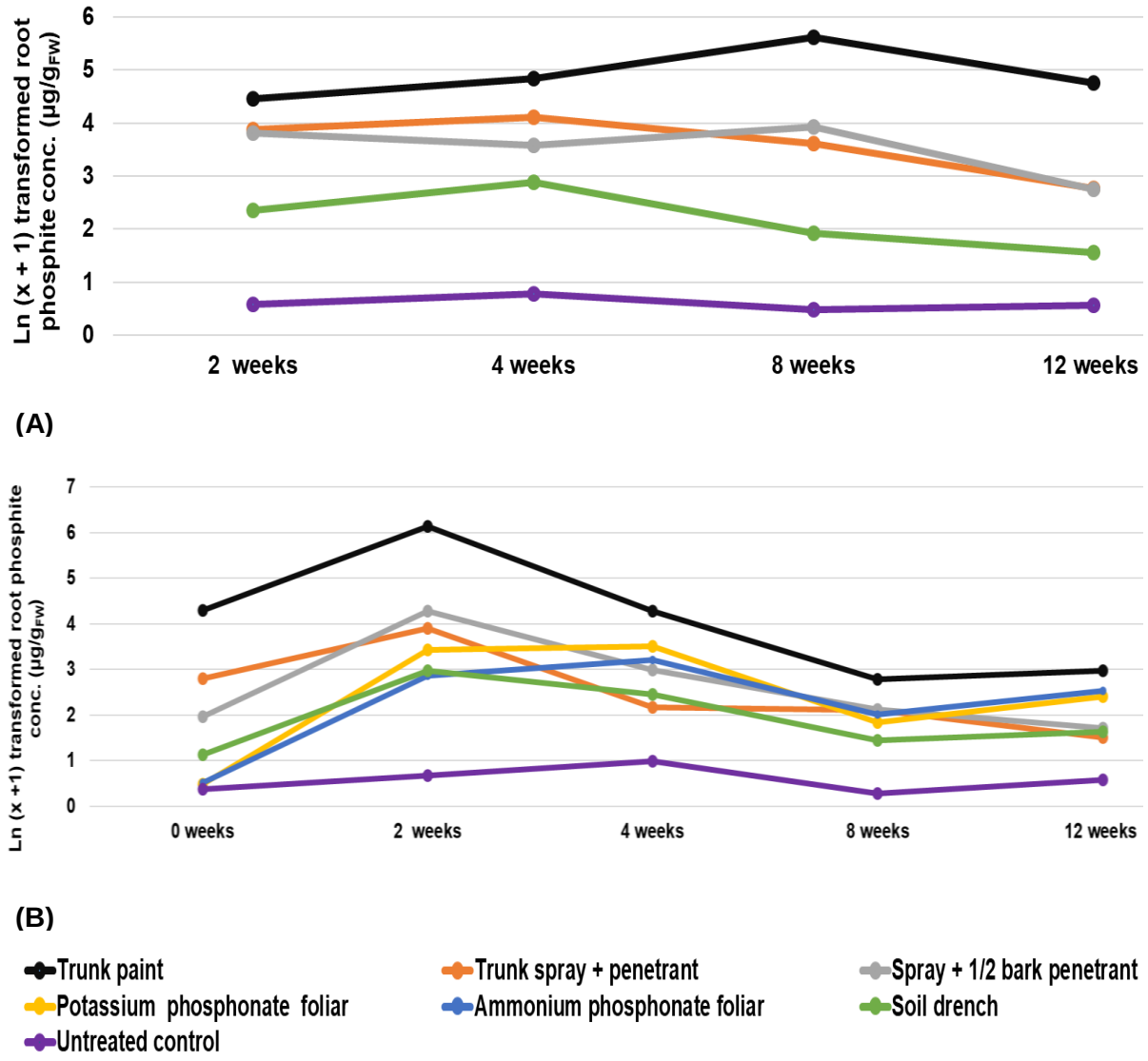

Fig. 1. Apple root phosphite concentrations (µg/mg $_{\text{fresh weight (FW)}}$) at the Paardekloof orchard following phosphonate applications using different application methods to apple trees in (A) winter and (B) summer. Root phosphite concentrations were determined 2, 4, 8 and 12 weeks after the summer and winter phosphonate applications. For summer one sampling before applications (0-week) was also conducted. Most phosphonate treatments were potassium phosphonate treatments, except for a foliar ammonium phosphonate spray treatment. Foliar sprays were only applied in summer. The trunk spray treatment was evaluated with a full- and half dosage bark penetrant (Charge, a.i. 1000 g/l polyether-polymethylsiloxane-copolymer). Phosphonate treatments were applied in winter (June, 2015) and subsequently in summer (November, 2015). Values are the average of six replicates (4 trees per replicate).

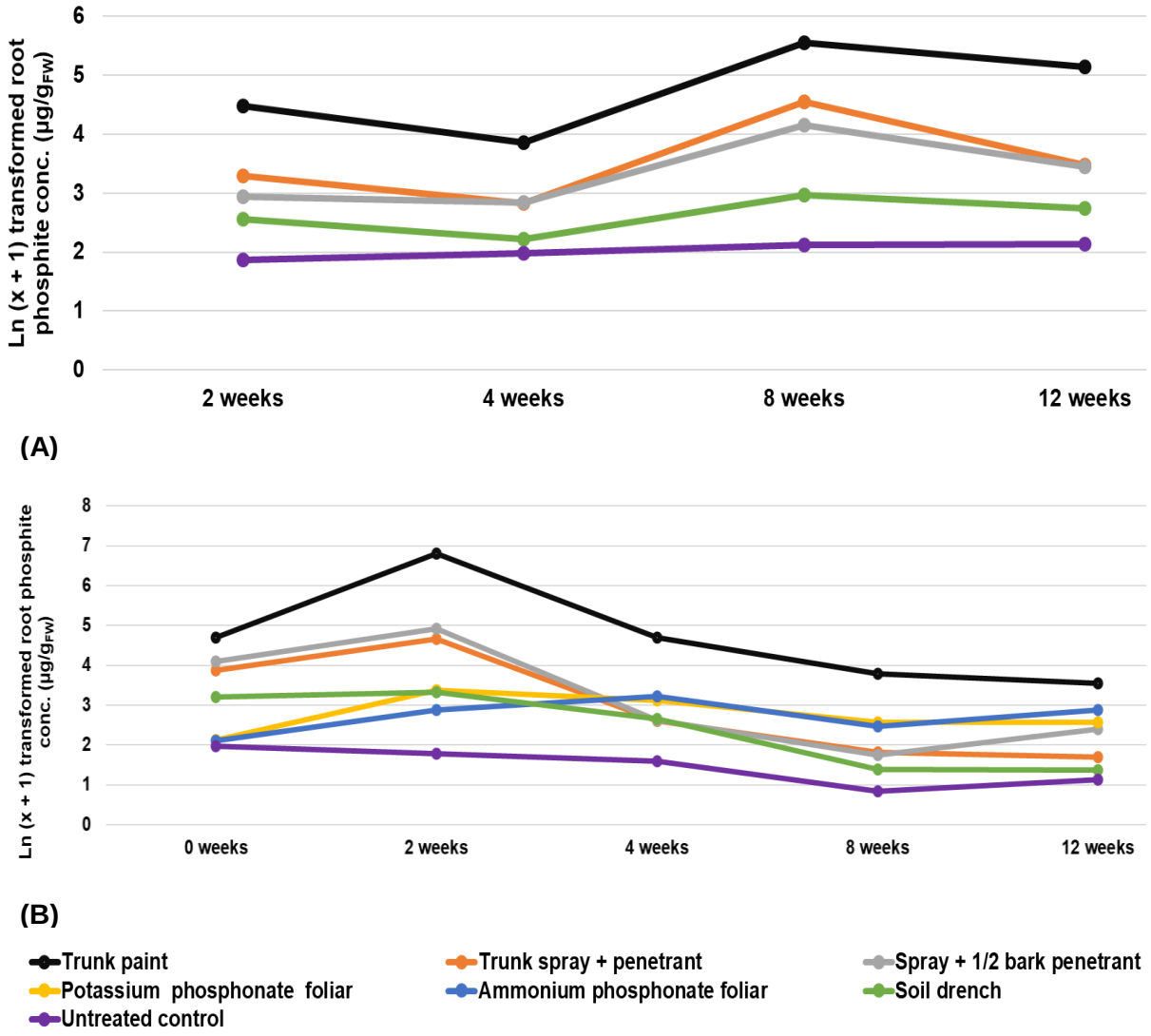

Fig. 2 Apple root phosphite concentrations (µg/mg $_{\text{fresh weight (FW)}}$) at the Vastrap orchard following phosphonate applications using different application methods to apple trees in (A) winter and (B) summer. Root phosphite concentrations were determined 2, 4, 8 and 12 weeks after the summer and winter phosphonate applications. For summer one sampling before applications (0-week) was also conducted. Most phosphonate treatments were potassium phosphonate treatments, except for a foliar ammonium phosphonate spray treatment. Foliar sprays were only applied in summer. The trunk spray treatment was evaluated with a full- and half dosage bark penetrant (Charge, a.i. 1000 g/l polyether-polymethylsiloxane-copolymer). Phosphonate treatments were applied in winter (June, 2015) and subsequently in summer (November, 2015). Values are the average of six replicates (4 trees per replicate)

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CHAPTER 4

Evaluating phosphonate application methods for managing *Phytophthora* root rot on young apple trees

ABSTRACT

Phosphonates are widely used for the management of *Phytophthora* diseases world-wide. In South Africa, no phosphonate products are registered on apple for managing Phytophthora root rot, caused by *Phytophthora cactorum.* The disease is especially problematic on young apple trees. Therefore, the curative efficacy of different phosphonate application methods (foliar sprays, trunk sprays and trunk paints) were evaluated in two orchard trials in the Grabouw region and one in the Koue Bokkeveld region. In the Grabouw trials, all application methods resulted in a significantly higher shoot growth after 12 months, relative to the untreated control, but not in the Koue Bokkeveld trial. However, in all trials the phosphonate applications did not significantly reduce the quantity of *P. cactorum* DNA detected in roots, and the pathogen's ability to produce zoospores from roots, relative to the control treatment. Pathogen quantification data did show that for all treatments, zoospore production from roots under laboratory conditions was significantly higher in autumn than in summer. Furthermore, that the pathogen colonized fine feeder roots and secondary roots to the same extent. In the two Grabouw trials, all application methods at their highest dosage yielded relative high root phosphite concentrations (40 – 187 $\mu g/g_{FW}$) 13-weeks after autumn phosphonate applications. In comparison, the Koue Bokkeveld trial yielded lower root phosphite concentrations (22 – 54 $\mu g/g_{FW}$) for autumn applications. A low dosage ammonium phosphonate foliar spray (2 g a.i./L) application yielded significantly lower root phosphite concentrations than a high dosage foliar spray (5 g a.i./L) of this formulation. The latter treatment was comparable to potassium phosphonate trunk spray and –paint treatments in yielding root phosphite concentrations. Irrespective of these differences in root phosphite concentrations between application methods, this did not result in differences in tree growth between treatments. Yet, root phosphite seemed important since there were significant low to moderate positive correlations (r = 0.384 – 0.536; $P \leq 0.012$) between root phosphite concentrations 13-weeks after the autumn application and shoot length in the Grabouw trials. Fruit phosphite residues were significantly lower in fruit from the trunk spray and -paint applications, in comparison to the foliar spray applications.

INTRODUCTION

Several *Phytophthora* spp. have been reported to cause crown- (bark rot below ground level), collar- (bark rot above ground level) and root rot on apple (Latorre *et al*., 2001; Welsh 2011, Naffaa and Rashid, 2017). However, in most apple production regions world-wide, including South Africa, *Phytophthora cactorum* (Leb and Cohn) Schroeter is the main causative species. The symptoms caused by *P. cactorum* ultimately result in chlorosis and premature purpling of leaves in autumn, stunted tree growth, poor yields, or tree death in extreme cases (Sewell and Wilson, 1964; McIntosch, 1975; Wilcox, 1993). In South Africa, young orchards in their second or third year of growth often only suffer from root rot symptoms, but do not exhibit crown- or collar rot symptoms. In several cases, a substantial number of trees within an affected orchard will die within a year of symptom appearance (personal communication, J.P.B. Wessels, ProCrop, Wellington, South Africa). The inoculum is likely introduced into these orchards through nursery material or irrigation water, since most new apple orchards in South Africa are established on fumigated soil.

The management of *P. cactorum* on apple is challenging, due to its soilborne nature. An integrated management strategy must be employed including the use of cultural practices such as irrigation management, planting on ridges and tolerant rootstocks, as well as select chemical treatment (McIntosch, 1975; Browne and Mircetich, 1988; Carisee and Khanizadeh, 2006). Although some of the Malling rootstocks (M.7, M.9) and Malling-Merton (M.111) are less susceptible than others (M.7, MM.104 and MM.106), no rootstock is immune to *Phytophthora* infections (McIntosch, 1975; Carisse and Khanizadeh, 2006). Optimal irrigation scheduling and planting on ridges can reduce the severity of the disease, but these tactics are not sufficient as standalone management practices. Therefore, chemicals are important for use in an integrated disease management strategy once trees start to express symptoms. A few studies have evaluated phenylamides (metalaxyl) for the curative management of crown and root rot on apple, with variable success (Ferree and Ellis, 1984; Autio *et al*., 1991, Utkhede and Smith, 1992; Utkhede and Smith, 1993). Problems associated with the use of phenylamides include pathogen resistance development, and microbial breakdown in soil due to repeated use (Jeffers, Schnabel and Smith, 2004, Hwang and Benson, 2005). Therefore, registered products recommend alternating phenylamides with compounds from unrelated chemical groups, and that applications should only be made in alternate years. In South Africa, in general, only one application is recommended on apples (personal communication J.P.B. Wessels). Phosphonates, usually applied in autumn and spring, have shown some efficacy under orchard conditions against crown and root rot on old trees (Utkhede and Smit, 1991) and on

newly planted apple trees (Utkhede and Smith, 1993). Microbial breakdown and pathogen resistance development have not been associated with phosphonates.

Phosphonates are alkali metal salts of phosphorous acid (H_3PO_3), and contain cations (e.g. K+, Na+, or NH4+) and any of the hydrogen phosphite (HPO_3^{-2}) or dihydrogen phosphite (H^2PO^3-) anions (Mc Donald *et al*., 2001). Phosphonates are an important class of agricultural fungicides, with the earliest examples of its use being about 20 years ago as a systemic fungicide, plant activator and plant strengthener (Thao and Yamakawa, 2009, Dalio *et al*., 2014). Although claims have been made for its use as a fertilizer, this is speculative and strong evidence exists against its role as a fertilizer (MacDonald *et al.*, 2001). Since their discovery as oomyceticides, phosphonates have been developed into various commercial fungicides, such as potassium, calcium- and sulphur phosphonates (Ouimette and Coffey*, 1989*, González *et al, 2017)*. In South Africa, an ammonium phosphonate is also registered on several crops other than apple. The first phosphonate formulation that was used for the management of *Phytophthora* diseases was an alkyl-phosphonate known as fosetyl-Al (Guest and Grant, 1991).

Phosphite can be translocated acropetally and basipetally in plants, which allows for various application methods including foliar sprays, soil drenches and trunk paints or sprays (Schutte *et al*., 1991, Fairbanks *et al*., 2000). For the purpose of this study, phosphonates will be used when referring to fungicide formulations, and phosphite when referring to the breakdown product of phosphonate fungicides in plants. Aside from the application method, the time of phosphonate application is also important since phosphite translocates in a source-sink manner (Ouimette and Coffey, 1990, Whiley *et al*., 1995, Fairbanks *et al*., 2000). Application method and time of application will thus likely determine the amount of phosphite translocated to roots, where root rot pathogens must be controlled. In South Africa, young apple trees in the Grabouw production region have been shown to have two main root flushes, one in autumn and the other in summer (personal communication, E. Lotze, Department of Horticulture, Stellenbosch University). In previous studies on apple, only fosetyl-Al foliar sprays and trunk/soil drench application methods were evaluated. The trunk/soil drench application was applied as a drench to the trunk and soil, but it was not specified how much of the product was applied to the trunk as opposed to the soil (Utkhede and Smith, 1991, 1993).

A few studies have shown a significant negative correlation between plant tissue phosphite concentrations and disease symptoms. These studies were conducted on avocado, tobacco, lupine and pawpaw (Smillie *et al*. 1988, El-Hamalawi *et al*. 1995). Therefore, root phosphite concentrations are likely important in the suppression of root rot pathogens. The only study on apple that has evaluated the effect of tissue phosphite

concentration on disease development was a study by Long *et al.* (1989) in which the translocation of phosphite following fosetyl-Al trunk injections was investigated. No correlation was found between fruit phosphite concentrations and fruit lesion size caused by *P. cactorum* in fruit bioassays (Long *et al.*, 1989).

Limited information is available on the effect of phosphite on *Phytophthora* root colonization and sporangial production *in planta*. It is well known that *in vitro,* sporangia of several *Phytophthora* spp. are sensitive to phosphite, with EC_{50} values for sporangial production being lower than for mycelial growth (Coffey and Bower, 1984; Coffey and Joseph, 1985). In contrast, only one study has investigated whether phosphite treated roots limit pathogen tissue colonization, and the production of sporangia and subsequent zoospore release. Wilkinson *et al.* (2001) reported that phosphite significantly reduced *Phytophthora cinnamomi* zoospore production in native Australian tree species (*Banksia grandis* and *Eucalyptus marginata*). Dalio *et al.* (2014) furthermore used confocal laser-scanning microscopy to show that in phosphite treated *Fagus sylvatica* seedlings, *Phytophthora plurivora* was restricted in roots to the outer cortex tissue, in contrast to untreated plants where the pathogen was present in the central cylinder, phloem tissue and pith in roots. Most studies on *Phytophthora* colonization in phosphonate treated plants were conducted in native Australian plant species, where results were highly influenced by plant species (Shearer and Crane, 2012).

Phytophthora infections in plants can be quantified using various approaches. Wilkinson *et al.* (2001) investigated zoospore production from phosphite treated plants by quantifying zoospores microscopically in floodwater of the treated plants. Alternatively, leaf baits were floated on the floodwater and the percentage infected baits were determined (Wilkinson *et al.*, 2001). Although baiting is known as a semi-quantitative method (Erwin and Ribeiro, 1996), a good correlation was found between zoospore microscopic counts and percentage baits colonized (Wilkinson *et al.*, 2001). Rollins *et al.* (2016) also found a good correlation between percentage leaf discs colonized by *Phytophthora ramorum* and zoospore quantity (determined by direct plating), but only up to a concentration of 451 zoospores/L. In the advent of molecular biology, quantitative real-time PCR (qPCR) is increasingly being used for quantification of *Phytophthora* spp. directly from roots (Spies *et al*., 2011, Tewoldemedhin *et al*., 2011a). This is especially useful for *P. cactorum,* since isolations can be difficult due to the fact that the species is sensitive to hymexazol, an important compound for suppressing fast growing *Pythium* spp. on selective media (Jeffers and Martin, 1986).

This study aimed to evaluate the efficacy of different phosphonate application methods (foliar sprays, trunk paints and –sprays) for the curative management of

Phytophthora root rot on young apple trees. Mainly potassium phosphonate formulations were evaluated, with an ammonium phosphonate formulation only evaluated for foliar sprays. Two orchard trials were conducted in the Grabouw production region, and one trial in the Koue Bokkeveld region. All phosphonate treatment methods were applied in autumn followed by a summer application. The efficacy of phosphonate treatments were evaluated through (i) determination of root phosphite concentrations at 12-weeks after autumn and summer applications, and 20 weeks after the autumn applications just before the summer applications were made, (ii) tree growth responses 14-months after the first phosphonate applications, (iii) yield in only one trial after the first year of treatment and (iv) the change in *P. cactorum* root quantities prior to phosphonate application and 11 months after the first phosphonate applications. *Phytophthora cactorum* root quantities were determined using qPCR analyses targeting the fine as well as secondary roots. Additionally, the potential of roots to produce sporangia that release zoospores were also investigated through qPCR analyses of leaf discs used in root baitings. The effect of different phosphonate application methods on fruit residues was also investigated, since for apples a maximum phosphite residue value of 75 mg/kg is enforced (EFSA, 2014).

MATERIALS AND METHODS

Orchard trial layout and treatments

Two apple orchard trials were established in May 2016 in the Grabouw production region (Breevlei A and Breevlei B), and another trial in the Koue Bokkeveld region (Langrivier). The trials were all planted on soil fumigated pre-plant with chloropicrin/1,3-dichloropropene. The trees were in their 3rd year of growth. In each of the three trials, the only symptoms that were observed consisted of root rot, and no crown- or collar rot symptoms were evident. At the Langrivier, Breevlei A and Breevlei B trials there were 2, 1 and 0 dead trees respectively at the start of the trials. The trials contained a total of 652 trees (Breevlei A and Breevlei B) or 612 trees (Langrivier orchard). The rootstocks in the trials consisted of MM109 for Breevlei A and Breevlei B, and M9 for the Langrivier orchard. The soil types varied from sandy loam to sandy clay loam (Table 1).

In each of the trials, different phosphonate application methods and dosages were evaluated along with an untreated control. Application methods consisted of foliar sprays, trunk sprays and trunk paints. Foliar sprays included potassium- and ammonium phosphonate formulations, whereas the trunk applications were all potassium phosphonate. Trunk sprays were evaluated with or without a bark penetrant (Charge, polyether-polymethylsiloxane-copolymer 1000g/l, Villa Crop Protection) (Table 2). All phosphonate treatments were applied in autumn (May 2016) and summer (November 2016). The last

application of all treatments was carried out on the same day. The same treatments were applied at the Breevlei A and Breevlei B orchards, whereas at Langrivier the 2g a.i./L ammonium phosphonate treatment was excluded (Table 2).

Each treatment was replicated six times, with each replicate containing six trees. The Breevlei A and Breevlei B orchards were both situated on a slight slope, and therefore the trial utilized a completely randomised block design. At the Langrivier orchard, there was no visible variation across the trial site, and therefore a completely randomised design was used.

Tree growth and yield

Tree growth was evaluated by determining the shoot length, and increase in trunk diameter. Measurements were taken at the four centre trees of each replicate treatment. Shoot length was determined by measuring one shoot per tree (four trees per replicate) of the current year growth on 4 June 2017. The shoots were randomly selected within 1.6 -2 m tree height. The increase in trunk diameter (mm) was determined from the start of the trial (30 May 2016) to the end of the trial (4 June 2017).

Apples were harvested only at the Langrivier trial on 18 March 2017. Fruits were harvested from the same trees where tree growth measurements were made. Yield was expressed as kg/tree. At the Breevlei A and Breevlei B orchards, the grower stripped the fruits from the trees early in the season, due to the low bearing nature of the orchards.

Phosphite fruit residues

Phosphite fruit residues were only determined for the Langrivier trial. Approximately 2 kg fruit were randomly selected from the harvested fruit of each replicate treatment. The fruits were sent to a commercial laboratory, Hearshaw and Kinnes Analytical laboratory (Pty) Ltd (Cape Town, South Africa), for phosphonic acid quantification (synonym of phosphite).

Root sample collection

Root samples were taken from all six replicates for each treatment, at the four centre trees within each replicate block. From each replicate, a total of approximately 45 g of roots were sampled consisting of a mixture of fine feeder roots and secondary roots (< 5 mm dia.). Sampling was conducted at four different times, of which three were used for *P. cactorum* quantification and three for phosphite quantification. The first sampling time point for phosphite quantification was at 13-weeks after the autumn applications (20 or 25 August 2016), this was followed by a 20- weeks after autumn application time point (12 or 15 October 2016) and the last time point was 13-weeks after the summer applications (1 or 3

March, 2017). *Phytophthora cactorum* quantification was conducted from the last two time points used for collecting roots for phosphite quantification (October 2016 and March 2017). Additionally a root sample was also collected in May (2 or 4 May 2016) before any phosphonate applications were made. Sampled roots were transported to the laboratory and thoroughly washed free of soil under running tap water. The roots were divided into three groups for subsequent use in avocado leaf disk baitings (13 g), phosphite extraction (20 g) and root DNA extractions (approximately 5 g) where appropriate.

Phosphite root quantifications

Root samples, approximately 10 g per replicate treatment, were dried in an oven for 3 days at 60˚C. The dried roots were ground to a fine powder and phosphite was extracted and purified from roots as described in Chapter 3.

A phosphite standard curve was constructed as described in Chapter 3 using a stock solution of 200 g/l phosphorous acid (Sigma-Aldrich-Aldrich, Oakville, ON). The standard curve contained ten serial dilution data points from 0.05 to 30 µg/ml.

All root extracts, only one sample per replicate, were analysed by the Central Analytical Facility Mass Spectrophotometry division at Stellenbosch University (Stellenbosch, South Africa). LC-MS/MS analyses were conducted as described in Chapter 3 using the European Commission Reference Laboratories for residues of pesticides Single Residue Methods: Quick method for the analyses of numerous highly polar pesticides in foods of plant origin; method 1.3 “Glyphosate and Co. AS 11-(HC (http://www.crl-pesticides.eu/library/docs/srm/meth_QuPPe.pdf). 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). A Thermo Hypercarb (100 x 2.1 mm, 5 µM particle size) (Thermo Fisher Scientific, Waltham, USA) column was used for LC separation. The flow rate, mobile phase and MS parameters were as described in McLeod *et al.* (2018).

Quantification of Phytophthora DNA in roots

DNA quantity in roots.

Root samples were dissected into fine feeder roots and secondary roots (smaller than 2 mm dia.). For each replicate, a 2 ml centrifuge tube was filled for each of the two different root orders (approximately 2 g), and the roots were lyophilized. The roots were ground to a fine powder and DNA was extracted using the NucleoSpin PLANT II kit (Macherey-Nagel GmbH and Ko, Duren, Germany) as described in Chapter 2.

Primers Yph1F and Yph2 (Schena *et al.,* 2008) were used to amplify the genus *Phytophthora* using a Syber Green ® based assay. A standard curve was constructed using *P. cactorum* culture DNA. qPCR reaction and amplification conditions were as described in Chapter 2. *Phytophthora* was amplified from root DNA samples (fine feeder roots and secondary roots) using 2 µl of root DNA diluted 1:5, in a total reaction volume of 20 µl. The roots from each replicate treatment were analysed in duplicate. In each qPCR run, a duplicated standard curve control calibrator sample was included in order to quantify *Phytophthora* DNA through importation of the standard curve.

The efficacy of phosphonate treatments in reducing *Phytophthora* DNA concentrations relative to the untreated control, was assessed by determining the change in *Phytophthora* DNA concentration. The change in *Phytophthora* DNA concentration was determined by subtracting the concentration obtained at the last sampling time point (March 2017) from the concentration obtained at the start of the trials before treatments were applied (May 2016).

Since the qPCR assay was a *Phytophthora* genus specific assay, the *Phytophthora* species involved was determined by sequencing a random selection of qPCR products from each trial. Three samples were selected from each of the fine- and secondary roots for each trial. The Yph1F/Yph2R primer pair yields an approximate 470 bp fragment size, which was sequenced in both directions. Sequencing was conducted as described in Chapter 2. The identity of sequences was confirmed through BLAST analyses in the Phytophthora-ID database (version 2.0) (Grunwald *et al.,* 2011; http://phytophthora-id.org/index.html).

Sporangia and zoospore quantification indirectly through root baiting.

Root baiting was conducted by adding 12 g of fresh roots (mixture of secondary and feeder roots) from each replicate to a plastic container (12 cm x 10 cm). Subsequently, 350 ml distilled water was added, and six square avocado leaf discs (1 cm^2) were floated on the water. Avocado leaves were first surface sterilised by briefly dipping the leaves in 70% ethanol, followed by air drying in a laminar flow. Baiting containers were left uncovered near a window providing natural day light, on a bench in an air-conditioned laboratory at 21 ± 4˚C. Baited avocado leaf disks were removed from the containers after 72 hours, and were blotted dry using a sterile paper towel. The leaves were placed in 2 ml centrifuge tubes and lyophilized.

The leaf disks were ground to a powder using a sterile plastic pestle, and DNA was extracted from 50 mg leaf disks using the NucleoSpin PLANT II kit (Macherey-Nagel GmbH and Ko) according to the manufacturer's protocol. A slight modification was made in that 0.5 g glass beads (2mm) were added to 50 mg leaf powder for further shaking (Retsch MM301

mixer mill, GmbH and Co, Haan, Germany). In the last step, DNA was eluted in 100 µl water. The extracted DNA was diluted 1:5, and 2 µl was used in 20 µl qPCR reaction volume. The qPCR reaction conditions and amplification with primer pair Yph1F/Yph2 was as described in Chapter 2. The identity of the *Phytophthora* spp. involved in each trial was determined from a random selection of three samples per trial, as described for the direct root DNA quantifications.

Statistical Analyses

Root and fruit phosphite concentrations, yield, shoot length and increase in trunk diameter were subjected to analysis of variance (ANOVA) using the GLM (General Linear Models) Procedure of SAS statistical software (Version 9.4; SAS Institute Inc, Cary, USA). The colonization of roots by *P. cactorum* was investigated by calculating the change in pathogen DNA concentrations, i.e. the quantity of *P. cactorum* DNA at the end of the trials was substracted from the pathogen DNA quantities at the start of the trials. The change in *P. cactorum* DNA quantity data was also subjected to ANOVA analyses. To test for significant interactions via ANOVA analyses between *P. cactorum* DNA concentrations obtained from the different tissue types (feeder roots, secondary roots and leaf disk baiting), sampling times (March, August and October) and treatments, a model consisting of a completely randomized/randomised block, split, split plot was setup. Means for all data sets were separated using Fisher's least significant difference (LSD) test at the 95% significance level. The Shapiro-Wilk test was used to test for deviation from normality for all data set (Shapiro and Francia, 1972). The root phosphite and *Phytophthora* concentration data deviated significantly from normality. Therefore, the root phosphite data was Ln (DW+1) transformed, and the pathogen quantities were square root transformed to improve normality. Pearson's correlation analyses and the significance level of correlations were determined between root phosphite concentrations and shoot length and increase in trunk diameter. The analyses were conducted in XLStat (Version 2014, Addinsoft, New York, USA).

RESULTS

Tree growth and yield

Phosphonate applications resulted in a significantly higher ($P \leq 0.0012$) shoot length than the untreated control at the two Grabouw orchards (Breevlei A and Breevlei B), but not at the Koue Bokkeveld orchard (Langrivier; $P = 0.2275$) (Table 3). At the Grabouw orchards, all phosphonate application methods yielded similar shoot growth, which did not differ significantly from each other. At the Koue Bokkeveld orchard, there was only a trend for

improved shoot growth, since all phosphonate treatments had a higher shoot length than the untreated control.

None of the three trials showed a significant increase in trunk diameter in response to phosphonate applications ($P \leq 0.560$) (Table 3). However, for all three trials there was a trend towards a higher increase in trunk diameter, relative to the untreated control.

Yield, which could only be obtained for the Langrivier orchard, did not differ significantly between any of the treatments ($P = 0.2035$).

Phosphite fruit residues

The phosphonate application methods differed significantly ($P < 0.0001$) in translocation of phosphite to apple fruits at the Langrivier orchard (Table 3). Foliar spray applications, either ammonium or potassium phosphonate, resulted in significantly higher fruit residues (12.88 – 15.60 mg/kg), than the trunk spray (5.63 – 6.92 mg/kg) and paint (3.70 mg/kg) applications. All the trunk paint and spray treatments yielded fruit residues, which did not differ significantly from each other. Only the trunk paint treatment did not differ significantly from the untreated control (Table 3).

Phosphite root quantifications

In all three trials root phosphite levels exhibited a significant treatment x time point interaction ($P < 0.0001$), and therefore the data of the different time points [autumn 1 (13 weeks after autumn applications), autumn 2 (20 weeks after autumn applications) and summer (13-weeks after summer applications)] were investigated separately. The root phosphite concentrations of the untreated controls in all the trials were significantly lower than all the phosphonate treatments at all time points (Fig. 1).

There was no phosphonate application method that consistently yielded significantly higher root phosphite concentrations across all trials and time points (total of nine time points when considering the three time points for each of the three trials) (Fig. 1). It was, however, evident that the lowest foliar spray concentration (2 g/L potassium phosphonate) evaluated only at the two Grabouw trials (Breevlei A and Breevlei B), yielded significantly lower root phosphite concentrations (6.71 – 32.33 µg/g) than the other treatments; the exception was for the autumn 1 time point at the Breevlei A trial (8.23 µg/g), since the trunk paint treatment (4.85 µg/g) was significantly lower than the 2 g/L potassium phosphonate foliar spray treatment.

The addition of the bark penetrant to the trunk spray treatment, did not significantly increase root phosphite concentrations (Fig. 1). This treatment commonly resulted in significantly lower root phosphite concentrations than the trunk spray treatment without the

bark penetrant, which was observed at the summer time point at Breevlei B, and at Langrivier for the autumn 1 and 2 time points.

Although there was a trend that the trunk spray (without bark penetrant) yielded significantly higher root phosphite concentrations than the foliar sprays (5 g/L potassium and ammonium phosphonate), this was not consistent for all trials and time points (Fig. 1). The trunk spray was only significantly higher than the ammonium phosphonate foliar spray treatment in root phosphite concentration at five of the nine time points across all trials and time points; these included the autumn 1 and summer time points at Breevlei A, for Breevlei B the autumn 1 time point and for Langrivier the autumn 1 and 2 time points. The potassium phosphonate foliar spray (5 g/L) performed somewhat poorer than the ammonium phosphonate foliar spray relative to the trunk spray (without bark penetrant). The trunk spray (without bark penetrant) yielded significantly higher root phosphite concentrations than the potassium phosphonate foliar spray (5 g/L) for seven of the nine time points considering all of the time points at all three trials. These included all three time points at Breevlei A, at Breevlei B the autumn 1 and summer time points, and at Langrivier the autumn 1 and 2 time points (Fig. 1).

The trunk paint treatment resulted in significantly higher root phosphite concentrations than the foliar sprays in four (ammonium phosphonate foliar) or five (potassium phosphonate foliar) of the nine time points across all three trials; Breevlei A at the summer time point, Breevlei B at the autumn 1 and summer (only potassium phosphonate foliar) and Langrivier the autumn 1 and 2 time points (Fig. 1).

Although there was a trend for the 5 g/L ammonium phosphonate foliar spray to outperform the potassium phosphonate foliar spray based on root phosphite concentrations in comparison to the trunk spray and trunk paint treatments; this was not evident when comparing the root phosphite concentrations of the two foliar formulation sprays with each other. The ammonium and potassium phosphonate foliar sprays did not differ significantly from each other in yielding root phosphite concentrations; the only exception was summer time point at Breevlei B, where the two treatments differed significantly from each other (Fig. 1C).

The trunk spray treatment (without bark penetrant), considering all nine time points across the three trials, were only significantly more effective in yielding elevated root phosphite concentrations than the trunk paint in four of the nine time points; Breevlei A the autumn 1 and 2 time points, and at Langrivier the autumn 1 and 2 time points. At Langrivier the root phosphite summer time point was actually significantly lower for the trunk spray than the trunk paint (Fig. 1).

The root phosphite concentrations remaining at the autumn 1 time point (13-weeks after autumn applications), was significantly higher for all the treatments in comparison to the summer time point (13 weeks after summer applications) for the two Grabouw trials (Fig. 1). The exceptions were at Breevlei A for the trunk spray + bark penetrant and trunk paint treatments, which were not significantly different after autumn 1 and summer time points. At the Koue Bokkeveld trial (Langrivier) the higher foliar spray dosage (5 g/L ammonium- and potassium phosphonate) yielded significantly higher root phosphite concentrations for the summer time point, in comparison to the autumn 1 time point.

At the two Grabouw trials (Breevlei A and Breevlei B), the root phosphite declined significantly from the autumn 1 (13-weeks after application) to the autumn 2 (20-weeks after application) time points for all the phosphonate treatments (Fig. 1). However, in the Koue Bokkeveld trial (Langrivier), most of the phosphonate treatments showed a significant increase in root phosphite at the autumn 2 time point, when compared to the autumn 1 time point. The only exceptions were the potassium phosphonate foliar spray and trunk spray + bark penetrant treatments. For all phosphonate treatments in the Koue Bokkeveld trial at the autumn 1 time point, the root phosphite concentrations were more than half of that attained for the corresponding treatments in the two Grabouw trials. Considering only the highest dosage treatment for all the application methods, the Grabouw trials had root phosphite concentrations at the autumn 1 time point that ranged from 40 to 85 µg/g for Breevlei A, 69 – 187 µg/g for Breevlei B, and for the Koue Bokkeveld trial 22 to 54 µg/g (Fig. 1).

Correlation between root phosphite concentrations and tree growth

Correlation analyses showed that at Breevlei A and Breevlei B, there were significant positive correlations between shoot growth and root phosphite concentrations. The root phosphite concentration 13-weeks after the autumn applications had a significant low to moderate Pearson's correlation with shoot length at both Grabouw trials [Breevlei B (r = 0.536; P = < 0.0001) and at Breevlei A (r = 0.384; P = 0.012)]. Subsequently, at Breevlei B, root phosphite concentrations at 13 weeks after the summer application and at 20 weeks after the autumn application correlated moderately with shoot growth (r = 0.475, P = 0.001; and r = 0.497, P = 0.001), respectively.

Correlation analyses at the Koue Bokkeveld Langrivier trial unexpectedly showed that root phosphite concentrations at some of the time points were negatively correlated with shoot length and increase in trunk diameter. The-13 weeks after summer application root phosphite concentration had a low to moderate negative correlation with shoot length (r = - 0.343; P = 0.040) and increase in trunk diameter (r = - 0.453; P = 0.005).

Quantification of *Phytophthora* DNA in roots

DNA quantity in roots.

qPCR quantification of *Phytophthora* spp. from the primary and secondary roots of all three trials yielded amplification products across all treatments and time points. Based upon sequence analysis, all amplification products were indicative of root infection by *P. cactorum.*

At all three trials there were no significant differences ($P \geq 0.0500$) in the change in *P. cactorum* root DNA concentrations among treatments when assessed at the start of the trial and 11 months later. This was true for the feeder root quantifications (P = 0.0634 – 0.8082), as well as the secondary root quantifications (P = 0.0500 - 0.6854). Although a significant difference (P = 0.005) was observed for the secondary roots at the Breevlei A orchard, this was not associated with a reduction in *P. cactorum* in response to phosphonate treatments (data not shown).

There were no significant interactions (P = 0.1039 - 0.9415) between treatment x tissue type x time for all three trials (Table 5), and therefore treatment could be ignored for investigating the time x tissue type interaction. The time x tissue type (feeder roots, secondary roots or leaf disks) interaction was significant at all three trials (P = < 0.0001 - 0.0379) (Table 5). This interaction was investigated further. At Langrivier and Breevlei B there were no significant differences in *P. cactorum* quantities in the feeder roots and secondary roots for the different sampling time points (May 2016, October 2016 and March 2017) (Fig. 2). At Breevlei A, feeder roots and secondary roots (but not leaf disks) sampled in March 2017 had significantly higher *P. cactorum* DNA quantities than feeder and secondary roots sampled in October 2016.

Sporangia and zoospore quantification indirectly through root baiting.

Phytophthora was amplified from the leaf disks used in root baitings from all the phosphonate treatments and the untreated control in all three trials (data not shown). Sequencing of the qPCR product of representative samples, indicated that all infections were caused by *P. cactorum.* qPCR quantification of *Phytophthora* from leaf discs used in root baiting at the start and end of the trials, did detect a significant change (P = 0.4207 to 0.6751) in *P. cactorum* quantities between any of the treatments for all three trials (data not shown).

There were no significant differences (P = 0.24 – 0.55) in the *P. cactorum* DNA quantities determined at the start of the trial and 11 months later between the phosphonate treatments and the untreated controls in all three trials.

Since there was no significant treatment x time x tissue type (feeder and secondary roots and leaf disks) interaction in any of the three trials (P = 0.1039 - 0.9415) (Table 4), the

effect of sampling time point could also be investigated for the zoospore quantities detected in the leaf disks. There was a significant tissue type x time interaction for all three trials (P = < 0.0001 - 0.0379). At all three trials, *P. cactorum* quantities detected in leaf disks in May was significantly higher than in October (Fig. 2). At two of the trials (Langrivier and Breevlei A) quantity of *Phytophthora* spp. detected at the March sampling was also significantly higher than the October sampling, but did not differ significantly from the May sampling point (Fig. 2).

DISCUSSION

The present study showed that curative phosphonate treatments can improve the growth (shoot length) of young apple trees suffering from Phytophthora root rot, within one year following the first applications. However, the pathogen was not suppressed in roots by phosphonate treatments. Different phosphonate application methods (trunk spray, trunk paint and foliar sprays) were equally effective in improving tree growth. This was observed irrespective of the fact that several of the application methods yielded significantly different root phosphite concentrations. Root phosphite concentrations decreased in summer (October) following the application of phosphonates in autumn (May), but this was only evident in the Grabouw production region, not the Koue Bokkeveld region. Phosphite fruit residues were lower following phosphonate applications to trunks, in comparison to foliar applications.

Only a few studies have previously evaluated the efficacy of phosphonates (alkyl-phosphonate fosetyl-Al), against *P. cactorum* on apple. These trials were all conducted on trees showing root and crown rot symptoms, unlike the current study where the young trees only showed root rot. Utkhede and Smit (1991) evaluated fosetyl-Al on relatively old trees (25-, 10- and 7 years) using either foliar sprays or a trunk/soil drench. A significant increase in trunk diameter and yield was only obtained with foliar phosphonate sprays in the youngest orchard. The foliar phosphonate spray rates applied in autumn and summer in the previous study (Utkhede and Smit, 1991) were comparable to that used in the trials from the current study (9 to 15 g a.i./tree annually), since it amounted to a total of 10 to 15 g a.i./tree annually. However, due to differences in tree size (7- to 25-year-old trees in low density orchards versus 3 year old trees in high density orchards), the dosages of the current trial would have likely resulted in a higher root phosphite concentration. Fosetyl-Al applied as a drench/trunk treatment was ineffective when evaluated by Utkhede and Smit (1991). Their trunk/soil drench application is difficult to compare to the current study, since it isn't clear from the published work how much of the product was applied to the trunk versus the soil, only that it was applied as a trunk spray (60 cm area) at 5 L per tree. Nonetheless, even if

most of the product was applied to the trunk, the rate was quite low being 20 g a.i./tree annually for relative large trees (Utkhede and Smith, 1991), as opposed to the 40 to 80 g in the current trials for relatively small trees. A second study by Utkhede and Smith (1993), evaluated the effect of a similar trunk/soil fosetyl-Al application against *Phytophthora* crown rot in one trial that contained newly planted apple trees, which were artificially inoculated annually with *P. cactorum* at the crown region. Fosetyl-Al was applied for 7-years in the orchard, using 10g a.i./tree annually for the first 4-years, and subsequently 5g a.i./tree annually for 4-years. Over the 7 year trial period, significant increases in trunk diameter were only seen for cumulative tree growth data, not for individual years. The same observation was made for cumulative yield over 3-years (Utkhede and Smith, 1993). Utkhede and Smith (1995) also reported that two annual 2g a.i. /l foliar sprays controlled *P. cactorum* in newly planted trees, resulting in enhanced tree growth and yield. Orlikowsski *et al.* (1986) also found, similar to the current study, that although high dosages are used in trunk paints (5% to 10%), the lower foliar spray dosages (0.25% to 0.5%) yielded similar inhibition of lesion lengths on the trunks of apple inoculated with *P. cactorum*.

Although in the current study foliar phosphonate treatments (ammonium and potassium phosphonates; 5 g/L and 2 g/L) improved shoot growth to a similar level than the trunk spray and –paint treatments at the Grabouw trials, the treatments often differed significantly in their root phosphite concentrations. Phosphonates applied to foliage and the translocation of phosphite to roots appeared to be more effective than when phosphonates are applied as trunk paint or –sprays based on root phosphite concentrations. This is evident from the fact that the 5 g/L foliar sprays were applied as a total of 9 to 15 g a.i./tree annually, as opposed to the 40 to 80 g a.i./tree applied annually for the trunk paint and -sprays respectively. Yet the foliar spray treatments often did not differ significantly in root phosphite concentrations from the trunk applications, especially the trunk paint treatment. It is likely that phosphonates cannot effectively penetrate the bark of young trees. Attempts to improve phosphite uptake by trunks through the addition of a bark penetrant (polyether-polymethylsiloxane-copolymer) to trunk sprays, was ineffective based on a comparison of root phosphite concentrations.

Foliar sprays would be more cost effective for growers due to the reduced product cost, and reduced labour required for treatment in comparison to trunk applications. The only benefit from the trunk applications would be the significantly lower fruit residues relative to the foliar sprays. If applications have to be made to bearing trees, trunk applications would be advisable for summer applications when fruit or flowers are present on the trees. Fruit is known to be a strong sink for phosphite when applied to trees having flowers or small fruits,

due to the source-sink translocation of phosphite (Whiley *et al*., 1995). Since autumn applications are made after harvest, this treatment is unlikely to contribute to fruit residues.

There is very limited information available regarding the phosphite concentration required in plant tissue for suppression of *Phytophthora* spp. Phosphite concentrations previously reported in literature for suppressing *Phytophthora* spp. include 21 µg / g_{FW} for suppression of *Phytophthora citricola* in avocado trunk cankers (El-Hamalawi *et al*., 1995). Furthermore, in avocado, non-peer reviewed articles also reference concentrations of 25−40 µg/g_{FW} for suppressing root rot caused by *Phytophthora cinnamomi* (Giblin *et al,* 2007). In contrast, for different native Australian plant species, a wide range of phosphite concentrations (26 to 265 µg/g_{DW}) have been reported as being required for 50% suppression of *P. cinnamomi in planta* (Shearer *et al.*, 2012). Thus, different phosphite concentrations are likely required for suppression of *Phytophthora* spp. in different host plant-pathogen interactions. This is likely due to the mode of action of phosphonates consisting of a direct and indirect mode of action. Efficacy and mode of action is likely to be influenced by the host's ability to launch a defence response and the toxicity of phosphite towards the pathogen (Guest and Grant, 1991). It can thus not be hypothesised whether the root phosphite concentrations obtained in the current study would have been adequate for *P. cactorum* suppression. The measured *P. cactorum* quantifications from roots (direct or the ability to produce zoospores) in the trials showed that there were no significant reductions in pathogen quantities. This could suggest that the root phosphite concentrations were inadequate. However, it is possible that more time will be required for significant pathogen suppression in the trials, since the analyses were conducted only 10 months after applications were first made to symptomatic trees. Furthermore, it has been reported that phosphite does not kill *P. cinnamomi* inoculum on the surface of roots for up to 48 hours after inoculation (Van der Merwe and Kotze, 1994). Since the roots used for *P. cactorum* quantifications in the current study were not surface sterilized, this could have resulted in an overestimation of the pathogen in phosphonate treated roots due to the presence of surface propagules that would not be able to infect the root. The fact that tree growth was significantly improved by phosphonate treatments does suggest that adequate root phosphite was present in at least the Grabouw trials (Breevlei A and Breevlei B). In these trials, it was found that a significant but low, positive correlation existed between autumn root phosphite concentrations and shoot length. High root phosphite concentrations were obtained in the trials 13-weeks after the autumn application (Breevlei A, 40 - 85 µg/g_{FW}; Breevlei B, 69 -187 µg/g_{FW}). In contrast, at the Koue Bokkeveld orchard, where tree growth was not significantly improved by any of the phosphonate treatments, unexpected significant negative correlations were obtained between root phosphite and tree growth. This could be

due to the much lower root phosphite concentrations (22 – 54 µg/g_{FW}) achieved in this orchard 13-weeks after the autumn applications.

The difference in the efficacy of the translocation of phosphite to roots 13-weeks after the autumn application in the Langrivier orchard in comparison to the other two orchards (Breevlei A and Breevlei B), could possibly be explained by differences in tree phenology and rootstocks. The two Breevlei orchards were established on rootstock MM109, whereas Langrivier was on a M9 rootstock. MM109 is a highly vigorous rootstock (Costa, 2008), which could result in more photosynthate being allocated to this rootstock, and consequently root phosphite, than the less vigorous M9 rootstock (Grant and Hammatt, 1999). Although it has been hypothesized that rootstock can influence phosphite root quantities, this has not been demonstrated in any plant species. Another reason for the discrepancy in the root phosphite concentrations between the trials could have been differences in the root flush versus shoot flush in the orchards. Phosphite is known to be translocated in source-sink relationship, thus if applied during root flushes, higher concentrations are expected in roots (Whiley *et al.,* 1995, Giblin *et al.,* 2007b). The root flush was not monitored in the trials, but preliminary data show that it might differ in the two production regions (Grabouw and Koue Bokkeveld) in South Africa (E. Lotze, personal communication).

The root phosphite concentrations achieved 13-weeks after the autumn application were superior to those obtained after the summer application in the two Grabouw orchards. Root phosphite concentrations furthermore decreased in summer following the autumn application. Apple roots are a key metabolic sink in autumn months due to the fact that photosynthates are translocated to roots in order to prepare the trees for winter dormancy (Heide and Prestrud, 2005; Tartachnyk and Blanke, 2001). Phosphite will travel along with the photosynthates to roots. Following the translocation to roots in autumn, root phosphite concentrations will decrease in spring when trees resume growth. The summer growth involves a number of source-sink relations and competition for photosynthates and consequently phosphite. During active growth, phosphites have been quantified in high concentrations in floral sets, young fruit, mature fruit and shoots (Malusa and Tosi, 2005). Furthermore, phosphites are known to decrease in concentration due to plant growth and the consequent dilution effect (Tynan *et al*., 2001, Hardy *et al*., 2001). Due to the decrease in root phosphite after the autumn applications, it seems likely that a summer application, as was applied in the current study, would be required to sustain root phosphite concentrations year long. This, however will require further investigation since some studies have reported that fosetyl-Al provided protection against *P. cactorum* crown infections for up to 15 months (Long *et al.*, 1989; Orlikowski *et al*., 1986).

None of the phosphonate treatments resulted in a significant reduction in *P. cactorum* quantities in roots, nor in the pathogens ability to produce sporangia and zoospores. It is not unexpected to isolate *Phytophthora* spp. from phosphonate treated plants (Wilkinson *et al*., 2001a, Shearer and Fairman, 2007b, Reglinski *et al*., 2009, Hardy *et al*., 2001; Pilbeam *et al*., 2000; Wilkinson *et al*. 2001c). This is due to the fact that phosphite is only fungistatic, and does not eradicate *Phytophthora* from plants (Pilbeam *et al*., 2001a; Garbelotto *et al.,* 2009). The ability of phosphonates to reduce the asexual phase of the pathogen *in planta,* has been reported by Wilkinson *et al.,* (2001) for native Australian plant species. It will be important to determine in the future whether sporangia and zoospore production are reduced if phosphonate treatments are continued. A reduction in the potential of the pathogen to produce inoculum could have a significant effect on disease progress and a reduction in inoculum potential will be required for effective long term disease control.

Quantification of *Phytophthora* spp. by qPCR provided important information regarding colonization patterns of the pathogen in different root orders and in different seasons. The pathogen colonized fine feeder and secondary roots to the same extent since DNA quantities in these root orders did not differ significantly from each other in all three trials. However, it was noted that the incidence in secondary roots is less than in fine roots (data not shown). Based on the root baiting results, pathogen inoculum production in roots exhibited seasonal variation when assessed under laboratory conditions. Roots produced significantly higher zoospore quantities in May (autumn) than in October (late spring) in all the orchards. This could suggest that host resistance is lower during the autumn to winter period. However, this disagrees with previous reports in that apple rootstocks are more susceptible to *P. cactorum* in spring (Utkhede and Quamme 1988; Browne *et al*. 1990; Browne and Mircetich 1996, Zondo, *et al* 2007). In South Africa specifically, it was shown using artificial trunk inoculations, that apple rootstocks have a low susceptibility to *P. cactorum* during dormancy in winter. The rootstocks were highly susceptible during active growth in spring and summer (Zondo *et al*., 2007). The importance of inoculum production during spring was also shown by Horner and Wilcocks (1996). They investigated apple soils in New York, USA and found that *P. cactorum* produces most sporangia and zoospores from soil in spring, which coincided with high soil temperatures (~ 20˚C) and high precipitation. In South Africa, rain events occur predominantly in winter, which could explain the seasonality of *P. cactorum* inoculum production observed in the current study. Furthermore, inoculum production during this period would be favourable for the pathogen in South Africa as it would correspond with one of the main root flushes of young apple trees. In South Africa, young apple trees show continuous growth, but the two main root flushes occur in summer and autumn months in the Grabouw region. All apple productions in the Grabouw and Koue

Bokkeveld region are conducted using irrigation in summer (personal communication Elmi Lortze, Stellenbosch University, Department of Horticulture).

In conclusion, this study has shown the potential of phosphonates to manage Phytophthora root rot curatively on apple trees based on increase in shoot length observed during the initial year of treatment applications. However, the pathogen was not significantly reduced in the roots of phosphonate treated trees. The continuation of phosphonate applications and evaluation of tree growth responses will be important to draw definitive conclusions from these trials. In ARD replant trials where *P. cactorum* plays a causative role, the highest increase in growth relative to untreated control trees is observed from the second year onwards (Chapter 2). Since it is known that phosphonates are only fungistatic, continued evaluation of the trials after conclusion of phosphonate will also be important. Current grower practices and observations are that a 2-year treatment program for young root rot affected trees is sufficient, since trees seem to become more tolerant to the pathogen as they age (personal communication, J.P.B. Wessels). This is provided that other management practices such as good irrigation scheduling, ridging and mulching are followed. These anecdotal observations will have to be confirmed to ascertain the viability of phosphonate treatment programs. Foliar phosphonate applications will be the most cost effective for growers, although it has a risk for higher fruit residues on bearing trees if applied in summer during fruit development. Although fruit phosphite residues were below the maximum residue limit set for apples (75 mg/kg, EFSA, 2014) for foliar sprays in the current study, this requires further investigation due to the fact that in the harvested trial, a small crop was set, which probably minimized the sink for phosphite. In avocado, a significant positive correlation has been found between crop load and fruit residues (Whiley, 2001). Future work should further investigate the root phosphite concentration required for suppression of *P. cactorum* in apple, as well as determining elevated periods of host susceptibility in terms of infection and inoculum production. Such information will be instrumental in defining appropriate periods for treatment application to optimize protection against the pathogen.

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Table 1. Soil characteristics for three ale orchard trials where different phosphonate alication methods were evaluated for the management of Phytophthora root rot.

Orchard	pH	Classification	Resistance (Ohm) [a]	CEC (Cmol (+)/kg [b]	Clay %	Silt %	Sand %	Water holding Capacity (mm/m) [c]
Breevlei A	5.5	Sandy loam	270	4.37	13	10	77	66.36
Breevlei B	4.8	Sandy clay loam	280	9.50	21	16	63	70.07
Langrivier	5.2	Sandy loam	830	5.77	19	14	67	107.42

Soil sampling was conducted to a depth of 30 cm. Ten random soil samples were collected and thoroughly mixed for each orchard. The composite sample of each orchard was sent for soil analysis at Bemlab (Somerset, South Africa).

[a] Resistance (Ohm) indicates the presence of large quantities of salts in the soil

[b] CEC (Cmol (+)/kg is the total exchangeable cations, a measure of the soil's ability to retain and suly nutrients, specifically the positively charged nutrient ions called cations. These include the cations calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^{1+}), ammonium (NH_4^+), and many of the micronutrients**.** The higher the CEC the harder it becomes to change factors such as pH.

[c] Water holding Capacity (mm/m) is the depth of water held between field capacity and permanent wilting point per metre depth of soil.

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Table 2. Phosphonate treatments and date of alication for summer and autumn phosphonate alication at three ale orchard trials (Breevlei A, Breevlei B and Langrivier) aimed at managing Phytophthora root rot.

Treatment name [a]	Product [b]	Phosphorous acid concertation	Volume alied per tree	Autumn alication dates	Summer alication dates	Total amount of phosphorous acid alied per tree (summer + autumn alications)
Untreated control	Water			25 May 2016	30 November 2016	0
Foliar potassium phosphonate (5 g/L)	potassium phosphonate	5 g / 100L	300 – 500 ml	11,18 and 25 May 2016	16,23 and 30 November 2016	9 – 15 g
Foliar potassium phosphonate (2 g/L)	Potassium phosphonate	2 g / 100L	300 – 500 ml	11,18 and 25 May 2016	16,23 and 30 November 2016	3.6 – 6 g
Foliar ammonium phosphonate (5 g/L)	Ammonium phosphonate	5 g / 100L	300 – 500 ml	11,18 and 25 May 2016	16,23 and 30 November 2016	9 – 15 g
Trunk spray + bark penetrant	Potassium phosphonate + polyether-polymethylsiloxan-copolymer	400 g / L	50 ml	18 and 25 May 2016	23 and 30 November 2016	80 g
Trunk spray	Potassium phosphonate	400 g / L	50 ml	18 and 25 May 2016	23 and 30 November 2016	80 g
Trunk paint	Potassium phosphonate	200 g / L	100 ml	18 and 25 May 2016	30 November 2016	40 g

[a] Foliar spray alications were alied with a knapsack sprayer. The pH of the 5 g/L foliar sprays were adjusted to 7.2 using potassium hydroxide. Trunk paints were alied using a 100 mm width paint brush. Trunk sprays were alied using a trunk sprayer containing three nozzles located in a semi-enclosed circle. All treatments were alied at the Breevlei A and Breevlei B orchard trials. At the Langrivier orchard 2g/L ammonium phosphonate foliar was excluded, other treatments were similar to Breevlei A and B.

[b] The potassium phosphonate product used was Phosguard (Nulandis, Phosguard 400 SL, Witfield, South Africa; 400 g phosphorous acid/L), and the ammonium phosphonate was Brilliant (Arysta, Brilliant SL, South Africa, 300g phosphorous acid/L). The bark penetrant, was Charge (Villa Crop, South Africa, 1000 g/l polyether-polymethylsiloxane-copolymer). The penetrant was added at a concentration of 0.50 ml / L.

Table 3. Effects of different phosphonates alication methods on tree growth, yield and fruit residues at three trial sites (Breevlei A, Breevlei B and Langrivier), following autumn and summer alications.

Treatments [a]	Breevlei A		Breevlei B		Langrivier			
	Increase in trunk diameter (mm) [b]	Shoot length (cm) [b]	Increase in trunk diameter (mm) [b]	Shoot length (cm) [b]	Increase in trunk diameter (mm) [b]	Shoot length (cm) [b]	Yield (kg/tree) [c]	Phosphite fruit residues (mg/kg) [c]
Potassium phosphonate trunk paint	16.14	33.32a	19.29	32.71a	10.79	34.38	3.98	3.70bc
Potassium phosphonate trunk spray	15.95	31.88a	18.27	33.06a	11.25	32.25	3.94	5.63b
Potassium phosphonate trunk spray+charge	15.13	33.06a	19.47	36.00a	11.09	33.47	3.79	6.92b
Ammonium phosphonate foliar(2g/l)	19.07	35.39a	19.92	31.69a	N/A	N/A	N/A	N/A
Ammonium phosphonate foliar(5g/l)	15.65	32.83a	22.69	31.97a	10.90	33.73	2.56	15.60a
Potassium phosphonate foliar(5g/l)	19.79	30.18a	19.03	31.40a	16.57	30.64	4.31	12.88a
Untreated control	10.03	21.36b	13.4	18.76b	8.73	20.92	3.84	0.62c
***P* value**	0.1305	0.0012	0.560	< 0.001	0.138	0.2275	0.2035	<0.0001

[a] All phosphonate treatments were alied in autumn (May 2016) and again in summer (November 2016). The dosages and number of alications for each treatment are shown in Table 2.

[b] Shoot length and increase in trunk diameter was measured 10 months after phosphonate alications. Values are the average of six replicates, with each replicate containing 4 to 5 trees that were measured. For each orchard, values followed by the same letter to not differ significantly ($P > 0.05$) according to Fisher's least significant difference test.

[c] Yield was determined at harvest (March 2017), while increase in trunk diameter and shoot were measured in (June, 2017). Ale fruit phosphite residue was quantified from 2 kilograms of randomly selected harvested fruit per replicate..Values in columns are the average yield or fruit residue of six replicates. For yield six trees per replicate were harvested. For each orchard, values followed by the same letter to not differ significantly ($P > 0.05$) according to Fisher's least significant difference test.

Table 4. Analyses of variance of the effect of tissue type (fine roots, secondary roots and leaf disks) and month of sampling (March, October and May) on *Phytophthora cactorum* DNA quantities in three trials (Breevlei A, Breevlei B and Langrivier) where different phosphonate treatments were alied.

	Breevlei A				Breevlei B			Langrivier			
Source	Df	MS	F	P	MS	F	P	Df	MS	F	P
Rep	5	3.132	0.98	0.4456	3.388	1.69	0.1677	NA	NA	NA	NA
Treat	6	7.248	2.27	0.0634	6.815	3.40	0.0112	5	15.088	2.54	0.0498
Treat (rep)	30	3.194			2.006			30	5.947		
Time	2	3.214	1.04	0.3599	7.813	8.47	0.0005	2	5.614	1.18	0.3157
Treat x time	12	1.589	0.51	0.8998	1.657	1.80	0.0655	10	1.738	0.36	0.9573
Treat x time (rep)	70	3.099			0.922			60	4.776		
Tissue type [a]	2	15.592	6.20	0.0024	2.530	3.33	0.0376	2	151.416	31.35	< 0.0001
Treat x tissue type	12	1.669	0.66	0.7851	0.499	0.66	0.7901	10	6.595	1.37	0.2000
Time x tissue type	4	23.606	9.39	< 0.0001	1.966	2.59	0.0379	4	16.229	3.36	0.0112
Treat x time x tissue type	24	2.659	1.06	0.3958	1.071	1.41	0.1039	20	2.646	0.55	0.9415
Error	209	2.515			0.759			169	4.829		
Corrected total	376	1132.367			420.775			312	1871.870		

[a] Tissue type refers to whether *P. cactorum* was qPCR amplified from fine feeder roots, secondary roots or leaf disks used in root baitings.

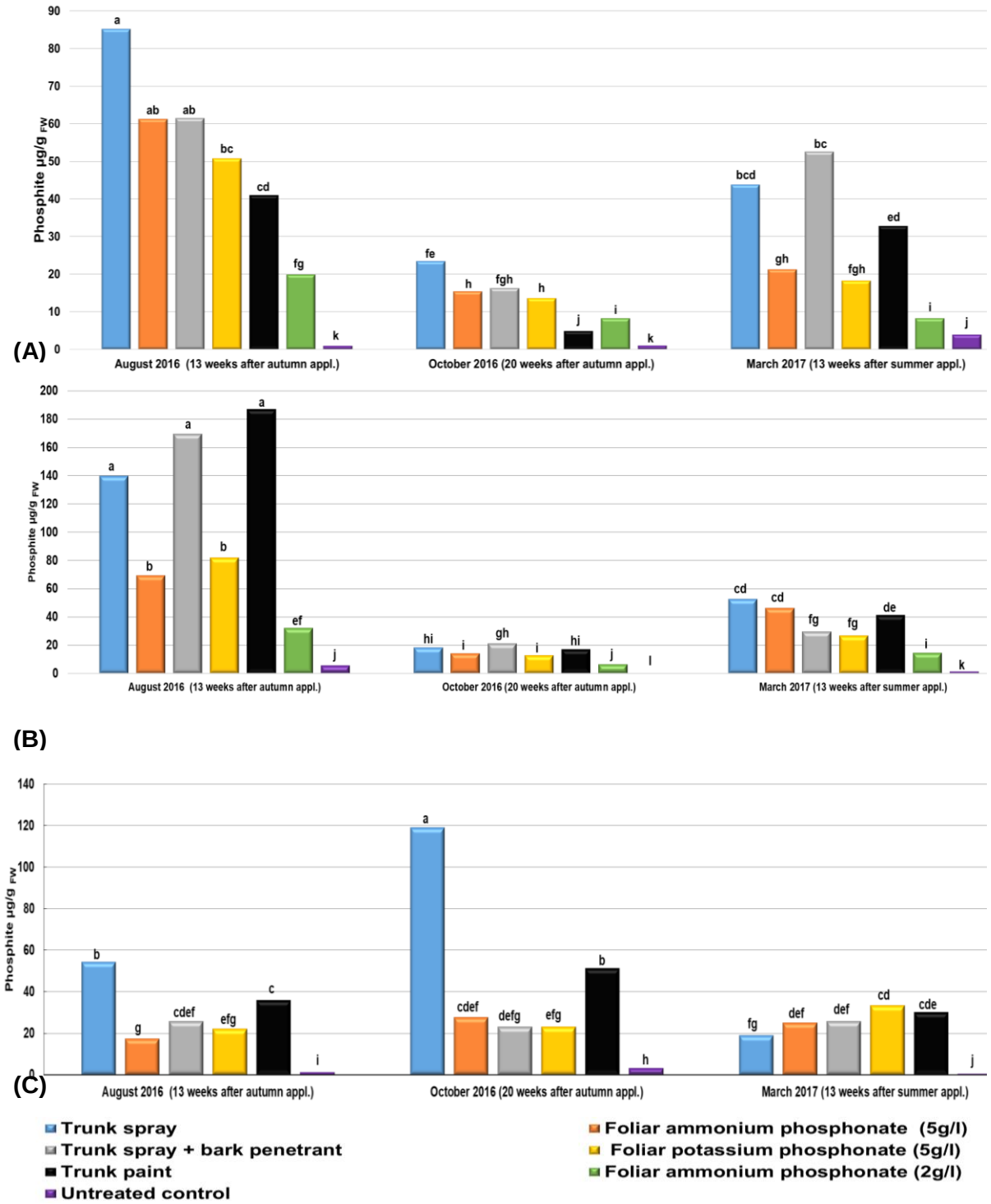

Fig. 1. Root phosphite concentrations (µg/mg $_{\text{fresh weight (FW)}}$) in three trials [(A) Breevlei A (B) Breevlei B and (C) Langrivier] where ale trees were treated with phosphonates using different alication methods. Most phosphonate treatments were potassium phosphonate treatments, except for the one foliar spray that was an ammonium phosphonate treatment. The same treatments were mostly alied at all three trials, except for the Langrivier trial where the ammonium phosphonate foliar spray was excluded. Foliar sprays were alied at two different concentrations (2 and 5 g/L). The trunk spray treatment was evaluated with and without a bark penetrant (Charge, a.i. 1000 g/l polyether-polymethylsiloxane-copolymer). Phosphonate treatments were alied in autumn (May, 2016) and subsequently in summer (November, 2016). Root phosphite concentrations were determined at three time points including (i) autumn 1, which was 13 weeks after the autumn alications, (ii) autumn 2, which was just before alication of the summer phosphonate treatments were made and 20 weeks after the autumn alications (iii)) summer, which was 13-weeks after the summer alications were made. Values are the average of six replicates (4 trees per replicate). For all three trials there was a significant treatment x season interaction ($P < 0.0001$).

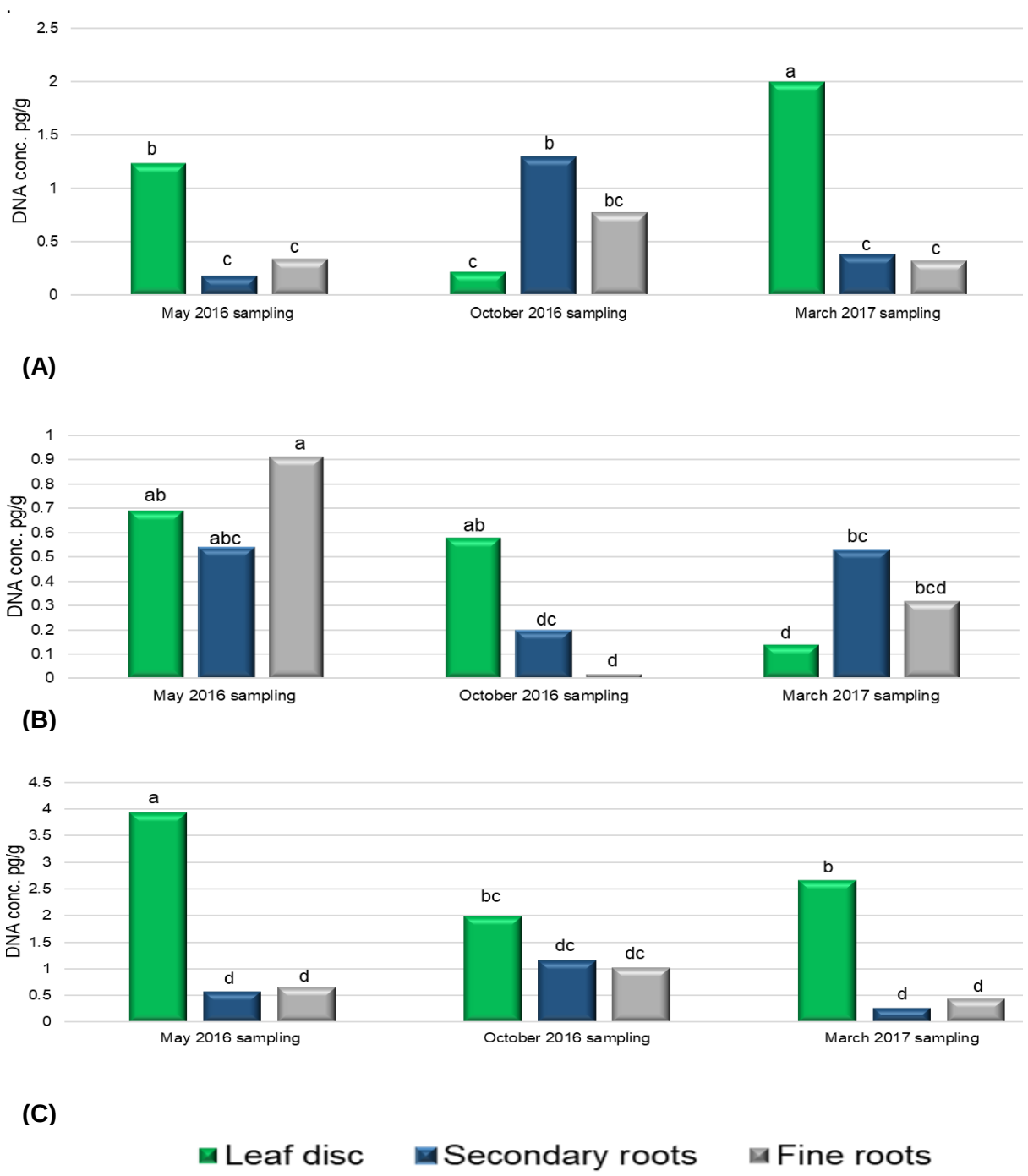

Fig. 2. *Phytophthora cactorum* DNA quantities in the feeder- and secondary roots of ale trees, during different months of samplings in three ale orchard trials (A) Breevlei A, (B) Breevlei B and (C) Langrivier. The sporangial and zoospore production of roots was also assessed using avocado leaf disks in ale root baitings. DNA quantities in roots and leaf disks were determined using quantitative real-time PCR. Values are the mean of six (Langrivier) or seven (Breevlei A and Breevlei B) treatments, each having six replicates (4 - 5 trees per replicate). For all three trials, there were no significant (P = 0.1039 - 0.9415) treatment x time x tissue type (feeder- and secondary roots and leaf disks) interaction. There were significant (*(P = < 0.0001 - 0.0379)* tissue type x time interactions for all three trials. Bars followed by the same letter do not differ significantly (P > 0.05) according to Fisher's least significant difference test.

CHAPTER 5

CONCLUSION

This study was the first to evaluate the co-alication of semi-selective chemicals, under ale orchard conditions. The study demonstrated that the independent use of semi- selective chemicals has potential for managing ale replant disease. It was furthermore shown that *P. cactorum* was an important component of ARD in South African orchards. A synergetic relationship was found between *Pratylenchus* species and *P. cactorum* (Chapter 2). It was also evident that phosphonate can suress *P.cactorum* and *P.irregulare* in asymptomatic orchards. *In vitro* assays showed variation in the phosphite sensitivity of *P.cactorum*, *P.vexans* and *P.irregulare* (Chapter 3). qPCR can be utilised in evaluating the efficacy of phosphonates and semi-selective chemicals in *Phytophthora* root rot affected and ARD orchards (Chapter 2 ,3 and 4) .*Phytophthora cactorum* in the roots of phosphite treated ale trees were shown to still have the ability to produce zoospores (Chapter 4). LC-MS/MS analysis was successfully used in quantifying phosphite in roots of symptomatic and asymptomatic ale orchards (Chapter 3 and 4).

Some areas for future research should be directed towards the co-alication of phosphonates with other biocides to manage ARD since fenamiphos is likely to be phased out in South Africa in the near future. Evaluation of the co-alication of phosphonates with other biocides that suress '*Cylindrocarpon*'-like s. and *Pratylenchus* s., should be investigated with an emphasis on alications being made more than once. There furthermore is a need to develop qPCR assays that can quantify pathogenic '*Cylindrocarpon'* -like s. on ale. This will allow for better investigations into the efficacy on chemicals that suress '*Cylindrocarpon'*-like s. *In planta* studies using different *P. cactorum* inoculum concentrations and root phosphites concentrations should be investigated. Similar studies should also be conducted with *Pythium irregulare.* This knowledge may provide an understanding regarding the suression of these pathogens in symptomatic orchards that may contain high inoculum levels. In future, phosphonate treatments in symptomatic orchards should be evaluated for 2 to -3 years. This will allow for a better understanding of *Phytophthora cactorum and Pythium irregulare* epidemiology in the presence of phosphite. Although phosphite is not expected to control the pathogen in soil, it is expected that a significant reduction in the pathogens ability to produce inoculum and survive in infected roots, will be reflected in reduced soil inoculum levels. Lastly, it is recommended that more studies should be conducted on the temporal nature of root growth in young ale trees in South Africa.