ASSESSING *PHYTOPHTHORA CINNAMOMI* SEASONAL ROOT COLONISATION PATTERNS AND PATHOGEN RESPONSE TO MANAGEMENT PRACTICES (PHOSPHONATES AND ROOTSTOCK TOLERANCE) IN SOUTH AFRICAN AVOCADO ORCHARDS

by

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SUMMARY

Phytophthora root rot (PRR), caused by *Phytophthora cinnamomi* (Pc), is a destructive soilborne disease that can cause major economic losses in commercial avocado orchards. Despite this, there is limited information on the pathogen's seasonal colonisation patterns, as well as which sampling strategy and quantification method would be best for assessing it. Current limitations in Pc quantification methods can lead to inaccurate assessments of PRR management strategies including phosphonate fungicides and PRR-tolerant rootstocks.

The current study was able to identify a peak in seasonal Pc root colonisation in late autumn (May) in mature avocado orchards situated in two main production regions (Mooketsi and Letaba) in the Limpopo province of South Africa. During two investigated growing seasons (2017 and 2018), Pc root quantities were significantly higher in May 2018 than in March (early autumn), August (late winter) and October/November (late spring) of the same season (2018). In 2017, colonisation trends were less evident, which is likely due to the less conducive PRR conditions that prevailed, especially in the Mooketsi region. In Letaba (2017), August and May yielded the highest Pc root quantities in most orchards, but these did not differ significantly from the other months (March and October/November). In May, Pc root quantities were furthermore significantly positively correlated with the number of hours at soil temperatures of 15-19°C, but negatively with 20-24°C. Soil moisture fluctuations were not associated with Pc root quantities. Evaluation of two sampling strategies consisting of four tree groups (each containing five trees) and one tree group (20 trees), showed that both approaches are suitable for investigating Pc colonisation patterns. A traditional root baiting method, where leaf baits were plated onto selective media, was as effective in identifying colonisation trends as a molecular approach using small-scale root DNA extractions and quantitative real-time PCR (qPCR) analysis. A large-scale root DNA extraction and qPCR analysis method was deemed less effective.

A molecular quantification (qPCR) approach was shown to be ineffective for evaluating two management strategies (phosphonate treatments and rootstock tolerance) in avocado orchards showing no obvious aboveground symptoms of PRR decline. Although root phosphite (breakdown product of phosphonates) concentrations of a 2x trunk injection treatment applied at the preventative dosage (0.3 g a.i./m^2) were significantly higher than the untreated control, the Pc root and soil DNA concentrations were not significantly reduced by the phosphite, relative to the untreated control. This was for quantifications conducted in either May or October 2018 and using the best of three evaluated Pc-specific qPCR assays. The potentially more PRR-tolerant R0.06 rootstock yielded higher Pc root DNA concentrations than the Dusa® rootstock in November 2017, but not in the other two sampling months (March and May 2018).

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The identification of effective sampling strategies, Pc root quantification methods and the Pc root colonisation patterns in avocado orchards in the current study is important. Since May had the highest root colonisation levels, PRR management practices should be put in place to achieve optimal root protection during, or just prior to, this period (late autumn). The effective sampling and quantification methods that were identified for studying seasonal root colonisation patterns in avocado, will be useful for other studies that are conducted on the over 5000 host plant species of Pc. Alternative quantification methods to qPCR for assessing management strategies must be investigated. However, it is possible that qPCR analysis may be successful for evaluating management strategies if improvements are made to the trial design, and if analyses are conducted in diseased rather than asymptomatic orchards.

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OPSOMMING

Phytophthora wortelvrot (PW) wat deur *Phytophthora cinnamomi* (Pc) veroorsaak word, is ‘n vernietigende grondgedraagde siekte wat grootskaalse ekonomiese verliese in kommersiële avokado-boorde veroorsaak. Ten spyte hiervan, is daar ‘n gebrek aan kennis oor die patogeen se seisoenale kolonisasie patrone, asook watter monsternemingstrategie en kwantifiseringsmetode die beste sou wees om dit te bepaal. Huidige tekortkominge in Pc kwantifiseringsmetodes kan tot onakkurate toepassing van PW bestuurstrategie lei, insluitende fosfonaat swamdoders en PW-bestande onderstamme.

Die huidige studie kon ‘n piek in seisoenale Pc wortelkolonisasie identifiseer, naamlik láát herfs (Mei) in volwasse avokado-boorde, wat in twee hoof produksiestreke geleë is (Mooketsi en Letaba) in die Limpopo provinsie van Suid-Afrika. Gedurende twee groeiseisoene (2017 en 2018) wat ondersoek is, was Pc wortelhoeveelhede betekenisvol hoër in Mei 2018 as in Maart (vroeë herfs), Augustus (láát winter) en Oktober/November (láát lente) van dieselfde seisoen (2018). In 2017 was kolonisasie-neigings minder opmerklik, wat moontlik ‘n gevolg was van minder gunstige toestande vir PW wat geheers het, veral in die Mooketsi-streek. In Letaba (2017), het Augustus en Maart die hoogste Pc wortelhoeveelhede in die meeste boorde opgelewer, maar dit het nie betekenisvol van die ander maande (Maart en Oktober/November) verskil nie. In Mei was Pc wortelhoeveelhede verder betekenisvol positief gekorreleer met die hoeveelheid ure by grondtemperature van 15-19°C, maar negatief gekorreleer met 20-24°C. Grondvog fluktuasies het nie met Pc wortelhoeveelhede verband gehou nie. Evaluasie van twee monsternemingstrategieë wat uit vier groepe bome (elke groep bestaande uit vyf bome) en een groep met twintig bome bestaan het, het getoon dat beide benaderings geskik is om Pc kolonisasie patrone te ondersoek. ‘n Tradisionele wortellokaasmetode, waar blaarlokaas op selektiewe media geplaas is, was net so effektief om kolonisasie neigings te identifiseer as ‘n molekulêre benadering waar kleinskaal wortel DNS ekstraksies en kwantitatiewe real-time PKR (qPKR) analise gebruik is. ‘n Grootskaalse wortel DNS ekstraksie en kwantitatiewe polimerase kettingreaksie (qPKR) analisemetode was minder effektief geag.

Dit het geblyk dat ‘n molekulêre kwantifiserings (qPKR) benadering oneffektief was om twee bestuurstrategieë te evalueer (fosfonaatbehandelings en onderstam bestandheid) in avokado-boorde wat geen duidelike bogrondse simptome van PW-agteruitgang getoon het nie. Hoewel wortelfosfiet (afbraakproduk van fosfonate) konsentrasies van ‘n 2x staminspuitingbehandeling wat teen die voorkomende dosis (0.3 g a.i./m^2) toegedien is, betekenisvol hoër was as die onbehandelde kontrole, was die Pc wortel- en grond DNS-konsentrasies nie betekenisvol deur die fosfiet verminder, relatief tot die onbehandelde

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kontrole nie. Dit was vir kwantifikasies wat óf in Mei óf in Oktober 2018 gedoen is, en deur die gebruik van die beste van die drie geëvalueerde Pc-spesifiek qPKR toetse. Die potensieel meer PW-bestande R0.06 onderstam het hoër Pc wortel DNS konsentrasies gelewer as die Dusa® onderstam in November 2017, maar nie in die ander twee monsterneming maande (Maart en Mei 2018) nie.

Die identifikasie van effektiewe monsternemingstrategieë, Pc wortelkwantifiseringsmetodes en Pc wortelkolonisasie patrone in avokado-boorde in die huidige studie is belangrik. Aangesien Mei die hoogste wortelkolonisasievlakke gehad het, behoort PW bestuurspraktyke in plek gestel te word om die optimale wortelbeskerming gedurende, of net voor, hierdie periode (láát herfs) te verkry. Die effektiewe monsterneming- en kwantifikasie-metodes wat geïdentifiseer is vir die bestudering van seisoenale wortelkolonisasie patrone in avokado, sal nuttig wees vir verdere studies wat op 5000 gasheerplantspesies van Pc uitgevoer word. Alternatiewe kwantifikasiemetodes as qPKR om bestuurstrategieë te evalueer, moet ondersoek word. Dit is egter moontlik dat qPKR ontleding suksesvol kan wees vir die evaluering van bestuurstrategieë indien daar verbeterings gemaak word aan die proef–ontwerp, en indien ontledings uitgevoer word in siek eerder as asimptomatiese boorde.

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CHAPTER 1

A review of Phytophthora root rot of avocado: the causal agent, its quantification, and management strategies

GENERAL INTRODUCTION

South Africa is considered as one of the largest producers of avocados (*Persea americana* Mill.) worldwide, while also being ranked amongst the top four largest exporters of avocados to Europe (Naamani, 2011). Approximately 55 000 tonnes of avocado fruit are exported annually, comprising roughly 50% of the total yield (Donkin, 2016). Fruit which are not exported are consumed locally, of which, 10% is further processed into avocado oil and guacamole (Donkin, 2007). Production areas are mainly concentrated in the warm subtropical regions of the Limpopo (60%) and Mpumalanga (29%) provinces; however, approximately 8% of production is also situated in KwaZulu-Natal, with the remaining 2% distributed between the Eastern and Western Cape (SAAGA, 2018).

Of the diseases affecting avocados worldwide, Phytophthora root rot (PRR) is considered as being the most destructive (Darvas *et al.*, 1983). Initially, during the 1920s, symptoms of PRR were thought to be caused by poor soil aeration and waterlogging (Coffey, 1987), due to its close association with wet soil conditions. As a result, the disease was referred to as ‘avocado decline’ (Zentmyer, 1953), or ‘melanorhiza’ (Horne, 1934). It was only in 1928 when *Phytophthora cinnamomi* (Pc) was isolated from avocado for the first time in Puerto Rico (Tucker, 1928), that a biological agent was implicated as the cause of PRR. These findings were later supported by Wager (1931, 1942) who isolated Pc from symptomatic avocado feeder roots in South Africa and California, USA. In South Africa, PRR was particularly destructive during the 1970s, when an estimated 20% of avocado trees were affected by PRR (Milne and Chamberlain, 1971). High soil temperatures, high summer rainfall and low soil calcium levels, inherent in South African avocado growing regions, are key factors that are known to contribute towards high PRR severity (Zentmyer, 1980).

Phytophthora cinnamomi primarily infects the white fleshy feeder roots of avocado, which subsequently turn necrotic and brittle, and, as a result, start to break away from the root system (Zentmyer, 1980). As the feeder roots are a critical means of nutrient and water uptake (Wolstenholme, 1981), symptoms are typically those of water-stress and nutrient deficiency. This includes stunted tree growth, chlorotic and abnormally small leaves, large numbers of small fruit, as well as premature leaf abscission which leads to a sparse tree canopy (Pérez-Jiménez, 2008). Low-hanging fruit are also at risk of developing rot due to pathogen infection from rain splash or direct contact with infested soils (Pérez-Jiménez, 2008). Cankers may

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develop on avocado tree trunks; however, this is more common in tropical production regions (Crandall, 1948). A decline in tree vigour and productivity typically occurs over a few years, leading to the eventual death of the tree (Zentmyer, 1953); however, a rapid decline is also known to occur in younger trees (Wager, 1942).

Management of PRR requires a comprehensive integrated management strategy that is best summarised by what is known as the 'Pegg wheel' (Wolstenholme and Sheard, 2010). The 'Pegg wheel' was developed by Ken Pegg in Australia and focuses on six main principles including soil selection, irrigation management, chemical control, inorganic nutrition, organic amendments and tolerant rootstocks. The combination of approaches is aimed at maintaining tree health and minimising the effects of pathogen colonisation (Wolstenhome and Sheard, 2010).

Two aspects of the 'Pegg wheel' that are most often applied by South African avocado growers include the use of phosphonate fungicides and tolerant rootstocks. Phosphonate fungicides, which are highly mobile in trees, are often applied twice a year during the two root flush windows as a preventative management strategy. However, due the enforcement of strict maximum residue limits (MRLs) (50 mg/kg) by the European Union (EU) for avocado fruit exports in 2014, the use of phosphonates has become problematic. Several fruit consignments have exceeded the MRL, irrespective of phosphonate applications abiding by the registered label recommendations (McLeod *et al.*, 2018). The use of phosphonates and/or their time of application will thus need to be reconsidered. South Africa has been a leader in developing PRR-tolerant rootstocks, of which, Dusa® is best known. Dusa® comprised approximately 50% of the total nursery sales made by South African Avocado Nurserymens' Association members during 2009 and 2010 (Retief, 2011), indicating its relevance as a PRR management tool. However, mechanisms associated with PRR tolerance in Dusa®, and several other PRR-tolerant rootstocks, remain controversial.

Several factors are known to affect pathogen infection and colonisation; having either a direct or indirect influence on the severity of disease development. Soil moisture and temperature are considered the most important factors, as they directly stimulate the destructive asexual life cycle stages (sporangial production and zoospore release) of the pathogen (Hardham, 2005; Hardham and Blackman, 2018). Optimal conditions for pathogen colonisation and disease development typically include high soil moisture levels and warm soil temperatures of between 24-27°C (Zentmyer, 1980; Nesbitt *et al.*, 1979). Interactions surrounding disease development are very complex, and several other factors also need to be considered. For example, the susceptibility of different rootstock varieties to pathogen colonisation due to differences in the chemotactic effect of root exudate composition on zoospore attraction, as well as biochemical and physical defence mechanisms (Kellam and Coffey, 1985; Botha *et al.*, 1989; Engelbrecht *et al.*, 2013; Van den Berg *et al.*, 2018), the

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effect of soil types in promoting optimal soil environments for pathogen infection (Ploetz and Schaffer, 1988), weakened plant defence responses due to plant stress factors (Chang-Ho and Hickman, 1970; Allen and Newhook, 1973; Blaker and MacDonald, 1986; Drew, 1997; Sanclemente *et al.*, 2014), isolate virulence (Dudzinski *et al.*, 1993; Linde *et al.*, 1999), and tree phenological events, such as root flush windows, which may encourage increased pathogen activity and infection (Ploetz *et al.*, 1992).

Pathogen quantification is an essential means for estimating pathogen levels within orchards, therefore making it a key tool in obtaining a better understanding of the factors influencing root colonisation, as well as for optimising the efficacy of management practices. In addition, pathogen quantification can help improve knowledge on pathogen behavioural patterns to assist in predicting critical periods of pathogen colonisation or disease development (Zentmyer, 1981; Shearer and Shea, 1987; Dirac *et al.*, 2003; Shearer *et al.*, 2010). Pathogen quantification, using either soil or roots, typically involves conventional isolation methods such as baiting techniques (Darvas, 1979; Tsao, 1983; Erwin and Ribeiro, 1996; Eden *et al.*, 2000) and direct plating methods (Johnson and Curl, 1972; Shew and Benson, 1982; Hüberli *et al.*, 2001), or molecular methods such as quantitative real-time PCR (qPCR) (Eshraghi *et al.*, 2011a).

This review presents an overview of PRR in avocado including aspects related to the host, pathogen and environment. The life cycle and distribution of the pathogen is discussed, with emphasis placed on factors that affect pathogen infection and colonisation. The review furthermore discusses pathogen identification methods, including morphological, immunological and molecular identification approaches, as well as quantification methods, including conventional and molecular methods. Lastly, a general discussion elaborates on the six main principles behind disease management; however, an emphasis is placed on phosphonates, their application methods, translocation and mode of action, as well as the history of rootstock selection in South Africa and possible mechanisms behind rootstock tolerance. The conclusion section highlights the most important aspects of the review and indicates the scope of this thesis.

THE AVOCADO TREE

Origin and Taxonomy

Avocado (*Persea americana* Mill.) is an evergreen tree that is indigenous to the tropical and subtropical regions of Mexico, Guatemala and Central America (Storey *et al.*, 1986; Bergh, 1992). The genus *Persea* belongs to the Lauraceae family, which comprises approximately 50 genera and 2500 species (Rohwer, 1993). *Persea americana* Mill. is a polymorphic species which can be subdivided into three horticultural races including Mexican, Guatemalan and West Indian (Popenoe, 1941; Storey *et al.*, 1986), with further classifications into the varieties,

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'var. *drymifolia*', 'var. *guatemalensis*', and 'var. *americana*', respectively (Bergh and Ellstrand, 1986). These three horticultural races hold significance as most commercial avocados exist due to extensive outcrossing and backcrossing between the races (Popenoe and Williams, 1947). The ease of interracial hybridisation in *P. americana* Mill. has been particularly beneficial in terms of enabling the selective breeding of desirable agronomic traits (Davis *et al.*, 1998; Ashworth and Clegg, 2003). Examples of such racial hybrids include Hass® and Fuerte which are both of Guatemalan and Mexican descent (Crane *et al.*, 2013); however, Hass® is predominantly Guatemalan, and Fuerte is predominantly Mexican (Chen *et al.*, 2008).

Tree phenology

Mature avocado trees, in general, exhibit rhythmic vegetative growth which typically involves alternations between shoot and root flushes (Wolstenholme, 1981; Whiley, 1994). However, there are exceptions, since Thorp *et al.* (1995) found that root growth coincided with shoot growth in avocado production regions in New Zealand. The frequency of vegetative flushes depends heavily on the climatic region (Kaiser and Wolstenholme, 1993; Ploetz *et al.*, 1992; Arpaia *et al.*, 1994) and nature of carbohydrate partitioning within the tree (Wolstenholme and Whiley, 1989), as a response to environmental changes (Whiley *et al.*, 1988), reproductive development (Davenport, 1982), and feedback interaction between the shoots and roots (Wolstenholme, 1981). A variety of factors can also influence the extent of growth during vegetative flushes, including soil nutrients (Wolstenholme, 1981), scion and rootstock variety (Mickelbart *et al.*, 2012), as well as soil type and irrigation practices (Salgado and Cautín, 2008).

In the subtropical regions of Queensland, Australia, similar to avocado growing regions in Limpopo, South Africa, two distinct root flushes occur annually; one from late spring to early summer and the other from autumn to early winter. The latter root flush is considered as being the main root flush period (personal communication E. Dann, Queensland University, Australia). However, different observations have been reported for the cooler avocado growing regions of KwaZulu Natal, South Africa, where Kaiser and Wolstenholme (1993) observed only one root flush which extended from early autumn into winter. Ploetz *et al.* (1992) found that avocado roots actively grew throughout the year in tropical regions of Florida, USA, however, this study used young avocado seedlings and it is thus possible that the seedlings had not yet developed the typical rhythmic growth cycles of mature trees (Whiley, 1994). Studies in semi-arid regions of California, USA, also observed two distinct annual root flushes (Arpaia *et al.*, 1994), as well as active root growth throughout the year (Mickelbart *et al.*, 2012). In Chile, similar bimodal periodicity in root growth patterns were found in the avocado growing regions (Hernández, 1991), however, Salgado and Cautín (2008) only observed one root flush during the autumn season.

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Root morphology

The avocado tree typically has a shallow root system, with the majority of its white fleshy feeder roots concentrated within the top 15-60 cm of soil (Gregoriou and Kumar, 1982; Salazar-García and Cortés-Flores, 1986; Michelakis *et al.*, 1993). This shallow feeder root distribution, which predominantly consists of low-absorbent suberised roots, subjects avocado to increased flood-sensitivity (Sanclemente *et al.*, 2014). The avocado root system does not contain any root hairs (Burgis and Wolfe; 1945; Ginsburg and Avezohar-Hershenson; 1965; Wolstenholme, 1981; Gregoriou and Kumar, 1982), and therefore relies fully on the feeder roots for adequate water and nutrient absorption (Coit, 1940; Wolstenholme, 1981). The feeder roots vary in thickness, with the majority of roots having diameters of 1-2 mm (Gregoriou and Kumar, 1982; Scora *et al.*, 2002), although diameters of up to 8 mm have also been reported (Salazar-García and Cortés-Flores, 1986). These variations could be explained by both soil type (Donnelly, 1941) and scion/rootstock combinations (Aloni *et al.*, 2010). Scion/rootstock interactions play a critical role in determining the nature of root morphology since auxins that are produced by the shoots are translocated to the roots for normal root function (Aloni *et al.*, 2010).

Spatial distribution of the root system

Avocado root distribution is greatly influenced by soil texture and irrigation practices (Durand and Claassens, 1987; Michelakis *et al.*, 1993; Salgado and Cautín, 2008), soil profile uniformity (Durand and Claassens, 1987), as well as cultivar types (Salazar-García and Cortés-Flores, 1986). The water holding capacity of the soil, in combination with the irrigation method, determines soil aeration levels and subsequently the area for optimal root growth (Michelakis *et al.*, 1993; Salgado and Cautín, 2008). Since avocado is a water-sensitive crop (Wolsteholme, 1981; Schaffer *et al.*, 1992), a well-aerated soil is essential for healthy root development. When comparing the effects of irrigation methods on root distribution, Salgado and Cautín (2008) found that avocado roots concentrated away from the emitters for micro-sprinkler irrigation, and beneath the emitters for drip irrigation. This phenomenon is caused by the combination of varying soil moisture accumulations associated with each irrigation method, and the sensitivity of avocado roots to the resulting soil aeration levels.

In fine-textured soils (i.e. clay soils) under micro-sprinkler irrigation, Salgado and Cautín (2008) found that the greatest concentration of avocado roots was located approximately 200 cm from the trunk and 50-60 cm deep, while in coarse-textured soils (i.e. sandy soils), this was 30 and 25 cm, respectively. Salgado and Cautín (2008) also observed that, under drip-irrigation, over 25% more roots developed in fine-textured soils than coarse-textured soils. Contrarily, Donnelly (1941) found that in fine-textured soils there was a general reduction in the number, yet increased thickness, of feeder roots. Salazar-García and Cortés-Flores (1986)

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attributed the reduced root growth in fine-textured soils to the negative impacts of soil compaction including both direct growth hindrances due to impenetrable soil layers, as well as waterlogging effects. In addition to soil textural class, profile uniformity was found to play a major role in avocado root distribution (Durand and Claassens, 1987), as avocado roots do not readily penetrate from one soil texture and structure into another. In deep uniform soils, even root distribution was found to occur at depths of up to 1.2 and 2.1 m (Correa *et al.*, 1984).

PHYTOPHTHORA CINNAMOMI

Origin and Taxonomy

Phytophthora cinnamomi is a soilborne oomycete that belongs to the Phylum Oomycota (Cooke *et al.*, 2000). Oomycetes are behaviourally similar to fungal organisms due to their fungal-like hyphae and nutrient acquisition (Hardham, 2005); however, they are phylogenetically very distant from the Kingdom Eumycota. Furthermore, organisms belonging to the Phylum Oomycota produce biflagellate, heterokont zoospores, therefore forming part of an assembly called Stramenopiles (Hardham, 2005; Tyler *et al.*, 2006; Beakes *et al.*, 2012).

The origin of Pc has been a matter of debate; however, Papua New Guinea is likely to be the centre of origin. Papua New Guinea was found to contain Pc populations with the greatest genetic diversity when several worldwide Pc populations were studied (Old *et al.*, 1988; Dobrowolski *et al.*, 2003). Studies on the population genetic structure of Pc in Australia, South Africa and other areas of the world, showed that low levels of genetic diversity occurred in populations outside of Papua New Guinea since only three clonal lineages were identified (Linde *et al.*, 1999; Dobrowolski *et al.*, 2003). However, variations within single clonal lineages were also found, with variation most likely arising from mitotic recombination through asexual growth and development (Hüberli *et al.*, 2001; Dobrowolski *et al.*, 2003). In a recent South African study by Engelbrecht *et al.* (2017), the clonality and lack of sexual reproduction in Pc populations were confirmed through analyses of 211 isolates from avocado orchards in the Limpopo and Mpumalanga production regions. The study used newly developed additional simple sequence repeat markers, which confirmed the clonality of the South African Pc populations since only two or three clonal lineages were evident (Engelbrecht *et al.,* 2017).

Lifestyle

Phytophthora cinnamomi has been reported as both a biotrophic (Crone *et al.*, 2013a, b) and necrotrophic pathogen (Cahill *et al.*, 2008), but is essentially considered as a hemibiotroph (Davison *et al.*, 1994; Shearer and Crane, 2012). During the biotrophic phase, Pc produces haustoria to gain nutrients from the host cells (Crone *et al.*, 2013b), with infection taking place in seemingly asymptomatic tissue (Davison *et al.*, 1994; Hüberli *et al.*, 2000). The pathogen then converts to necrotrophy, where the secretion of elicitins known as α- and β-cinnamomins

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(Duclos *et al.*, 1998; Horta *et al.*, 2008) are thought to play a major role in the induction of necrosis in the *Phytophthora*-host interaction (Kamoun, 2007).

Life cycle

Phytophthora cinnamomi produces three types of spores that are crucial for disease development and pathogen survival, including zoospores, oospores and chlamydospores (Zentmyer, 1980). The pathogen grows vegetatively as mycelium, producing hyphae with few or no septa (O'Brien and Hardy, 2014). Asexual reproduction involves the differentiation of these hyphae into sporangia in the presence of free water (Hardham, 2005; Hardham and Blackman, 2018). The sporangia form motile zoospores by means of compartmentalisation (Hyde *et al.*, 1991), with zoospore release likely being driven by the build-up of hydrostatic pressure in the sporangium (Gisi *et al.*, 1979). With the assistance of flagella, zoospores are able to actively swim over short distances to new host infection sites with the aid of chemo- and electrotaxis (Khew and Zentmyer, 1973, 1974). The zoospores encyst once contact is made with the host surface, subsequently forming germ tubes and producing penetration hyphae, before entering the root epidermis (O'Brien and Hardy, 2014). Thereafter, the pathogen invades the root cortex both inter- and intracellularly (Oßwald *et al.*, 2014), however, it is limited in its ability to invade further into the host's suberised roots (Zentmyer, 1980). Secondary sporangia and zoospores are also known to develop on root surfaces when free water becomes available, thus leading to multiple cycles of infection (Oßwald *et al.*, 2014).

Adverse conditions, such as mycelium starvation (Bartnicki-García and Wang, 1983), trigger the pathogen to produce survival spores known as chlamydospores, which can be either thick- or thin-walled (Zentmyer and Mircetech, 1966; Crone *et al.*, 2013a). These chlamydospores develop in living roots and are released into the soil as the roots begin to decay (Marais *et al.*, 2002; O'Brien and Hardy, 2014). The pathogen has been found to produce both thin-walled (< 1 µm) (Rands, 1922; Hemmes and Wong, 1975; Jung *et al.*, 2013) and thick-walled (> 1 µm) chlamydospores (Shew and Benson, 1982; Crone *et al.*, 2013b; Jung *et al.*, 2013; McCarren, 2006). However, the stimulation mechanisms behind the formation of thick- and thin-walled chlamydospores are not yet fully understood (McCarren *et al.*, 2005). *In vitro* studies done by Hemmes and Wong (1975), found that the thickness of chlamydospore walls can increase from 0.2 µm to 0.5-0.6 µm over a 2-week period in pure cultures, suggesting that wall thickness may be age-related. This was similar to findings for *P. ramorum*, where the chlamydospore wall thickness increased from 0.5 µm to 4.0 µm in 120-day old cultures grown in V8 broth (Smith, 2007). From these findings, it could be argued that chlamydospore wall thickness is dependent on the duration of chlamydospore exposure to specific chemical stimuli. Smith (2007) furthermore found that thin-walled chlamydospores of *P. ramorum* were more likely to germinate, suggesting that thick-walled chlamydospores have

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greater involvement in pathogen dormancy, while thin-walled chlamydospores may develop as a response to short-term fluctuations in adverse conditions.

The long-term survival of Pc in soil and root fragments are thought to be mediated by the formation of chlamydospores and/or stromata. *Phytophthora cinnamomi* can survive in the form of chlamydospores for 2 months up until six years (Zentmyer and Mircetech, 1966; Hwang and Ko, 1978; Brasier *et al.*, 1993; Pérez-Jiménez, 2008), until favourable conditions such as adequate exogenous nutrients (Mircetich *et al.*, 1968), stimulate chlamydospore germination and subsequently sporangial formation (Hwang and Ko, 1978). Apart from chlamydospores, it has also recently been found that Pc produces asexual survival structures known as stromata (Crone *et al.*, 2013b; Jung *et al.*, 2013). Stromata are dense intermingled hyphal aggregations that, once germinated, form multiple germ tubes that are able to produce chlamydospores and selfed-oospores (Crone *et al*., 2013b).

Phytophthora cinnamomi is considered to be heterothallic, requiring both A1 and A2 mating types for sexual reproduction and subsequent oospore formation (Galindo and Zentmyer, 1964). Oospore production is initiated by the induction of gametangial formation, through chemical exchanges made between the A1 and A2 mating types (Brasier, 1972). The oosphere within the oogonium is fertilised by the nucleus of the antheridium (Erwin and Ribeiro, 1996), with Pc producing both paragynous and amphigynous antheridia (Hüberli *et al.*, 1997). This leads to the production of thick-walled, resilient oospores which aid in the survival of the pathogen (Crone *et al.*, 2013b).

The A2 mating type dominates in the majority of the avocado growing regions around the world, while the A1 mating type has a much more limited distribution, having only been found in Papua New Guinea, China, Taiwan, South Africa, Madagascar, Australia and California, USA (Oudemans and Coffey, 1991). Consequently, oospores are not considered as being of major importance in the survival of the pathogen. In the Limpopo and Mpumalanga avocado production regions of South Africa, only the A2 mating type has been identified (Engelbrecht *et al.*, 2017). Although the A1 and A2 mating types have previously been isolated from the same area, there is still a lack of genetic evidence supporting sexual recombination in natural populations (Dobrowolski *et al.*, 2003).

Phytophthora cinnamomi has also been reported as being homothallic, producing selfed-oospores when given chemical stimuli (Brasier, 1971; Pratt *et al.*, 1972; Brasier, 1975; Zentmyer, 1979; Jayasekera *et al.*, 2007) or through mechanical damage (Reeves and Jackson, 1974). Selfed-oospore production was initiated in the A2 mating type of Pc in the presence of oleic acids from avocado root extracts (Zentmyer, 1979), unidentified volatile compounds produced by *Trichoderma viride* (Brasier, 1971; Brasier, 1975) and *T. koningii* (Pratt *et al.*, 1972), as well as *in planta* stimulation in *Acacia pulchella* (Jayasekera *et al.*,

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2007). Consequently, it is currently uncertain as to whether oospores, produced through selfing, play an important role in the survival of the pathogen.

Factors affecting root colonisation

The main abiotic and biotic factors affecting Pc root colonisation, that will be discussed in this section, include chemo- and electrotaxis, soil type, soil moisture and temperature, isolate virulence (severity of disease caused) and plant stress. Although several rootstock attributes, such as root exudate composition, root regenerative ability, structural barrier formation and biochemical defence responses can also affect root colonisation, these aspects are not discussed in this section but rather under the “Mechanisms associated with PRR tolerance” section of this literature review.

Chemotaxis and electrotaxis

Chemotaxis plays an essential role in enabling zoospores to locate new host infection sites (Khew and Zentmyer, 1973) and involves the movement of zoospores in response to a gradient of increasing concentration of stimulative chemicals such as root exudates (Dukes and Apple, 1961; Ho and Hickman, 1967; Zentmyer, 1970). Zentmyer (1961) found a noticeable increase in zoospore attraction of Pc to the elongation zone of avocado roots; a major site for root exudation (Pearson and Parkinson, 1960). Root exudates are mostly made up of organic compounds including amino acids, organic acids, sugars, phenolic acids and various secondary metabolites (Badri and Vivanco, 2009). Different root exudates were found to have varying chemotactic influences on *Phytophthora* spp. (Zentmyer, 1966; Bimpong and Clerk, 1970; Khew and Zentmyer, 1973). Aspartic acid and glutamic acid are both amino acids that were found to have the greatest chemotactic attraction for Pc (Khew and Zentmyer, 1973). As the concentration and composition of root exudates can vary between plant species and cultivars (Uren, 2000; Botha *et al.*, 1989), this would likely be a determining factor for cultivar susceptibility to root colonisation (Lapin and Van den Ackerveken, 2013).

Electrotaxis refers to the electrical currents and voltages that are present in the rhizosphere, and which have an influence on zoosporic behaviour. Protons and other ions generated by the electrical currents lead to the creation of ionic and pH gradients, which in turn result in either the repulsion or attraction of zoospores. Electric currents are present in the apical root region (root cap, meristematic and elongation zones) as well as at wound sites. The attraction of *P. palmivora* to root tips but not wounds, was reported as being mediated by natural-root-generated electric fields (Van West *et al.*, 2002).

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Soil type

Soil types differing in terms of matric potentials, pH, pore sizes and organic content can influence zoospore motility and Pc colonisation. Sterne *et al.* (1977) observed that pathogen colonisation was hindered at a matric potential of -0.25 bar for sandy loam soils, while minimal differences were found between -0.25 bar and 0 bar for clay soils. In addition to matric potential, Young *et al.* (1979) identified soil pore size as having an effect on zoospore motility. Zoospores of Pc were able to passively pass through pore necks of 25-35 µm; however, these confined spaces resulted in a lack of chemotactic response (Young *et al.*, 1979). In contrast, Allen and Newhook (1973) suggested that soil pore sizes of 190 µm or lower would hinder the active movement of zoospores due to the nature of their helical motion, rather than a lack of chemotactic response. Premature zoospore encystment may also be encouraged by the collision of zoospores with solid soil surfaces (Ho and Hickman, 1967), which may occur more frequently in soils that are inclined to faster soil drainage (i.e. sandy soils). Soils with higher clay content, especially in subsoil layers, are also more prone to waterlogging conditions which can exacerbate PRR development (Ploetz and Schaffer, 1988; Reeksting *et al.*, 2014a, b). Zentmyer (1976) found that soil pH influenced pathogen colonisation, with a soil pH of 6.5 being indicated as optimal for PRR development in avocado. In addition, soil factors, such as high organic content, can indirectly suppress Pc colonisation by providing an environment supportive of microbial communities antagonistic towards the pathogen (Broadbent and Baker, 1974; Broadbent *et al.*, 1989; Bonanomi *et al.*, 2007).

Soil moisture and temperature

Soil moisture and temperature are considered as the most important factors affecting Pc colonisation (Zentmyer, 1980), as they play key roles in providing reproductive development and survival strategy cues for the pathogen (Nesbitt *et al.*, 1979). Free water is essential for the stimulation of sporangial production as well as for zoospore release and dissemination (Hardham, 2005; Hardham and Blackman, 2018); both critical events in disease development (Zentmyer, 1980). Soil moisture, however, works in close association with soil temperature, and sporangial production may not be stimulated if the soil temperatures are not within the optimal pathogenicity range (Nesbitt *et al.*, 1979), regardless of free water availability. Nesbitt *et al.* (1979) found that maximal sporangial production of Pc occurred at 24˚C, while Zentmyer (1981) found that the highest level of Pc colonisation took place between 24-27 ˚C, noting that minimal pathogen colonisation took place below 15˚C or above 33˚C. Soil temperature is also critical since most oomycetes require cold shock treatments with temperatures as low as 12˚C to allow for the efficient production of zoospores (Ribeiro, 1983). However, this may vary among *Phytophthora* spp., as a temperature of 15°C was reported for the cold shock treatment

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of Pc (Khew and Zentmyer, 1973). Cold shock treatment initiates cytokinesis of the sporangial cytoplasm to compartmentalise single nuclei into each zoospore (Chen and Zentmyer, 1970).

Despite the requirement of free water for asexual reproduction, the pathogen still requires sufficient soil oxygen levels to survive (Dann *et al.*, 2013). This was confirmed by Nesbitt *et al.* (1979) who found that periods of prolonged waterlogging resulted in reduced disease development. Instead, Pc is favoured by short periods of soil saturation with aerated water, in combination with warm soil temperatures (Zentmyer, 1980; Dann *et al.*, 2013).

Seasonal variations in soil moisture and temperature can influence pathogen colonisation (Zentmyer, 1981), however, due to irrigation practices, soil moisture is usually not a limiting factor in commercial avocado production (Zentmyer and Richards, 1952). Zentmyer (1981) found that the highest level of Pc colonisation took place during the autumn season, which coincided with the cooling down of soil temperatures. Studies that were done on other *Phytophthora* spp. likewise found seasonal variations in pathogen colonisation, with *P. nicotianae* var. *parasitica* showing its greatest level of colonisation in summer roots of citrus, and *P. citrophthora* in winter roots, using excised root experiments (Dirac *et al.*, 2003). These findings were indirectly linked to soil temperature, with root colonisation having been influenced by phenological-related changes in starch and glucose composition of the roots (Dirac *et al.*, 2003).

Isolate virulence

Variation in virulence within Pc populations were discovered amongst several host species, including cinnamon (Rands, 1922), avocado (White, 1937), *Eucalyptus* spp. (Dudzinski *et al.*, 1993; Linde *et al.*, 1999), chestnut and red oak (Robin and Desprez-Loustau, 1998). Dudzinski *et al.* (1993) performed pathogenicity trials on *Eucalyptus marginata* using 42 Australian isolates, where noticeable variations were observed according to each isolate's extent of root damage. Linde *et al.* (1999) reported similar findings when comparing the pathogenicity of 59 South African isolates on *Eucalyptus smithii*. In addition, a positive correlation was observed between the *in vitro* growth rate of each isolate and their resulting virulence in field inoculation trials, with the fastest growing cultures showing the greatest virulence (Linde *et al.*, 1999). Contrarily, Hüberli *et al.* (2001) found no significant relationship between radial growth rates and the pathogenicity of the 73 isolates that were tested on *E. marginata* and *Corymbia calophylla*. Studies by White (1937) compared the pathogenicity of rhododendron and cinnamon isolates, with that of an avocado isolate, and found that the avocado isolate was less virulent on avocado. From these findings it could be suggested that root colonisation may differ according to the virulence of the Pc isolates present in avocado orchards.

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Plant stress

Environmental stresses, such as waterlogged conditions (Allen and Newhook, 1973; Sanclemente *et al.*, 2014), root wounds (Chang-Ho and Hickman, 1970; Drew, 1997) and salt stress (Blaker and MacDonald, 1986), place the plant under greater susceptibility to pathogen attack due to weakened plant defence responses. When soil becomes hypoxic or anoxic, the roots shift to anaerobic respiration, which leads to an increased production of ethanol (Kennedy *et al.*, 1992). Ethanol exuded by flood-stressed avocado roots was found to be a major chemical attractant for zoospores of Pc (Allen and Newhook, 1973). Anaerobic respiration can also create a build-up of lactic acid which can reduce the pH levels in root cells thus leading to cell death (Drew, 1997). This can subsequently exacerbate host susceptibility to disease development (Drew and Lynch, 1980). Soil salinity has been linked to increases in the susceptibility to PRR development in both citrus (Blaker and MacDonald, 1986) and Chrysanthemum (MacDonald, 1984) varieties. Blaker and MacDonald (1986) suggested that this may be due to either increased tissue susceptibility to pathogen infection or the inhibition of root growth and root regeneration due to a reduction in nutrient and water uptake by the plant. As avocado is a salt-sensitive crop (Ben Ya'acov and Michelson, 1995; Bernstein *et al.*, 2004), it would most likely be subject to similar disease development responses.

Pathogen dissemination

Phytophthora cinnamomi can disseminate by movement of soil particles containing either isolated propagules such as sporangia, chlamydospores and mycelial fragments, or detached diseased roots (Zentmyer, 1980). Dissemination is assisted by soil water movement which involves both sub-soil water drainage and surface run-off containing contaminated soil (Shea *et al.*, 1983; Marais and Hattingh, 1985; Kinal *et al.*, 1993; Grant and Barrett, 2001). Soils with poor water drainage can favour horizontal dissemination of Pc. For example, Weste and Taylor (1971) found that pathogen spread reached distances of up to 7 m per month in poorly drained soils, whereas for well-drained soils this was only 4 m per month. It has been noted, however, that the pathogen's horizontal distribution is typically very erratic (Pryce *et al.*, 2002).

Due to the premature encystment associated with solid surface collisions (Ho and Hickman, 1967), zoospores are most likely not associated with long-distance subsoil dispersal. Instead, zoospores can disperse by means of rain splash from Pc-infested soils (Kliejunas and Ko, 1976), where they may occasionally infect and colonise low-hanging avocado fruit resulting in seed transmission (Zentmyer, 1980; Pérez-Jiménez, 2008). Zoospores are mainly associated with short-distance dispersal which is driven by chemotaxis (Khew and Zentmyer, 1973). Pc soil propagules can also disseminate through human and animal activities (Ristaino and Gumpertz, 2000), by movement of infested soil attached to farm equipment, vehicle tyres, footwear and hooves of invertebrates (Kliejunas and Ko, 1976).

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In addition to pathogen dissemination through infested soil movement, Pc can spread through infected nursery trees and root-to-root contact. Zentmyer (1980) was one of the first researchers to recognise the importance of infected nursery trees and their link to widespread PRR incidence (Zentmyer, 1980). Furthermore, several researchers have also acknowledged that root-to-root contact made between infected and non-infected crops, can contribute to pathogen spread of Pc and other *Phytophthora* spp. (Shew, 1987; Hill *et al.*, 1994).

Phytophthora cinnamomi has been isolated from different water sources, which may account for its significantly widespread distribution. In South Africa, USA, Australia and China, Pc has been detected in natural waterways, reservoirs and irrigation channels (Kliejunas and Ko, 1976; Palzer, 1980; Von Broembsen, 1984; MacDonald *et al.*, 1994; Lauderdale and Jones, 1997; Oudemans, 1999; Von Broembsen and Charlton, 2000; Themann *et al.*, 2002; Smith *et al.*, 2009; Zeng *et al.*, 2009).

Spatial distribution of *Phytophthora cinnamomi* in soil

Several studies have investigated the spatial distribution of Pc in soil, to help predict high risk areas and prevent further spread of the pathogen (Weste *et al.*, 1973; Shea *et al.*, 1983; Marais and Hattingh, 1985; Pryce *et al.*, 2002; Shearer *et al.*, 2010; Shearer, 2014). From these studies it was reported that the spatial distribution of Pc in soil can be greatly influenced by both soil type (Weste *et al.*, 1973) and climatic region (Shearer *et al.*, 2010).

The distribution of Pc at the soil surface can vary, which is most likely due to differences in soil temperature and moisture levels. Shearer and Shea (1987) observed an increase in pathogen population densities in surface soil in diseased *E. marginata* sites situated in the Mediterranean regions of south-western Australia, during the higher winter rainfall periods. Similarly, Shea *et al.* (1980) found that in *E. marginata* sites in south-western Australia, the highest recovery of Pc from surface soil occurred during spring, when soil temperature and moisture levels were both higher. Conversely, Pc populations were lowest when the surface soil was exposed to harsher environmental conditions during the hot, dry summer (Shearer *et al.*, 2010).

In terms of vertical pathogen distribution, most studies have found that the highest soil inoculum levels are present within the upper soil layers (0-300 mm) but that the specific depths containing the highest Pc populations varied. *Phytophthora cinnamomi* was most frequently isolated from soil depths of 160-240 mm under *Eucalyptus* spp. located in the Mediterranean regions of south-western Australia (Weste *et al.*, 1973), and 240-320 mm from vineyards established in the Mediterranean climates of Western Cape, South Africa (Marais and Hattingh, 1985). In semi-arid regions in California(USA) Pc was isolated most frequently in the upper soil layers in avocado orchards (0-50 mm), although it could also be isolated in deeper soil layers (600-1050 mm) (Brodrick *et al.*, 1976). The variability in reports on the specific

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depths at which Pc is most prevalent, is likely due to the fact that soil type and drainage can influence vertical movement of the pathogen. Coarse-textured soils have a greater range of vertical pathogen distribution, compared to less permeable fine-textured soils, due to improved soil water drainage to deeper soil regions (Weste *et al.*, 1973).

Several studies have focused on the isolation of *Phytophthora* spp. populations from rhizosphere soil. Marais and Hattingh (1985) found that Pc soil inoculum levels were greatest near the root zone of grapevines (Marais and Hattingh, 1985). In addition, several other studies involving various *Phytophthora* spp. including *P. gallica* sp. nov. (Jung and Nechwatal, 2008), *P. quercina*, *P. cactorum* and *P. cambivora* (Jönsson *et al.*, 2003), have targeted rhizosphere soil for soilborne pathogen isolation experiments due to the typically high pathogen inoculum levels found in the rhizosphere.

IDENTIFICATION AND DETECTION OF *PHYTOPHTHORA CINNAMOMI*

Morphological

Phytophthora cinnamomi can be identified morphologically, from pure cultures, through it's sporangial, antheridial, oogonial and mycelial characteristics (Waterhouse *et al.,* 1983). However, it is most commonly identified through its globular hyphal swellings that create a distinctive coralloid-type appearance. In addition, its mycelial growth develops a camelloid or rosette-type pattern when grown on PDA (potato dextrose agar) medium (Dann *et al.*, 2013). Although the key hyphal growth characteristics of Pc are useful for identification, they cannot be the only characteristics used for identifying the pathogen since phylogenetically closely related species, such as *P. parvispora* (Scanu *et al.*, 2014) and *P. niederhauserii* (Abad *et al.*, 2014), have similar mycelial growth characteristics. Using sporangial, antheridial and oogonial characteristics to differentiate Pc from closely related species can also be difficult due to variation occurring between isolates of the same species, as well as when isolates are exposed to different growing conditions (Waterhouse *et al.*, 1983; Daniel *et al.*, 2003; Scanu *et al.*, 2014). Morphological identification can also be labour-intensive and time-consuming when identifying numerous isolates (Tsao, 1990; Dobrowolski and O'Brien, 1993).

Immunological

Immunological assays that can be used for the detection and identification of *Phytophthora* spp. include antibody-based assays such as zoospore trapping immunoassays, enzyme-linked immunosorbent assays (ELISA), and immunological dipstick assays (Gabor *et al.*, 1993; Cahill and Hardham, 1994, Pettitt *et al.*, 2002). Studies have shown that a positive correlation exists between pathogen detection using *Phytophthora* species-specific monoclonal antibodies (MAbs) and pathogen detection using PCR or conventional isolation methods (MacDonald *et al.*, 1990; Kox *et al.*, 2007). However, inconsistencies with regards to false-

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negative and false-positive detections of antigens have been reported for some *Phytophthora* spp. using MAbs assays (MacDonald *et al.*, 1990). For example, false-positives have been reported for some commercial *Phytophthora* spp. assays due to cross-reactivity with *Phytophthora* or *Pythium* spp. (MacDonald *et al.*, 1990; Bulluck *et al.*, 2006; Kox *et al.*, 2007); making detection only partially *Phytophthora*-specific. In contrast to these reports, Hardham *et al.* (1986) were able to develop a species-specific antibody-based assay for the identification and detection of Pc from plant tissue and soils. However, this assay is not widely used by research groups other than that of Hardham, since it is not commercially available. Laboratories wanting to use the assay would have to be equipped to produce and purify the antibody from laboratory animals.

Molecular

Molecular identification can provide more rapid, efficient and accurate identification of *Phytophthora* spp. compared to morphological and immunological identifications (Choi *et al.*, 2015). Molecular identification techniques can include several approaches such as DNA sequence analysis of barcoding genes (Schena *et al.*, 2008; Robideau *et al.*, 2011; Choi *et al.*, 2015; Miles *et al.*, 2017), PCR restriction fragment length polymorphism (RFLP) analyses (Martin and Tooley, 2004) and species-specific primers (Kong *et al.*, 2003; Schena *et al.*, 2008; Langrell *et al.*, 2011). Species-specific primers can be used for the detection of specific species from environmental samples, whereas PCR-RFLP analyses require *Phytophthora* genus-specific primers for the same purpose, as will be discussed in the following sections.

Sequence identification

Three barcoding gene regions have been suggested for the identification of oomycetes, in pure culture, to species level. These include the internal transcribed spacer (ITS) region of the rDNA (Robideau *et al.*, 2011), and the cytochrome *c* oxidase 1 (*cox*1) and 2 (*cox*2) gene regions of the mtDNA (Martin and Tooley, 2004; Robideau *et al.*, 2011; Choi *et al.*, 2015; Miles *et al.*, 2017). The ITS region is currently the most commonly used gene region for the identification of oomycete pathogens (Robideau *et al.*, 2011). This is due to the ease of *Phytophthora* spp. PCR amplification using universal ITS primers, as well as it being a multicopy gene region (White *et al.*, 1990). Although the ITS region typically possesses conserved and variable sequences among oomycete pathogens, there are several cases where ITS sequences provided insufficient variability for phylogenetic distinction and the identification of closely related *Phytophthora* spp. (Cooke *et al.*, 2000; Martin and Tooley, 2003a, b; Schena *et al.*, 2006; Jung and Burgess, 2009). Similar to the ITS gene region, *cox1* and *cox*2 are multiple copy genes (Miles *et al.*, 2017). Due to the shorter sequencing region of the *cox*1 gene (± 650bp), in comparison to the ITS region (± 900 bp) (Cooke *et al.*, 2000),

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sequence alignment is a lot simpler and easier (Robideau *et al.*, 2011). Robideau *et al.* (2011) found that *cox*1 sequencing performed similarly to ITS sequencing in terms of specificity and therefore recommended that both be conducted simultaneously as a method for multilocus identification. Recently, Choi *et al.* (2015) compared the *cox1* and *cox*2 gene regions for species-level identification in oomycetes. The *cox*2 region had a higher nucleotide diversity and interspecific divergence, which resulted in improved PCR performance; thus, suggesting *cox*2 as the preferable barcoding gene region to use.

In addition to the aforementioned proposed oomycete barcoding genes, Schena *et al.* (2006, 2008) showed that the Ras-related *Ypt*1 protein gene sequence contains highly conserved intraspecific coding regions flanked by highly variable introns in the genus *Phytophthora*. The non-coding regions of the *Ypt*1 gene were reported as being sufficient for differentiating between almost all known *Phytophthora* spp., including several closely related species (Schena *et al.*, 2006), however, in some instances, it lacked intraspecific variability (Schena and Cooke, 2006).

Phylogenetic analysis using multi-gene sequences has placed Pc along with the *Phytophthora* spp. *P. alni*, *P. cajani*, *P. cambivora*, *P. cinnamomi* var. *robiniae*, *P. europaea*, P*. fragariae*, *P. melonis, P. niederhauserii*, *P. pistaciae, P. rubi, P. sinensis*, *P. sojae, P. uliginosa P. vignae*, and *P. parvispora* (syn. *P. cinnamomi* var. *parvispora*) in clade 7 of *Phytophthora* (Cooke and Duncan, 1997; Robideau *et al.*, 2011). *Phytophthora parvispora*, which is the closest relative of Pc, along with *P. niederhauserii* and Pc, are the only *Phytophthora* clade 7 species known to occur in South Africa (Bezuidenhout *et al.*, 2010; Spies *et al.*, 2011; Oh *et al.*, 2013).

PCR-RFLPs identification and detection

PCR restriction fragment length polymorphism (RFLP) analyses of specific gene regions can be used for molecular identification of *Phytophthora* spp. (Ristaino *et al.*, 1998). Gene regions that have been used for the development of PCR-RFLPs for a wide range of *Phytophthora* spp. include the ITS (Cooke and Duncan, 1997; Ristaino *et al.*, 1998; Drenth *et al.*, 2006) and *cox*1-2 spacer regions (Martin and Tooley, 2004). PCR-RFLP analysis involves the amplification of a selected gene region, followed by the digestion of the resulting PCR products with restriction enzymes. Subsequently, gel agarose electrophoresis allows for the separation and visualisation of species-specific sized fragment patterns that can be used for species identification (Ristaino *et al.*, 1998; Drenth *et al.*, 2006). The employment of universal *Phytophthora* genus-specific primers targeting the ITS region allowed for the use of PCR-RFLP for species-specific identification and detection of *Phytophthora* pathogens from environmental samples such as plants, soil and water (Drenth *et al.*, 2006).

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Species-specific primers

Several studies have been aimed at developing species-specific primers for the accurate identification and detection of Pc from environmental samples and pure cultures, using conventional PCR. These assays have targeted various gene regions including the ITS region (Williams *et al.,* 2009; Langrell *et al.,* 2011), *Ypt*1 gene (Schena *et al.*, 2008), cinnamomin gene (Coelho *et al.*, 1997) and the *Lpv* putative storage protein gene (Kong *et al.*, 2003). Kunadiya *et al.* (2017) recently re-evaluated several published Pc-specific assays, to confirm their species-specificity. Here, it was found that many of the published conventional PCR assays were not species-specific. This was due to the validation studies excluding closely related *Phytophthora* spp. such as *P. parvispora*, as well as other species belonging to *Phytophthora* clade 7, during specificity testing (Kunadiya *et al.*, 2017).

The multicopy ITS region was targeted by two studies for the development of Pc-specific conventional PCR assays. Langrell *et al.* (2011) designed a nested PCR assay, which was used in a touchdown nested multiplex reaction, to simultaneously and specifically detect Pc and *P. cambivora*. The nested PCR assay consisted of a first round of amplification, with universal fungal primers (ITS5-ITS4), after which, the PCR product was used in a second round of amplification with Pc-specific primers (PciF2-PciR2) and *P. cambivora*-specific primers (PcaFshort- PcaR), respectively (Langrell *et al.,* 2011). Williams *et al.* (2009) also designed a nested PCR targeting the ITS region where Pc-specific primers were used in the first (CIN3A-CIN4) and second (CIN3B and CIN2R) round of amplification. However, Kunadiya *et al.* (2017) later reported that the assay of Williams *et al.* (2009) was not specific to Pc, whereas the assay of Langrell *et al.* (2011) was. Although nested PCR is known to improve the sensitivity of assays (Hayden *et al.*, 2006), the incorporation of additional pipetting and amplification steps make nested PCR more laborious, increases the chance of PCR product contamination of samples, and can furthermore result in increased human error (Schena *et al.*, 2008).

The *Ypt*1 gene was targeted by a nested conventional PCR assay developed by Schena *et al.* (2008). A nested PCR approach was used since the *Ypt*1 gene is single copy (Chen and Roxby, 1996), and is therefore limited in its sensitivity of detection. The assay consisted of *Phytophthora* genus-specific primers (YPh1F and YPh2R) which were used in the first round of amplification, followed by a second round of amplification using the Pc-specific Ycin3F and Ycin4R primers. The Ycin3F and Ycin4R primers were confirmed to be Pc-specific by Kunadiya *et al.* (2017).

Coelho *et al.* (1997) developed species-specific primers (95.422 and 96.007) targeting the elicitin cinnamomin (*Cina-6a*) gene of Pc. The cinnamomin elicitin genes are putative sterol carrier proteins, which are also required for virulence (Hardham, 2018), and are thus also likely to occur in other *Phytophthora* spp. The *Cina*-6a gene assay was not tested by Kunadiya *et*

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al. (2017), however, since closely related species, such as *P. parvispora* and *P. niederhauserii*, were not included by Coelho *et al.* (1997) during specificity testing, the assay could potentially be non-specific.

Kong *et al.* (2003) designed two species-specific primer pairs (LPV2F-LPV2R and LPV3F-LPV3R) targeting the *Lpv* gene region. The LPV3 primers showed greater specificity and sensitivity compared to the LPV2 primers, which cross-reacted with *Pythium* spp. It was therefore recommended that the LPV3 primers should be used for detecting Pc from environmental samples, while the LPV2 primers could be used as an effective alternative for the identification of Pc from pure culture (Kong *et al.*, 2003). Engelbrecht *et al.* (2013) subsequently developed the LPV3 primer pair into a nested qPCR assay by designing primers LPV3NF and LPV3NR that anneal internal to the LPV3 primers. Kunadiya *et al.* (2017) tested the *Lpv* primers (LPV3NF, LPV3NR, LPV3F and LPV3R) in the nested assay that was developed by Engelbrecht *et al.* (2013) and found that the nested assay was non-specific for Pc.

QUANTIFICATION OF *PHYTOPHTHORA CINNAMOMI*

Conventional methods

Direct plating

Phytophthora cinnamomi can be quantified from infected roots by directly plating the roots onto semi-selective media (Hüberli *et al.*, 2000), and then conducting morphological or molecular identification (Marks and Kassaby 1974; Eden *et al.*, 2000; Kox *et al.*, 2007). *Phytophthora* can be quantified from root samples by expressing the number of infected roots as a percentage of the total number of roots plated (Mazzola *et al.*, 2002). The disadvantage of direct root plating is that smaller sample volumes are used compared to root baiting (see below), due to the labour-intensive nature of plating root segments. This ultimately reduces the likelihood of detecting pathogens at low population densities (Ferguson and Jeffers, 1999). False-negatives may also occur due to the failure of the pathogen to grow out from infected roots (O'Brien *et al.*, 2009). This could be as a result of growth inhibitory substances present on the external surface of the roots (Hüberli *et al.*, 2000), antagonistic microorganisms within the root tissue (Malaczjuk, 1983), inhibition by plant phenolics (Hüberli *et al.*, 2000), overgrowth by fast-growing *Pythium* spp. (Nechwatal *et al.*, 2001; Bush *et al.*, 2003), or chlamydospore dormancy (McCarren *et al.*, 2005).

Soil inoculum can be measured by conducting a standard serial soil dilution plate method using semi-selective media (Johnson and Curl, 1972). This method involves making serial dilutions of soil using either water or water agar, followed by the direct plating of soil onto semi-selective media at the selected dilution rates (Tsao and Guy, 1977). After 2 or 3 days of incubation, soil inoculum is calculated as propagules per gram of soil. The disadvantage of

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this method is that it is only effective when inoculum density is relatively high, i.e. > 10 propagules per gram of soil (Tsao, 1983), and it is therefore unsuitable for pathogens, such as Pc, which have naturally occurring low soil inoculum densities (Hendrix and Kuhlman, 1965; Eden *et al.*, 2000). This can be attributed to the limited sample volumes associated with direct soil plating, in comparison to the larger volumes used during soil baiting (Dance *et al.*, 1975; Jeffers and Aldwinckle, 1987). To circumvent the aforementioned problem, Shearer (2014) incubated soil with water for a 24-hour period, before pouring the supernatant onto semi-selective media and subsequently calculating the soil propagules. Similar to soil and root baiting techniques, pathogen detection and quantification accuracies of the above-mentioned soil dilution approaches are also affected by pathogen dormancy and the overgrowth of hymexazol insensitive *Pythium* spp.

Some studies have focused on the recovery of Pc chlamydospores as a means of measuring soil propagule levels (McCain *et al.*, 1967; Hwang and Ko, 1978; Shew and Benson, 1982). Shew and Benson (1982) used a wet-sieving technique (125 µm over 38 µm), whereby chlamydospores were collected from a 38 µm sieve and subsequently made into a spore suspension, before being poured onto PCH (pimaricin, chloramphenicol, and hymexazol) medium. The spore suspensions were incubated in the dark for 2 to 3 days and quantified based on the colony-forming units (CFU). This soil sieving technique is advantageous, as it provides a quantitative measure of viable soil inoculum. However, there is still the possibility of propagule loss through the 38 µm sieve (Kliejunas and Nagata, 1979), and chlamydospore dormancy, which can both lead to an underestimation of pathogen propagule levels.

Baiting techniques

Phytophthora cinnamomi can be isolated from infected root tissue, or infested soil, using traditional baiting techniques (Zentmyer, 1980; Erwin and Ribeiro, 1996). These methods involve incubating the infected root or infested soil samples in water, along with baits that consist of susceptible plant tissue. Various baits may be used, including avocado fruit (Zentmyer *et al.*, 1960), lupine radicles (Chee and Newhook, 1965), pear fruit (Greenhalgh, 1978), whole rhododendron leaves (Themann and Werres, 1997, 1998), or leaf discs, such as those from citrus or pineapple (Erwin and Ribeiro, 1996). Baiting creates flooding conditions which initiate the production of sporangia and the release of zoospores, which subsequently locate the baits through chemotaxis (Khew and Zentmyer, 1973). After several days of incubation, the baits are plated onto semi-selective media, such as PARPH (pimaricin, ampicillin, rifamycin, PCNB and hymexazol) (Jeffers and Martin, 1986). Thereafter, the hyphal growth, which emerges from the plated bait tissue, is identified to species level using either morphology or DNA sequence analysis (Marks and Kassaby 1974; Eden *et al.* 2000; Kox *et*

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al., 2007). The amount of pathogen present in samples can be quantified by determining the percentage of either the number of pathogen-infected baits (Shearer *et al.*, 2010) or the surface area or number of lesions that have developed on the infected baits (Erwin and Ribeiro, 1996; Rollins *et al.*, 2016). However, the latter is not always possible, since not all bait types develop lesions during Pc infection (Podger, 1978; Hüberli *et al.*, 2000). The disadvantage of using traditional baiting techniques is that they are mainly semi-quantitative, due to their inability to give a definite pathogen concentration.

The success of baiting techniques can be influenced by several factors. Firstly, the bait susceptibility and incubation conditions can determine the quantity of pathogen detected (Erwin and Ribeiro, 1996). Furthermore, some baits have a greater vulnerability to contaminants, such as *Pythium* spp. For example, leaf disc baits that have wounded edges are more susceptible to contaminants than intact fruits or whole leaves (Jeffers and Aldwinckle, 1987; Ferguson and Jeffers, 1999). Since *Pythium* spp. generally grow more rapidly than *Phytophthora* spp. (Nechwatal *et al.*, 2001; Bush *et al.*, 2003), the presence of *Phytophthora* spp. can also often be masked by the presence of *Pythium* spp., thus leading to a false-negative diagnosis. Although baiting relies on semi-selective media, such as media amended with hymexazol for the suppression of *Pythium* spp., a few hymexazol insensitive *Pythium* spp. exist (Ali-Shtayeh *et al.*, 2003). Due to the electrotactic effect of positively charged ions on zoospore motility (Khew and Zentmyer, 1974), baiting water, other than deionised water, may also reduce the efficiency of zoospores in locating selected baits, thus also resulting in false-negative detections.

Other factors that may influence the quantification of *Phytophthora* spp. from soil samples include pathogen dormancy and the soil layer depths used in baiting containers, which can both lead to false-negative detections (Eden *et al.*, 2000; McCarren *et al.*, 2005). Studies have shown that for some *Phytophthora* spp. pathogen dormancy can be broken by subjecting soils to pre-moistening before baiting (Crone *et al.*, 2014), re-baiting after air-drying (Jeffers and Aldwinkle 1987; Bunny, 1996; Davison and Tay, 2005), or exposing the soil to cold storage treatments prior to baiting (Tooley and Carras, 2011); thus, increasing the likelihood of detecting and accurately quantifying the pathogen. Davison and Tay (2005) found that re-baiting after air-drying increased the recovery rate of Pc from 6.3 to 7.2 % for soil samples taken from jarrah dieback sites in Western Australia. However, contrarily, Ferguson and Jeffers (1999) observed that Pc had heightened sensitivity to soil air-drying procedures when compared to *P. nicotianae*, with air-drying almost always eliminating its detection. Similarly, Eden *et al.* (2000) discovered that Pc propagules were unable to survive two days of air-drying exposure. In addition, Eden *et al.* (2000) showed that an increase in soil depth during baiting, such as when higher soil volumes are used, inhibited zoospore movement and therefore resulted in reduced detection levels.

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The serial dilution end-point method (SDEM) is a soil baiting-based method that has been used to estimate the disease potential of *Phytophthora* spp. in soil. This is typically achieved by mixing infested soil with sterilised soil in a dilution series (Tsao, 1960). Each dilution undergoes soil baiting, after which, a disease potential index is calculated as a reciprocal of the highest dilution that gives a positive result (Eden *et al.*, 2000). The disease potential index, however, is only a semi-quantitative measure of disease potential. Eden *et al.* (2000) found that this method was unreliable, as trends for calculated infections per gram of soil were only apparent at very high soil inoculum levels. This was attributed to the variation associated with low soil propagule levels and their uneven distribution within soil samples.

Plant bioassays

Darvas (1982) developed a semi-quantitative lupine bait technique to determine the disease potential in avocado orchards in South Africa. This method involved planting healthy lupin seedlings in infested soil. The soil was sampled from underneath diseased trees varying in disease severity according to the PRR Ciba-Geigy tree health rating scale. A positive correlation was found between the percentage of infected lupins and the allocated tree health ratings; however, under severe disease development, the opposite was true. This was attributed to the absence of roots under severe root rotting conditions for trees ranked from 6 to 8 on the tree health rating scale (Darvas, 1982). Consequently, the lupine baiting technique is considered an unreliable and inconsistent method for measuring disease potential in orchards already in a severe state of decline. However, the method may hold potential for orchards where trees are at the early stages of decline (1 to 5 tree ratings), which is beneficial from a commercial grower's perspective (Darvas, 1979).

Molecular methods

Quantitative real-time PCR (qPCR) can be used to quantify pathogen DNA extracted directly from plant tissue and soil (Eshraghi *et al.*, 2011a). This method allows for rapid, sensitive and accurate detection and quantification of plant pathogens (Martin *et al.*, 2000; Li *et al.*, 2008). qPCR measures the intensity of fluorescent signals that are directly proportional to the amount of DNA generated during PCR amplification (Kubista *et al.*, 2006). There are two main approaches for creating such fluorescent signals. The first approach consists of fluorogenic probe-based assays, for example Taqman®, which involves target-specific hybridisation of the fluorescent-tagged probe sequence to single-stranded DNA (Livak *et al.*, 1995). Probe-based assays, although relatively expensive, are beneficial in that they can enhance the specificity of assays and can also be multiplexed to detect several target genes of interest in a single reaction (Polinski *et al.*, 2013). The second approach uses fluorescent DNA binding dyes, such as SYBR® Green, which bind indiscriminately to double-stranded DNA (Morrison *et al.*,

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1999). SYBR® Green-based assays require well-designed primers to prevent non-target DNA binding. Due to the likelihood of SYBR® Green-based assays being non-specific, melt curve analyses are always conducted on the amplified products to confirm the assay specificity. The melt curve analyses should show that the amplified products have the same melting temperature as the target species (Polinski *et al.*, 2013). Melt curve analyses are also useful for identifying primer-dimers which can affect the reaction efficiency and quantification results (Rodríguez *et al.*, 2011).

Factors affecting the sensitivity and accuracy of qPCR assays

A few other factors, in addition to the type of qPCR assay conducted (probe-based versus SYBR® Green-based), are known to influence the sensitivity and accuracy of quantification of qPCR-based assays. These include the selected target sequence (Spies *et al.*, 2011; Miles *et al.*, 2017), primer-design (Taylor *et al.*, 2010), reaction efficiency (Svec *et al.*, 2015), presence of PCR inhibitors (Daniell *et al.*, 2012; Wang *et al.*, 2017), gene copy number (Schena *et al.*, 2008) and whether a nested or regular qPCR assay is performed (Engelbrecht *et al.*, 2013; Kunadiya *et al.*, 2017).

The target sequence and selected primers are important components to consider when designing a qPCR assay since both can affect the specificity and accuracy of quantification. To ensure assay sensitivity, it is imperative that the selected target region must be conserved only in the target species and not in closely related species. Furthermore, it is important that primers designed for the targeted region should yield relatively short PCR amplicons (<100 bp), as this has been associated with improved reaction efficiencies. Reaction efficiency is defined as the fraction of target molecules that are copied in one PCR cycle, with the requirement that target molecule copies must increase at a constant exponential rate as the assay progresses (Svec *et al.*, 2015). qPCR assays should preferably have a reaction efficiency of between 0.95 and 1.05 for accurate quantification, however, qPCR assay efficiencies of 0.85 are still considered acceptable provided that the data is interpreted critically (Bustin and Huggett, 2017). qPCR efficiency is calculated using a standard curve (serial dilution of the target DNA) containing the target sequence. The standard curve is also used to determine the limit of detection (lowest concentration of pathogen DNA that can be detected) and limit of quantification (lowest concentration of pathogen DNA that can be quantified). An inability to optimise qPCR assays to an acceptable reaction efficiency, once all reaction and amplification conditions have been optimised, can be due to characteristics inherent to the targeted sequence region or the designed primers (Taylor *et al.*, 2010; Spies *et al.*, 2011; Miles *et al.*, 2017).

The sensitivity of qPCR assays may be linked to the gene copy number of the selected gene region of interest (Schena *et al.*, 2008). For example, when Kunadiya *et al.* (2017)

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evaluated two nested PCR assays targeting the single copy *Ypt*1 gene region or the multiple copy ITS region, the *Ypt*1 gene assay was less sensitive (15 fg) than the ITS assay (0.015 fg) (Kunadiya *et al.*, 2017). However, despite the advantage of having a lower limit of detection, multiple copy gene regions may result in inaccurate pathogen DNA quantification. For example, Spies *et al.* (2011) found that *Phytopythium vexans* isolates differed in terms of their estimated DNA quantities, despite using the same amount of genomic DNA during qPCR assays. This was likely due to differences in the ITS copy numbers between the different isolates (Spies *et al.*, 2011).

The accuracy of qPCR assays and impaired pathogen detection can also be caused by PCR inhibitory substances, such as humic acids and phenolic compounds, which are often co-extracted from soil and plant samples (Tsai and Olson, 1992; Zhou *et al.*, 1996; Jackson *et al.*, 1997; Okubara *et al.*, 2005). These substances inhibit key enzymatic reactions, such as those required for PCR amplification, which consequently leads to the complete or partial inhibition of target DNA fragment amplification (Daniell *et al.*, 2012). This can result in an underestimation of pathogen DNA concentrations or an increase in false-negative detections (McKee *et al.*, 2015). As a result, several studies have focused on reducing or eliminating PCR inhibitory substances for improved accuracy and sensitivity of qPCR assays. Pre-dilution of DNA extracts was found to successfully alleviate PCR inhibition, by lowering the concentration of inhibitory substances (Schneider *et al.*, 2009; McKee *et al.*, 2015; Wang *et al.*, 2017). Although five-fold and ten-fold dilutions have been recommended by researchers (McKee *et al.*, 2015), others have found that dilutions of 40 to 60-fold were required in order to completely eliminate PCR inhibition (Wang *et al.*, 2017). In addition, dilution requirements may differ according to the extraction material or source used, due to the variations found in the composition or concentration of inhibitory substances (McKee *et al.*, 2015; Hargreaves *et al.*, 2013). To counteract problems associated with inhibitor variation between samples, relative quantification, which involves the incorporation of an artificial foreign reference gene into DNA extractions, may be used. This allows for the accurate monitoring of dilution effects between samples, as well as assists in determining the dilution point at which minimal PCR inhibition occurs (Bürgmann *et al.*, 2001; Eshraghi *et al.*, 2011a; Daniell *et al.*, 2012; Catal *et al.*, 2013; Wang *et al.*, 2017).

The sensitivity of qPCR assays can be enhanced by incorporating a nested PCR approach, as discussed under the species-specific primer section. The use of nested qPCR assays has successfully been used for the quantification of Pc during early stages of infection in inoculated avocado seedlings under glasshouse conditions (Engelbrecht *et al.*, 2013).

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Expressing pathogen DNA concentrations from qPCR assays using relative and absolute quantification

For qPCR-based quantification, pathogen DNA concentrations can be estimated using either absolute (Daniell *et al.*, 2012) or relative (Eshraghi *et al.*, 2011a; Catal *et al.*, 2013; Wang *et al.*, 2017) quantification approaches. Absolute quantification involves the direct estimation of pathogen DNA concentrations in comparison to a standard curve generated by serial dilution of the target amplicon and amplified under the same reaction and amplification conditions (Daniell *et al.*, 2012). The pathogen DNA concentrations are then expressed as either the amount of pathogen present within fixed sample (plant or soil) weights or sample surface areas (Brouwer *et al.*, 2003; Eshraghi *et al.*, 2011a). Brouwer *et al.* (2003) found that measuring the amount of pathogen DNA relative to sample weights, was a reliable method for determining root colonisation levels in *Arabidopsis thaliana*. In contrast, Eshraghi *et al.* (2011a) found considerable variations between replicates of equal sample weights and similar disease levels in both *A. thaliana* and *Lupinus angustifolius*. This suggests that variable DNA extraction yields between sample replicates can lead to the inaccurate quantification of pathogen biomass (Gachon and Saindrenan, 2004). Another negative factor that might be associated with absolute pathogen quantification, is the inability to account for PCR inhibition (Wang *et al.*, 2017).

Relative quantification aims to account for PCR inhibition by normalising pathogen DNA concentrations to reference genes, such as host genes or foreign gene spiking (Eshraghi *et al.*, 2011a; Daniell *et al.*, 2012; Catal *et al.*, 2013; Wang *et al.*, 2017). Catal *et al.* (2013) found that relative quantification using host genes gave an accurate and less variable representation of soybean varietal reaction to *Phytophthora sojae* when compared to absolute quantification. However, Eshraghi *et al.* (2011a) observed that normalising pathogen concentrations to host genes can lead to an overestimation of pathogen biomass. This is due to host DNA degradation being associated with necrotrophic pathogen infection, i.e. host cell death during the later stage of Pc infection (Cahill *et al.*, 2008; Eshraghi *et al.*, 2011a). Eshraghi *et al.* (2011a) therefore used foreign gene spiking (pScFvB1 mouse gene located on an *E. coli* plasmid) during DNA extractions, and subsequent relative quantification using the foreign gene, to successfully amend problems associated with PCR inhibition, DNA extraction inefficiency, and host DNA degradation, leading to an improved accuracy of Pc quantification. Other studies have likewise incorporated *E. coli* plasmid DNA containing a foreign gene, to successfully monitor PCR inhibition (Park and Crowley, 2005; Wang *et al.*, 2017). The success of using plasmid DNA containing a foreign reference gene for relative quantification is partly attributed to its small size and, therefore, small amount (1 ng) required during spiking. It is also a more cost-effective method when compared to synthetic gBlocks (double-stranded DNA fragments).

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Phytophthora cinnamomi qPCR assays

Only a limited number of studies have been published that were aimed at developing qPCR assays specific to Pc. Engelbrecht *et al.* (2013) developed a nested SYBR® Green-based qPCR assay targeting the *Lpv* gene, for the detection of Pc from artificially-infected avocado rootstocks. The nested qPCR approach enabled a very low limit of detection of 20 fg (Engelbrecht *et al.*, 2013), in comparison to the 20 pg limit of detection initially reported for the conventional non-nested PCR assay targeting the *Lpv* gene (Kong *et al.,* 2003). Due to the increase in assay sensitivity, pathogen detection becomes more probable during early stages of infection (Engelbrecht *et al.*, 2013). However, Kunadiya *et al.* (2017), found that the nested qPCR assay developed by Engelbrecht *et al.* (2013) was not Pc-specific since the assay detected closely related *Phytophthora* spp., such as *P. parvispora* and *P. niederhauserii*. *Phytophthora niederhauserii* has recently been reported as a highly virulent pathogen of avocado (Rodríguez-Padrón *et al.*, 2018).

Phytophthora cinnamomi-specific qPCR assays targeting the *Ypt*1 gene were developed by Masikane (2017) and Trzewik *et al.* (2016). Trzewik *et al.* (2016) developed a SYBR® Green-based qPCR assay using primers Pcin59F and Pcin191R, however, the assay was also found to be non-specific to Pc by Kunadiya *et al.* (2017). This is likely due to a lack of specificity testing against closely related *Phytophthora* spp. In contrast, a probe-based qPCR assay targeting the *Ypt*1 gene was developed by Masikane (2017) which included specificity testing against the closely related species, *P. parvispora* and *P. niederhauserii*; the only clade 7 *Phytophthora* spp. known to occur in South Africa (Bezuidenhout *et al.*, 2010; Spies *et al.*, 2011; Oh *et al.*, 2013). The assay was found to be Pc-specific with a limit of quantification of 700 fg (Masikane, 2017).

The ITS region has also been targeted for the development of qPCR assays. Kunadiya *et al.* (2017) designed and evaluated two probe-based assays using primer pairs PcinF6-PcinR2 and PcinFF-PcinRF with probes PcinProbe1 and PcinProbeFP1, respectively. However, neither of the assays were found to be Pc-specific.

In addition to the abovementioned qPCR assays targeting nuclear genes, Bilodeau *et al.* (2014) published a Pc-specific Taqman® qPCR assay targeting a mitochondrial gene region (*atp9-nad9*). This assay used *Phytophthora* genus-specific primers (PhyG_ATP9_2FTail and PhyG-R6_Tail) with a Pc-specific probe (Pcinn_nad9sp_probe1) that had a limit of detection of 10 fg. The assay was reported to be specific to Pc (Bilodeau *et al.* 2014) and was subsequently optimised into a multiplex reaction, whereby the *cox*1 gene region of plants was co-amplified with Pc to monitor PCR inhibition and prevent false-negative detections (Miles *et al.*, 2017). The *atp9-nad9* qPCR assay was specifically developed for regulatory purposes, to allow for the sensitive detection of quarantine and destructive *Phytophthora* spp., however, the quantitative nature of the assay must still be evaluated. This is due to the fact that

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mitochondrial genes are multiple copy and may thus have similar problems to the ITS region in that copy numbers between different isolates may differ. Furthermore, the copy number in different morphological structures (zoospores, chlamydospores and oospores) may also differ, as well as in cells of different physiological status (Miles *et al.*, 2017).

MANAGEMENT OF PHYTOPHTHORA ROOT ROT

There are currently no management practices that can control PRR of avocado on their own. Consequently, focus has been placed on six main principles that form part of an integrated management strategy known as the 'Pegg wheel'. These six principles include soil selection, irrigation management, chemical control, inorganic nutrition, organic amendments and tolerant rootstocks (Wolstenholme and Sheard, 2010). The aim of the 'Pegg wheel' is to maintain tree health while minimising the effects of pathogen colonisation.

Soil selection

The most important soil characteristics to consider for soil selection include good soil drainage and soil aeration. In soils with poor drainage, ridges can be used to avoid oversaturation of the main feeder root zone. Ridges are usually prepared in soils that have a clay content of 15% or more (Wolstenholme and Sheard, 2010). These ridges are prepared prior to orchard establishment and typically fall within the dimensions of 1-1.5 m wide and 0.5-1 m high (Dann *et al.*, 2013). Apart from improved soil drainage, ridging increases rooting depth which can, therefore, help in increasing the overall tree productivity (Wolstenholme and Sheard, 2010). As avocado is a salt-sensitive crop (Steinhardt *et al.*, 1989; Ben Ya'acov and Michelson, 1995), it is also important to avoid establishing orchards in saline soils.

Irrigation management

Avocado is sensitive to both over- and under-irrigation (Pegg, 2010). As a result, soil moisture levels ranging between 0-10 kPa and -50 to -70 kPa should be avoided, as both conditions can predispose avocado roots to Pc infection. Soil moisture levels can be monitored using tensiometers or electronic soil capacitance probes (Wolstenholme and Sheard, 2010). This practice becomes essential for orchards already suffering from PRR, as water requirements in these orchards will be reduced due to impaired water uptake by the roots (Dann *et al.*, 2013). Reduced water uptake leads to moisture build-up in the soil which subsequently exacerbates PRR incidence. To avoid over-irrigation in diseased orchards, the number or output of micro-sprinklers and drippers may be reduced, as well as the duration of irrigation (Wolstenholme and Sheard, 2010).

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Inorganic nutrition

Maintaining a balanced soil nutritional status helps to improve tree health and subsequently improves host fitness to pathogen attack. This is accomplished by performing regular leaf and soil analyses (Dann *et al.*, 2013), which provide pre-warnings of possible deficiencies or toxicities in the orchard. Phosphorus, calcium and boron levels are particularly important, as these elements are known to have the greatest influence on healthy root development (Wolstenholme, 1981).

Calcium, specifically in the ionic form Ca^{2+}, has a mild fungicidal effect on Pc (Wolstenholme and Sheard, 2010). This, however, depends on the extracellular Ca^{2+} concentration present. A minimum level of extracellular Ca^{2+} is needed for sporangial production (Halsall and Forrester, 1977), adhesion of zoospore cysts to the host (Donaldson and Deacon, 1992; Gubler *et al.*, 1989), and the germination of zoospore cysts (Von Broembsen and Deacon, 1996). However, at higher concentrations, extracellular Ca^{2+} can reduce zoospore motility (Byrt *et al.*, 1982), and induce the premature germination of zoospore cysts in the absence of a host (Byrt *et al.*, 1982; Deacon and Donaldson, 1993). This explains why high soil calcium levels have often been associated with disease suppression (Von Broembsen and Deacon, 1997; Messenger *et al.*, 2000). Ca^{2+} is readily obtained from the ionisation of gypsum ($CaSO_4$) or calcium nitrate ($CaNO_3$) (Wolstenholme and Sheard, 2010). Gypsum applications are therefore recommended as part of an integrated management strategy for PRR suppression (Pegg, 2010). However, in soils that are more acidic, it is important to apply lime and/or dolomite since these can increase soil pH levels and reduce aluminium toxicity (Wolstenholme and Sheard, 2010).

Organic amendments

Several studies have shown that mulching is able to suppress PRR development (Broadbent and Baker, 1974; Turney and Menge, 1993; Downer *et al.*, 2001). Mulching suppresses PRR by promoting an environment suitable for a diverse range of antagonistic soil microbes (Wolstenholme and Sheard, 2010). As Pc is a moderately weak saprophytic competitor, it does not survive well in soils with high organic matter. Organic matter mainly comprises cellulose and consequently hosts soil microbes that require cellulose as their primary food source (Eriksson *et al.*, 1990). These soil microbes produce cellulase enzymes which are also harmful to oomycetes, such as Pc, due to their cell walls primarily containing cellulose (Downer *et al.*, 2001). Apart from pathogen suppression, mulching can also influence tree vigour, by improving the nutrient and water holding capacity of the soil, as well as improving the soil structure. Materials that are commonly used for mulching include avocado pruning chips, aged hardwood chips or pine bark, wheat straw and sorghum stubble (Dann *et al.*, 2013).

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Chemical control

Phenylamide fungicides

Metalaxyl (Ridomil®) is a phenylamide (acylalanine) that provides curative control against oomycetes (Schwinn and Staub, 1987), such as Pc. Metalaxyl acts directly on some, but not all *Phytophthora* spp., by inhibiting their growth and sporulation (Farih *et al.*, 1981). The fungicide is typically applied as a soil drench or in granular form, moving freely in soils and, once absorbed by avocado roots, moves rapidly within the xylem system (Dann *et al.*, 2013). Metalaxyl helps to maintain tree health (Whiley *et al.*, 1986) and encourages the recovery of trees that are already infected (Allen *et al.*, 1980; Pegg *et al.*, 1985). However, in South Africa, it was found that metalaxyl was unable to effectively control PRR after 2-3 years of repeated applications (Darvas *et al.*, 1984; McKenzie, 1984; Snyman and Kotzé, 1984). This loss in effectiveness was associated with the rapid biodegradation of metalaxyl in soil (McKenzie, 1984), as well as the development of metalaxyl-resistant Pc isolates (Darvas *et al.*, 1984; Snyman and Kotzé, 1984). Metalaxyl has since been replaced by mefenoxam (Ridomil Gold®), an R-enantiomer of metalaxyl (Parra and Ristaino, 2001). Mefenoxam provides the same level of efficacy as metalaxyl but at half of the application rate, since mefenoxam only contains the active R-enantiomer of metalaxyl (Nuninger *et al.*, 1996).

Phosphonates

Since the discovery of phosphonate trunk injections in avocado (Darvas *et al.*, 1984), phosphonate fungicides (fosetyl-Al and potassium phosphonate) have remained the most effective preventative and curative method for PRR control worldwide, including in South Africa (McLeod *et al.*, 2018). In South Africa, many growers apply phosphonates annually as a preventative management strategy (McLeod *et al.*, 2018). This, however, became problematic in 2014, when strict maximum fruit residue limits (MRLs) (50 mg/kg) were imposed by the European Union (EU) for phosphonate products used on avocado (McLeod *et al.*, 2018). Although phosphonate applications are applied according to label recommendations, many avocado producers are unable to meet the EU standards, often exceeding that of the MRL requirements. As the majority of South Africa's avocado exports (± 90%) are distributed amongst European countries (Donkin, 2007), this has resulted in substantial export losses and market access problems. New approaches, using phosphonates as a preventative management strategy against PRR, are therefore required to reduce fruit residues, but should not compromise disease management.

Translocation

In plants, phosphonates dissociate into the anion known as phosphite, which is directly involved in pathogen suppression (Cohen and Coffey, 1986). Phosphite is translocated

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systemically through the xylem and phloem vascular systems (Cohen and Coffey, 1986; Groussol *et al.,* 1986; Lüttringer and De Cormis, 1985), in accordance to a source-sink relationship found between shoots, roots and reproductive organs (Groussol *et al.,* 1986; Saindrenan *et al.*, 1988; Ouimette and Coffey, 1990; Guest and Grant, 1991). The timing of phosphonate applications are therefore critical; applications are made just after the summer and spring foliar flush has hardened off since this is when root flushing commences (Whiley *et al.,* 1986; Whiley *et al.*, 1995; Thomas, 2008). As phosphonates are required for root protection against PRR, it is essential that phosphonate applications take place during root flush windows, when roots are acting as a sink (Whiley *et al.*, 1995).

Plants are unable to metabolise phosphite (McDonald *et al.*, 2001); however, tissue concentrations are known to decline. This may be due to dilution through plant growth, harvesting of fruit, loss through root exudates, or loss of organs through plant abscission (Guest and Grant, 1991).

Application methods

Phosphonates can be applied as foliar sprays, trunk sprays, trunk injections, or soil drenches due to its high mobility in plants, both acropetally and basipetally (Hardy *et al.*, 2001; Giblin *et al.*, 2007). Soil drenches are considered as an ineffective application method due to low persistence in avocado roots, which subsequently leads to high rates of monthly re-applications (Dann *et al.*, 2013). Trunk sprays containing bark penetrants are an effective application method for younger avocado trees with green stems, however, these sprays are unable to efficiently penetrate the bark of mature avocado trees (Giblin *et al.*, 2007). Foliar sprays are also an effective application method on young and bearing avocado trees but can only work preventatively due to diseased trees having poor foliage (Pérez-Jiménez, 2008) and therefore providing inefficient phosphonate translocation to the roots. In contrast, trunk injections can be used for curative and preventative PRR control in bearing avocado trees (Darvas *et al.*, 1984).

Although trunk injections are currently considered the most effective application method, studies have shown that they can cause damage to the trunk wood with prolonged use (Robbertse and Duvenhage, 1999). Furthermore, due to the labour-intensity associated with trunk injection applications, and the recent increases in labour costs in South Africa, trunk injections have become an expensive means of PRR management. This has led to an increase in the popularity of preventative foliar phosphonate sprays as an alternative management approach (Thomas, 2008; McLeod *et al.*, 2018). Foliar potassium phosphonate sprays are registered in Australia (Whiley *et al.*, 2001), however, currently, only Aliette® (fosetyl-Al) is registered as a foliar phosphonate spray for avocado orchards in South Africa. Unfortunately, Aliette® foliar sprays are not economically viable for South African avocado

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producers, since five to six sprays are required annually according to label recommendations (McLeod *et al.*, 2015).

Mode of action

Phosphonates have a complex mode of action that involves: (i) directly suppressing the pathogen through phosphite concentrations found in plant tissue (ii) stimulating the pathogen to release stress metabolites, such as elicitors, that induce plant defence responses, and (iii) directly stimulating host plant defence responses (Coffey and Bower, 1984; Guest, 1986; Saindrenan *et al.*, 1988; Afek and Sztejnberg, 1989; Smillie *et al.*, 1989; Guest *et al.*, 1995; Jackson *et al.* 2000; Daniel and Guest, 2005). Direct suppression is thought to be due to the direct toxic effect of phosphite (Fenn and Coffey, 1984) on several targets of the pathogen, including the inhibition of vital phosphorylation reactions (Niere *et al.*, 1994), induction of chlamydospore dormancy (McCarren *et al.,* 2009) and damage to the cytoskeleton functionality and cell lysis (King *et al*., 2010). The concentration of phosphite required to have a direct effect on *Phytophthora* spp., under *in vitro* conditions, can vary greatly among different species as well as isolates within species (Guest and Grant, 1991).

Several studies have shown that host plant defence responses are stimulated by phosphonates during pathogen attack. This was supported by gene expression analyses in most studies, whereas the study of Massoud *et al.* (2012) also used the mutation of specific plant defence genes during phosphonate-mediated suppression of oomycetes. It has been reported that the salicylic acid (SA) and/or jasmonic acid/ethylene (JA/ET) signalling pathways are involved in phosphite-induced host defence responses. For example, SA-related defence responses were triggered in the Pc-*E. marginata, Hyaloperonospora arabidopsidis-A. thaliana* and Pc-*L. angustifolius* model systems (Molina *et al*., 1998; Jackson *et al*., 2000; Massoud *et al*., 2012; Groves *et al*., 2015). In contrast, the JA/ET pathways were involved in host defence responses in the Pc-*A. thaliana* model system (Rookes *et al*., 2008). Contrarily, studies have also supported the co-regulation of SA and JA/ET-mediated defence signalling during Pc and *Phytophthora plurivora* attack, under the Pc-*A. thaliana* and *P. plurivora-Fagus sylvatica* model systems, respectively (Eshraghi *et al.*, 2011b; Dalio *et al.*, 2014).

Recent studies also suggest that the mode of action *in planta* is dose-dependent, with high phosphite plant tissue concentrations directly suppressing the pathogen, and low phosphite plant tissue concentrations inducing host plant defence systems. This was demonstrated clearly in the *H. arabidopsidis-A. thaliana* model system, and to a lesser extent in the Pc-*E. marginata* model system (Jackson *et al.*, 2000; Massoud *et al.*, 2012). Although several studies have investigated phosphite levels in plant tissue, the results have been variable as to whether a direct correlation exists between root phosphite concentrations and pathogen suppression (Van der Merwe and Kotzé, 1994; Massoud *et al.*, 2012; Dalio *et al.*,

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2014; Groves *et al.*, 2015). Furthermore, the exact phosphite concentration required in different host-pathogen systems to suppress *Phytophthora* spp. is unknown. In avocado, the Australian industry considers root phosphite concentrations of 25-30 $\mu g/g_{FW}$ to be sufficient in suppressing Pc under orchard conditions, although no experimental data has been published to support this assumption (Giblin *et al.*, 2007). Van der Merwe and Kotzé (1994) reported in a non-peer reviewed study, conducted under glasshouse conditions, that only 9.5 $\mu g/g_{FW}$ was required for the suppression of Pc in avocado seedlings.

Phytophthora spp. have been isolated from plant tissue previously exposed to phosphonates. Therefore, the mode of action, being it direct or indirect suppression, is said to be fungistatic rather than fungitoxic (Guest and Grant, 1991; Dobrowolski *et al.*, 2008).

Rootstock tolerance and clean planting material

The importance of using pathogen-free planting material was well-recognised during the 1970s, with the first disease-free nursery being established in 1974 at Westfalia Estate (Kotzé *et al.*, 1987). To this day, avocado producers are encouraged to only purchase clean planting material from certified disease-free nurseries which conduct regular Pc monitoring tests. In addition, the commercial avocado industry has recognised rootstock tolerance as being one of the most important management strategies for PRR control (Coffey, 1992; Zentmyer *et al.*, 1994).

History of Phytophthora root rot tolerant rootstock development

Due to the destructive nature of PRR on avocado production worldwide (Pegg *et al.*, 2002), many studies have focused on finding resistant rootstocks (Zentmyer and Thorn, 1956; Zentmyer *et al.*, 1963; Botha *et al.*, 1989; Menge *et al.*, 1992; Kremer-Köhne and Duvenhage, 2000; Smith *et al.*, 2011), however, only highly tolerant rootstocks have been found thus far. While resistance refers to the plant's ability to prevent pathogen infection, tolerance refers to the plant's ability to reduce the effects of pathogen colonisation by improving plant fitness (Roy and Kirchner, 2000).

Zentmyer initiated the search for PRR-resistant rootstocks during the 1940s and 1950s in California, USA, which led to the selection of a moderately tolerant Mexican seedling known as Duke 7 (Zentmyer *et al.*, 1963). Up until the 1970s, avocado production was based on seedling rootstocks, however, in 1975, Duke 7 became the first commercially available clonal rootstock (Ben Ya'acov and Michelson, 1995). Clonal rootstocks were preferred over seedlings due to their genetic uniformity and, therefore, improved ability to tolerate PRR (Menge *et al.*, 1992). Furthermore, clonal propagation is required to maintain PRR tolerance since, in general, the inheritance of PRR tolerance in rootstocks is less than 1%, thus making

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seed propagation of tolerant rootstocks more likely to yield rootstocks that are PRR susceptible (Coffey, 1992).

During the 1980s, a highly tolerant Mexican rootstock, known as Dusa®, was identified in South Africa. Dusa® was a seedling of Duke 7 which had survived despite growing in a heavily Pc-infested orchard at Westfalia Estate (Roe *et al.*, 1995). Tolerance is typically sourced from 'escape trees' which have survived and shown reasonable tree health despite experiencing high inoculum pressure (Kotzé *et al.*, 1987; Zentmyer and Schieber, 1987). Over the years, studies have proven that Hass® grafted onto Dusa® rootstocks are able to out-perform Duke 7 with regards to tree health, yield and PRR tolerance (Roe *et al*, 1997; Roe *et al.*, 1999; Menge *et al.*, 2002; Kremer-Köhne and Mukhumo, 2003; Smith *et al.*, 2011). More recently, the R0.06 rootstock has been identified as potentially superior to Dusa® in terms of its PRR tolerance and yield (Van Rooyen, 2017).

History of rootstock selections used in South Africa

During the 1920s, the majority of avocado production in South Africa involved the use of Mexican seedlings, due to their hardier and slightly less vigorous nature (Kremer-Köhne and Köhne, 2007). However, their use became limited after an epidemic of sun-blotch viroid affected most of the mother block trees. This led to the introduction of a new Guatemalan seedling known as Edranol during the 1950s (Kremer-Köhne and Köhne, 2007). It was later found that Edranol had a greater susceptibility to PRR than the Mexican race, which resulted in a large-scale PRR decline in avocado orchards (Gaillard, 1987).

After the discovery of the first PRR-tolerant Duke 7 rootstock in California (USA), the South African avocado industry turned their focus towards the importation of PRR-tolerant rootstocks. Out of the PRR-tolerant rootstocks imported, Duke 7 performed the best under South African growing conditions, with similar results reported in California, USA (Arpaia *et al.*, 1992) and Australia (Young, 1992). Due to its uniformity, productivity and ability to provide reasonably healthy trees, Duke 7 became the industry standard rootstock in South Africa during the 1970s. However, since the selection of the highly tolerant Dusa® rootstock, the avocado industry has seen a substantial decrease in the use of Duke 7. Since its commercialisation in 2001, Dusa® comprised 50% of nursery sales made between 2009 and 2010 by South African Avocado Nuserymens' Association members (Wolstenholme, 2003; Retief, 2011). The tolerance of Dusa® to high soil salinity conditions is another favourable characteristic which has contributed to its widespread planting (Menge *et al.*, 2002; Crowley *et al.*, 2003). Rootstocks that are also currently used in South Africa include Bounty and Velvick seedling rootstocks (Wolstenholme and Sheard, 2010).

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Mechanisms associated with Phytophthora root rot tolerance

The mechanisms behind PRR tolerance are not yet fully understood, however, some evidence has been found to suggest that tolerant rootstocks produce structural barriers (Cahill and Weste, 1983; Phillips *et al.*, 1991; García-Pineda *et al.*, 2010, Van den Berg *et al.*, 2018), and biochemical defence responses (Sánchez-Pérez *et al.*, 2009; Acosta-Muñiz *et al.*, 2012; Van den Berg *et al.*, 2018) during pathogen infection and colonisation. Additionally, root regenerative ability and root exudates are also possible mechanisms involved in PRR rootstock tolerance (Kellam and Coffey, 1985; Aveling and Rijkenberg, 1989; Botha *et al.*, 1989; Gabor and Coffey, 1990; Aveling and Rijkenberg, 1991).

Structural barriers have been identified as important suppressants of Pc infections in *Eucalyptus* spp. and avocado. Callose deposits or papillae formation have been observed in resistant *Eucalyptus* spp. inoculated with Pc (Cahill and Weste, 1983; Cahill *et al.*, 1989), while, in avocado, callus tissue derived from the tolerant rootstocks Duke 7 and Martin Grande were found to have a greater inhibitory effect on Pc fungal growth, *in vitro*, compared to the less tolerant Topa Topa rootstock (Phillips *et al.*, 1991). Phillips *et al.* (1987) also showed that the moderately tolerant Duke 7 rootstock was able to hinder pathogen spread by developing necrophylactic periderm and periclinal cell division, suggesting a critical involvement of structural barrier formation in PRR tolerance. Van den Berg *et al.* (2018) found that the highly tolerant R0.06 rootstock produced impermeable callose deposits at the site of host plant cell penetration 24 hours post-inoculation, whereas the susceptible R0.12 rootstock was only able to produce lignin which did not prevent pathogen colonisation (Van den Berg *et al*., 2018).

Several biochemical defence gene products and reactive oxygen species have been shown to be involved in PRR tolerance responses in avocado. García-Pineda *et al.* (2010) showed that reactive oxygen species (ROS) and nitric oxide (NO) were activated in avocado roots in response to Pc infection, suggesting an important role of NO production during pathogen infection. Reactive oxygen species and NO are well known for their involvement in early defence responses in plants at the site of infection, subsequently leading to the downstream activation of several host plant defence systems (Bellin *et al.*, 2013). In terms of biochemical defence responses, several root proteins are known to be induced by Pc infection in highly tolerant rootstocks, including isoflavone reductase, glutathione S-transferase, cinnamyl alcohol dehydrogenase, cinnamoyl-CoA reductase and cysteine synthase (Acosta-Muñiz *et al.*, 2012). Engelbrecht and Van den Berg (2013) found, through gene expression studies, that phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX) were both associated with rootstock tolerance in Dusa® and R0.06. Subsequently, Van den Berg *et al.* (2018) reported that a stronger activation of β-1,3-glucanase and catalase was observed in the tolerant rootstock R0.06 and not in the susceptible R0.12 rootstock during early stages of

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pathogen infection. Contrarily, the susceptible rootstock R0.12 produced phenolic compounds which were unable to prevent pathogen colonisation (Van den Berg *et al*., 2018).

Differences in root exudate composition and root regenerative ability have been identified for rootstocks varying in tolerance. Botha *et al.* (1989) and Aveling and Rijkenberg, (1991) observed a noticeable reduction in zoospore attraction for avocado rootstocks Duke 7, G6 and G755 in comparison to the more susceptible Edranol rootstock. Kellam and Coffey (1985) showed that the tolerant rootstocks G6 and Duke 7 had a greater root regenerative ability than the less tolerant Walter Hole and Topa Topa, which correlated with reduced pathogen colonisation in G6 and Duke 7. Kellam and Coffey (1985) also observed a difference in Pc colonisation between the tolerant rootstocks G6 and Duke 7, with Duke 7 not only having better root regenerative ability than G6 but also sustaining relatively higher amounts of pathogen colonisation. This suggests that a good root regenerative ability is important for tolerant rootstocks and can compensate for susceptibility to pathogen infection and colonisation.

CONCLUSION

Phytophthora cinnamomi is a well-known destructive soilborne pathogen of many native plant species and agricultural crops. During the past three decades, our understanding of the pathogen's biology and its management has increased substantially. There are, however, still some knowledge gaps. For example, how the pathogen survives in soil and detached root fragments, since the role and existence of thick- versus thin-walled chlamydospores, selfed-oospores and stromata in pathogen survival is unclear (McCarren, 2006; Crone *et al*, 2013b; Jung *et al.*, 2013). Furthermore, effective pathogen quantification methods that can be used in natural environments are lacking. The survival, spatial distribution and seasonal population fluctuations of the pathogen have been studied to a limited extent under orchard and forest conditions using different culture-based quantification methods (Shea *et al.*, 1980; Zentmyer, 1981; Shearer and Shea, 1987; Shearer *et al.*, 2010). Although DNA-based quantification methods are available to investigate Pc population sizes in soil and roots, most of these have been limited to glasshouse evaluations. Therefore, investigations into improved quantification methods of the pathogen under natural environmental conditions are required. This can ultimately be used to create a better understanding of the survival and dissemination of the pathogen and improve management strategies.

The integrated management of PRR in avocado is heavily reliant on the use of phosphonate fungicides and tolerant rootstocks. The availability of tolerant rootstocks has increased steadily over the past few decades, with ongoing research aiming to help improve rootstock tolerance. The management of PRR in avocado has also significantly improved with the discovery of phosphonate trunk injections by Darvas *et al.* (1984). Phosphonates and

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tolerant rootstocks are both used in an integrated management strategy that can be accurately summarised by the six main components of the 'Pegg wheel' (Wolstenholme and Sheard, 2010).

Many studies have focused on phosphite, the breakdown product of phosphonates, and its complex mode of action (Coffey and Bower, 1984; Guest, 1986; Saindrenan *et al.*, 1988; Afek and Sztejnberg, 1989; Smillie *et al.*, 1989; Guest *et al.*, 1995; Jackson *et al.* 2000; Daniel and Guest, 2005). The mode of pathogen suppression is currently theorised as being dose-dependent (Jackson *et al.*, 2000; Massoud *et al.*, 2012). However, previous studies have only focused on the *A. thaliana* and *E. marginata* crop systems, and it would thus be important to assess the relationship between phosphonate dosage and resultant root phosphite concentrations with Pc suppression under avocado orchard conditions.

In South African avocado production systems, where PRR is highly destructive if not controlled, phosphonate trunk injections are an essential component in managing the disease preventatively (McLeod *et al.*, 2018). However, the use of phosphonates has become problematic due to the recently imposed MRLs made by the EU in 2014, South Africa's biggest export market. Avocado producers often do not meet the EU standards, despite following the label recommendations of registered phosphonate products (McLeod *et al.*, 2018). Therefore, the timing and/or dose of phosphonate applications need to be re-evaluated.

Phosphonate trunk injections must be applied in a manner that will ensure that root phosphite concentrations are sufficient during periods when Pc infection and reproduction are at their highest. It is therefore important to establish whether seasonal pathogen colonisation patterns exist for Pc. This will ultimately help in determining when critical colonisation periods occur during the year, and, therefore, when management practices such as phosphonate trunk injections are most fundamental. Studies that have focused on seasonal fluctuations of soil population levels found that Pc was typically more active during higher rainfall periods of the winter and spring seasons in Mediterranean climates (Shea *et al.*, 1980; Shearer and Shea, 1987). However, studies have yet to determine seasonal pathogen behavioural patterns of Pc under irrigated orchard conditions using root colonisation levels; a direct indication of PRR disease potential.

To accurately and reliably monitor pathogen population levels, many factors must be taken into consideration. Two of the most important factors are the sampling strategy and quantification method (conventional versus molecular) (Zentmyer, 1980; Erwin and Ribeiro, 1996; Eshraghi *et al.*, 2011a). In order for avocado producers to accurately monitor pathogen colonisation levels during the avocado growing season, quantification methods must be reliable. The reliability of molecular methods, such as qPCR analyses, can be limited by varying performances of different DNA extraction scales and DNA extraction substrates (e.g. roots versus soil) (Hargreaves *et al.*, 2013; McKee *et al.*, 2015) in terms of DNA yield, and with

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varying efficiencies and sensitivity of qPCR assays targeting different gene regions (multiple versus single copy genes) (Schena *et al.*, 2008; Kunadiya *et al.*, 2017). Problems may also arise when PCR inhibitory factors, co-extracted with roots and/or soil, cause false-negative detections (Daniell *et al.*, 2012; McKee *et al.*, 2015). Currently, there are no monitoring systems available for avocado producers to assess Pc population levels in their orchards, therefore, phosphonates are applied as a preventative management strategy without gauging whether it is necessary or not. It is thus essential to develop a cost-effective, reliable and accurate means of quantifying Pc from avocado orchards.

The overall aim of this study was to improve our understanding of the seasonal colonisation patterns of Pc in avocado orchards and to determine whether two management strategies (phosphonate treatments and rootstock tolerance) can affect Pc root and soil quantities. As there is a lack of information regarding seasonal colonisation patterns of Pc, the first aim of this study was to develop a reliable and accurate quantification approach to determine whether critical colonisation periods occur during the growing season. Two different quantification methods were investigated, including a root baiting technique and qPCR analyses (small-scale and large-scale DNA extractions). Both quantification methods were assessed and compared using two sampling strategies (4x5 [four groups of five trees each] and 1x20 [one group of 20 trees] tree group). To better understand the colonisation patterns, this study also determined whether correlations existed between Pc seasonal root colonisation levels and soil moisture and temperature levels prevailing in the investigated orchards. The second aim of this study was to evaluate whether qPCR analysis was effective for evaluating different management practices (phosphonates and rootstock tolerance). Pathogen quantification was performed during the critical colonisation period (May) identified from the first aim, as well as during the months of March (rootstock tolerance) and October/November (both strategies) when pathogen colonisation levels were typically low, based on the findings made in the first aim. Phosphite root concentrations were determined and compared to Pc soil population and root colonisation levels. Three qPCR assays targeting three different gene regions (*Ypt*1, ITS and mitochondrial) were evaluated for their sensitivity, efficiency and linearity. The assay which performed the best was developed into a multiplex qPCR assay to better assess the quantification of Pc from rhizosphere soil. The knowledge gained from this thesis will assist growers in understanding whether critical colonisation periods exist, and thus contribute towards the improvement of PRR management. The quantification approaches developed will also provide growers with an accurate and reliable means for testing Pc population levels during these critical colonisation periods.

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CHAPTER 2

Seasonal root colonisation patterns of *Phytophthora cinnamomi* in avocado orchards in South Africa

ABSTRACT

Phytophthora cinnamomi (Pc) is a destructive soilborne pathogen for which information is limited on whether seasonal root colonisation patterns exist. Investigations into six asymptomatic avocado orchards situated in two production regions (Mooketsi and Letaba) in Limpopo, South Africa, showed that seasonal colonisation patterns do exist. In both production regions, in 2018, Pc root quantities were significantly higher in May (late autumn) than in March (early autumn), August (late winter) and October/November (late spring) in almost all of the orchards, when quantitative real-time PCR (qPCR) (five orchards) and root baiting (four orchards) analyses were conducted. There was an exception for one orchard in Mooketsi, where Pc root quantities did not differ significantly throughout the year, irrespective of the sampling month and pathogen quantification method used. In 2018, in three of the six orchards, the August Pc root baiting quantities were significantly higher than October/November. In 2017, root colonisation patterns, only evaluated using qPCR analysis, yielded less clear colonisation patterns. In Letaba (2017), August and May yielded the highest Pc root DNA quantities in two orchards, but these did not differ significantly from the other months. In Mooketsi, no significant differences were evident in monthly Pc root DNA quantities in 2017. The May Pc root quantities were significantly positively correlated with the number of hours accumulated in May at soil temperatures that were 15-19°C, but negatively with 20-24°C. In May, Pc root quantities were furthermore significantly positively correlated with the February 16-24°C and 20-24°C soil temperature ranges. This study further showed that two sampling strategies consisting of either four tree groups (each containing five trees) or one tree group (20 trees) yielded Pc root quantities that were significantly correlated (qPCR and root baiting). However, the four tree groups were more sensitive in detecting the pathogen in root baiting analyses. The small-scale DNA extraction method (50 mg roots), used in all of the monthly quantifications, could not be improved by using a large-scale DNA extraction (2.5 g roots); the large-scale extraction yielded significantly lower Pc root DNA quantities. This study has aided in identifying important Pc root quantification approaches and seasons for when optimal disease management strategies are most critical.

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INTRODUCTION

Phytophthora root rot (PRR), caused by *Phytophthora cinnamomi* (Pc), is a destructive soilborne disease that has caused devastating economic losses in avocado orchards worldwide (Milne and Chamberlain, 1971; Darvas *et al.*, 1983; Coffey, 1992). The pathogen destroys the white fleshy feeder roots of trees, which in turn leads to symptoms of water-stress, nutrient deficiency, and ultimately tree death (Zentmyer, 1953; Pérez-Jiménez, 2008). Although the devastating effects of PRR are well known, there is still a poor understanding of the seasonal behavioural patterns of Pc in soil and roots, as well as the factors that can influence this.

Phytophthora cinnamomi root colonisation and PRR severity are influenced by several biotic and abiotic factors. Two of the most important abiotic factors include soil moisture and temperature which, if optimal, can directly stimulate the pathogen's asexual life cycle (sporangial production and release of zoospores) (Hardham, 2005; Hardham and Blackman, 2018). A relatively wide range of soil temperatures, ranging from 24-30°C, have been reported as being optimal for the production of sporangia (Byrt and Grant, 1979; Nesbitt *et al.*, 1979; Shearer, 2014). The release of zoospores from sporangia requires a reduction in soil temperatures, with optimum temperatures for release being between 15-18°C (Khew and Zentmyer, 1973; Hwang *et al.*, 1975; Byrt and Grant, 1979; Zentmyer, 1980; Hardham and Blackman, 2018). Soil type and cultural practices can also influence pathogen activity indirectly by influencing soil moisture and temperature (Broadbent and Baker, 1974; Turney and Menge, 1993; Messenger *et al.*, 2000), microbial competition (Downer *et al.*, 2001; Wolstenholme and Sheard, 2010) and mineral composition of the soil (Von Broembsen and Deacon, 1997; Messenger *et al.*, 2000). Several factors related to the host plant can likewise influence root colonisation. These include plant stress factors that can compromise the plant's defence system (Chang-Ho and Hickman, 1970; Allen and Newhook, 1973; Blaker and MacDonald, 1986; Drew, 1997; Sanclemente *et al.*, 2014), the susceptibility of rootstock varieties to pathogen colonisation (Kellam and Coffey, 1985; Botha *et al.*, 1989; Engelbrecht *et al.*, 2013; Van den Berg *et al.*, 2018), and phenological events, such as root flushes, which may amplify pathogen activity and infection levels (Ploetz *et al.*, 1992). In addition, characteristics related to the pathogen itself, such as the virulence of Pc isolates, can influence disease severity (Dudzinski *et al.*, 1993; Linde *et al.*, 1999).

Only a few studies have investigated the seasonal fluctuations of Pc populations in soil, as well as identified soil depths at which the pathogen is most frequently detected. Most studies have been conducted on the seasonal fluctuations of Pc soil populations in the *Banksia* woodland and *Eucalyptus marginata* forest biomes of south-western Australia (Shea *et al.*, 1980; Shearer and Shea, 1987; Shearer *et al.*, 2010). These studies reported that soil population densities were typically at their lowest during the dry and hot summer months in

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Mediterranean climates and that viable inoculum levels were particularly low in near-surface soil layers (Shearer *et al*., 2010). In the Australian woodland and forest biomes, Pc has been detected at soil depths of up to 1 m underneath dead trees (Shearer *et al.*, 2010). In contrast, studies on avocado (Brodrick *et al.*, 1976) and grapevines (Marais and Hattingh, 1985) have shown that Pc soil populations were highest at relatively shallow soil depths of 0-50 and 240-320 mm, respectively.

The effect of season on Pc root colonisation has only been studied in avocado and not in any other hosts of the pathogen. Zentmyer (1981) performed a temporal analysis on Pc root colonisation by planting avocado seedlings, on a bimonthly basis, in avocado orchards. Root infections were assessed using a direct root plating method, whereby roots were plated onto semi-selective media and the percentage of Pc-infected roots were subsequently calculated. The colonisation of roots by Pc was shown to peak during the late summer (July to September) and autumn (September to November) months in semi-arid climates of California, USA (Zentmyer, 1981).

In addition to using root plating for pathogen root colonisation assessments (Darvas, 1982; Tsao, 1983; Erwin and Ribeiro, 1996; Eden *et al.*, 2000), root baiting can also be used as a conventional semi-quantitative approach. Root baiting is based on the principle that infected roots, which are submerged in water, release zoospores that are attracted to plant baits floating on the water surface. The presence of the pathogen is assessed by plating out the baits onto semi-selective media (Erwin and Ribeiro, 1996). Disadvantages of using conventional isolation methods for pathogen quantification is that some methods, such as baiting, are semi-quantitative (Erwin and Ribeiro, 1996; Shearer *et al.*, 2010; Rollins *et al.*, 2016), more labour-intensive, and isolations can furthermore be limited by pathogen dormancy (McCarren *et al.*, 2005).

Molecular methods, such as real-time quantitative PCR (qPCR) analysis, in general, provide a more accurate, sensitive and quantitative measure of pathogen root colonisation levels than isolation approaches (Martin *et al.*, 2000; Li *et al.*, 2008). A limited number of studies have attempted to quantify Pc from avocado roots using qPCR analysis. Engelbrecht *et al.* (2013) developed a nested qPCR assay, targeting the *Lpv* putative storage protein gene, which was used to quantify Pc from artificially infected avocado seedling roots (rootstocks R0.12 and Dusa®). However, Kunadiya *et al*. (2017) found that the assay was not Pc-specific when tested against the closely related *Phytophthora* spp., *P. parvispora* and *P. niederhauserii*. Recently, Masikane (2017) developed a Pc-specific qPCR assay targeting the Ras-related *Ypt*1 protein gene. The *Ypt*1 assay was able to reveal significant differences in Pc root colonisation levels in avocado trees treated with phosphonates. The root colonisation levels were assessed by using a root baiting technique, whereby Pc was quantified from infected leaf disc baits using qPCR analysis. Subsequently, it was found that qPCR analysis

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of the leaf disc baits was less sensitive and reliable than direct qPCR analysis of the roots, as well as when the leaf disc baits were plated onto semi-selective media (Masikane, 2019).

To ensure that accurate monitoring of seasonal colonisation patterns is achieved, reliable sampling approaches and pathogen quantification methods are required. Sampling strategies that are appropriate for assessing Pc quantities in avocado orchard tree roots or other perennial hosts in natural field locations have not yet been investigated. In contrast, a few studies have investigated the effect of soil sampling strategies on *Phytophthora* soil populations for perennial hosts (Podger, 1978; Balci *et al*., 2007). In terms of pathogen quantification using qPCR analysis, many factors may influence the accuracy of quantification, including the variation between pathogen DNA quantities that are extracted from the same plant sample (Gachon and Saindrenan, 2004; Eshraghi *et al.*, 2011). This becomes especially problematic when the pathogen is present at low levels since false-negative detections are more probable when there is an uneven distribution of the pathogen within samples. The use of a larger root sample weight during DNA extractions may alleviate variation and contribute towards a more accurate pathogen quantification. However, extracting DNA from larger root samples can be difficult when trying to use DNA extraction kits since these kits are typically limited to smaller dry root weights (i.e. 20-200 mg) (Demeke and Jenkins, 2010). Small root weights may also be inadequate for providing accurate representations of Pc root colonisation levels in avocado orchards, especially under circumstances where sample pooling is practised.

The presence of PCR inhibitors that have been co-extracted with DNA from environmental samples, may impair qPCR quantification due to the complete or partial inhibition of target DNA fragment amplification (Daniell *et al.*, 2012). This is especially true for avocado roots, where high polysaccharide contents within avocado root samples can make DNA extraction challenging (Crandall *et al.*, 2017). As a result, an underestimation of pathogen DNA concentrations or an increase in false-negative detections may occur (McKee *et al.*, 2015). These shortcomings can be overcome by monitoring PCR inhibition through the spiking of DNA extraction buffers with an internal foreign reference gene. The foreign gene can subsequently be quantified from the final DNA extract using qPCR analysis (Daniell *et al.*, 2012).

An additional factor which could influence the accuracy of pathogen quantification for seasonal colonisation evaluations is the use of PRR symptomatic avocado trees. This is due to the fact that avocado trees which are severely diseased, are known to have reduced pathogen quantities due to increased root loss (Darvas, 1982). As a result, seasonal colonisation patterns of Pc can potentially be masked by PRR symptomatic trees.

The main aim of this study was to determine the seasonal colonisation patterns of Pc in avocado roots in orchards located in the Limpopo Province of South Africa. Since this relies

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upon accurate quantification of Pc in roots, it was also necessary to evaluate orchard tree sampling approaches and to optimise and investigate root DNA extraction and Pc quantification methods. To better understand seasonal colonisation patterns, another aim of this study was to determine whether correlations existed between Pc seasonal root colonisation levels and soil moisture and temperature levels prevailing in the investigated orchards. The knowledge gained from this study can be used to optimise PRR management practices by protecting avocado tree roots during the periods when the pathogen is most actively colonising the roots.

MATERIALS AND METHODS

Orchard sites, tree selection and soil probe data collection

Six asymptomatic avocado orchards (without obvious aboveground symptoms of PRR decline) were selected in January 2017 from two production regions (Mooketsi and Letaba) situated in Limpopo, South Africa. Both production regions are characterised by a subtropical climate with cool, dry winters and warm, wet summers. However, the Mooketsi region is known to be less conducive to PRR than the Letaba region (McLeod *et al.*, 2018). Three of the orchards (AM, CM and FM) were situated in Mooketsi and another three (BL, DL and EL) in Letaba. For each orchard, a total of 20 asymptomatic trees were randomly selected and tagged within a one-hectare area. The orchards were established on either moderately tolerant Duke 7 rootstocks or highly tolerant Dusa® rootstocks. The scion/rootstock combinations included Carmen/Dusa® (BL), Hass/Dusa® (CM), Maluma-Hass/Duke 7 (AM, EL, FM) and Pinkerton/Duke 7 (DL). Rows of trees were established on ridges in all orchards, however, they varied in terms of their irrigation management; i.e. drip irrigation (Mooketsi region) versus micro-sprinkler (Letaba region). In terms of topography, orchards were typically positioned on relatively flat ground, except for orchard BL, which had a 10% slope. Soil types also varied from loamy sand to sandy clay loam soils, and basic physico-chemical properties that were analysed are summarised in Table 1. The 20 tagged trees from each of the six orchards were left untreated with phosphonates for the duration of the study.

In each of the six orchard blocks, one DFM™ continuous logging soil probe (DFM Technologies, Pretoria, South Africa) was installed at a depth of 0-80 cm. The soil probe collected soil moisture and temperature measurements on an hourly basis over the two-year period (2017 to 2018). The soil moisture and temperature data were measured for the 0-20 cm soil depths. The average percentage of soil moisture was calculated for each month. The temperature data were summarised as temperature ranges on a monthly basis using Burgess *et al.* (2017) and Zentmyer (1981) as guidelines for the temperature range selections. The temperature ranges of 10-14°C, 15-19°C, 20-24°C and 25-29°C were selected based on Zentmyer (1981), whereas the 16-24°C temperature range was based on Burgess *et al.*

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(2017). The approach of Zentmyer (1981) was furthermore selected for summarising the monthly data, where the number of hours at specific temperature ranges were used, rather than calculating the average soil temperatures per month.

Root sampling and tree root groups

Roots were sampled from the 20 trees selected in each orchard over four sampling months (March, May, August, October/November) and for two consecutive years (2017 and 2018). Each sampling took place within the first two weeks of the sampling month. Approximately 50 g of roots, including a mixture of white root tips and older suberised feeder roots, were sampled from four sides of each tree at a depth of 0 to 20 cm. The roots, from which loosely adhering soil was shaken off, were stored at 4°C until analysed.

The first step in assembling the tree groups consisted of washing each of the trees' roots free of soil, using tap water, followed by air drying the roots on paper towels for ±10 min at 24°C. For each orchard, the 20 washed root samples were pooled into groups using two approaches. In the first approach, the roots of five trees were pooled into one sample, resulting in four pooled samples per orchard, hereafter referred to as the 4x5 tree group. In the second approach, the roots of all 20 trees were pooled into one sample per orchard, hereafter referred to as the 1x20 tree group (Figs. 1 and 2). A specific amount of roots, which depended on the Pc quantification method used (see Pc quantification method sections), was taken from each tree to compile the different tree groups (Figs. 1 and 2). Once pooled into the various groups, the roots were surface-sterilised for ±30 s in 70% ethanol and placed onto paper towels to air dry for ±15 min at 24°C.

***Phytophthora cinnamomi* qPCR quantification from roots**

Small-scale DNA extraction

Small-scale DNA extractions were conducted for all of the 4x5 tree group samples for each of the four sampling months in 2017 and 2018. For each orchard, a subsample of 1 g of roots was taken from each of the 20 trees and placed into a 15 ml Falcon tube (according to the tree group), thus 5 g of roots per group of five trees (Fig. 1). DNA was also extracted from the roots of the 1x20 tree group for the August 2017, May 2018 and August 2018 sampling months. The 1x20 tree group was compiled for each orchard by taking a subsample of 1 g of roots from each of the 4x5 tree groups' 5 g root mixtures (Fig. 1). The roots were placed into a 15 ml Falcon tube to yield a total of 5 g of roots per group of 20 trees.

The roots were stored at -80°C overnight and lyophilised in a condenser vacuum (VirTis®; SP Scientific, Warminster, USA) for 24 hours at a chamber temperature of -52°C to -55°C. The roots were fragmented using a sterile scalpel and a subsample of 50 mg of roots was transferred into a 2 ml Eppendorf® tube for each tree group. Another pulverising step was

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conducted, whereby 0.5 g of glass beads (2 mm diameter) was added to each tube. The tubes were shaken at 30 Hz for 5 min using a Retsch® MM400 mixer mill (Retsch GmbH, Haan, Germany).

DNA was extracted from the pulverised roots using the Nucleospin® PLANT II kit (Macherey-Nagel GmbH and Co., Düren, Germany) according to the manufacturer's instructions, with slight modifications. Firstly, prior to the start of the extractions, the required amount of the kit's PL1 DNA extraction buffer was spiked with a plasmid containing a mutated *Escherichia coli* gene (Daniell *et al.*, 2012) to a final concentration of 1.25 x 10^6 copies/µl. The plasmid was extracted from *E. coli* cells using the QIAGEN® Plasmid Midi Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions with one exception: in the final step, the plasmid DNA pellet was re-dissolved using nuclease-free water instead of TE water. The plasmid was extracted using the Qiagen®-tip 100 volumes. Prior to spiking the DNA extraction buffer, the plasmid was linearised using the restriction enzyme NCO1 (Thermo Fisher Scientific (NHK) Ltd., Waltham, USA) and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega Co., Madison, USA) kit. Other modifications that were made to the Nucleospin® Plant II kit protocol were as follows: 800 µl of the PL1 DNA extraction buffer was used instead of the prescribed 400 µl; the RNAse and binding buffer (PC) volumes were increased to 20 µl and 900 µl, respectively; the lysing mixture was not vortexed as advised by the protocol, but shaken at 30 Hz for 5 min using a Retsch® MM400 mixer mill (Retsch GmbH, Haan, Germany); an additional centrifugation step was conducted for 2 min at 11000 rcf (g) using an Eppendorf® 5424 Microcentrifuge (Eppendorf AG, Hamburg, Germany) following the first warm bath (65°C) incubation step; for the final step, DNA was eluted using one 50 µl aliquot of elution buffer (PE) and stored at -20°C.

For most of the 4x5 tree group samples, only one root DNA extraction replicate was conducted per group of five trees. However, exceptions were made for the August 2017, May 2018 and August 2018 sampling months, where two root DNA extraction replicates were conducted. For the analysis of the 1x20 tree groups (August 2017, May 2018 and August 2018), four root DNA extraction replicates were conducted per group of 20 trees (Fig. 1).

Large-scale DNA extraction using a CTAB buffer

Large-scale DNA extractions were only conducted for the August 2017, May 2018 and August 2018 sampling months, using the 4x5 and 1x20 tree groups (Fig.1). For the 4x5 tree group, a subsample of 5 g of roots was taken from each of the 20 trees and placed into a 50 ml Falcon tube (according to the tree group), thus 25 g of roots per group of five trees. The 1x20 tree group was compiled for each orchard by taking a subsample of 5 g of roots from each of the 4x5 tree groups' 25 g root mixtures (Fig. 1). The roots were placed into a 50 ml Falcon tube to

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yield a total of 20 g of roots per group of 20 trees. The tree group roots were stored at -80°C, lyophilised and fragmented as described for the small-scale DNA extractions.

The large-scale DNA extraction method was conducted similarly to the small-scale DNA extraction method, with a few modifications. Firstly, 2.5 g of lyophilised roots were used for extractions; in addition, the roots were pulverised in a 50 ml Falcon tube with 6 g of glass beads (2 mm diameter); the 2.5 g of pulverised roots were lysed using 30 ml of a CTAB-based DNA extraction buffer (1.4 M NaCl, 2% (w/v) CTAB, 0.1 M Tris pH 8.0, 0.02 M EDTA pH 8.0 and 1% (w/v) PVP); a final concentration of $1x10^6$ copies/µl of the mutated *E. coli* plasmid was used; and 500 µl of RNAse was used. The 50 ml Falcon tubes were shaken on the Retsch® MM400 mixer mill (Retsch GmbH, Haan, Germany) at 30 Hz for 5 min using a large tube Retsch® adaptor. An additional centrifugation step was conducted for 2 min at 11000 rcf (g) using an Eppendorf® 5810R Centrifuge (Eppendorf AG, Hamburg, Germany) following the first warm bath (65°C) incubation step; the total lysate volume was transferred into a new 50 ml Falcon tube and vortexed for 5 s whereafter, 700 µl of the lysate was loaded onto the Nucleospin® Filter (violet ring).

For each of the 4x5 tree group samples, two root DNA extraction replicates were conducted per group of five trees, whereas for the 1x20 tree group, four root DNA extraction replicates were conducted per group of 20 trees (Fig. 1).

Comparison of small-scale DNA extractions using a CTAB buffer and PL1 buffer

Ten root samples were randomly selected to determine whether the CTAB-based buffer, used for the large-scale DNA extractions, yielded similar Pc root DNA quantities to the PL1 plant DNA extraction kit buffer that was used for the small-scale DNA extractions. The root DNA extractions were conducted as described under the small-scale DNA extraction method. For each root sample, one extraction was performed using the CTAB-based buffer and another using the PL1 buffer.

Comparison of DNA quantities extracted from different aged roots

Since white root tips were not always available during all sampling months, DNA extractions were performed on samples containing only white root tips and compared with samples containing only older suberised feeder roots. Ten root samples were selected for each root age group and DNA was extracted as described for the small-scale DNA extractions.

qPCR analyses of root DNA samples

A standard curve was first constructed using genomic DNA extracted from the STEU-8674 Pc isolate from the Stellenbosch University culture collection. DNA was extracted from the isolate as described under the small-scale DNA extraction method. The extracted genomic DNA was

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quantified using a NanoDrop™ Spectrophotometer (NanoDrop Technologies LLC, Wilmington, USA). The genomic DNA used for the standard curve consisted of eight five-fold dilutions with a concentration range of 54.74 ng/µl to 0.0007 ng/µl. The serial dilution of the genomic DNA was carried out using the elution buffer (PE) from the Nucleospin® PLANT II kit (Macherey-Nagel GmbH and Co., Düren, Germany). All standards were analysed in triplicate, with a no template control (NTC) also included in the analysis. The Pc species-specific probe-based assay of Masikane (2017) was used for qPCR analysis. The primers and probes were synthesised by Inqaba biotec™ (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa). Each 20 µl qPCR reaction consisted of 2 µl genomic DNA, 1x SensiFast™ Probe mix (Bioline GmbH, Luckenwalde, Germany), 400 mM each of primers Pc5R (CAGCACCATATATTTGTTCAGTCAG) and Pcy3F (AGCTTCCAACAGGCGAATAGGACC) and 200 mM of probe PcP5 (FAM/AGCTTCCAACAGGCGAATAGGACC/BHQ1). The amplification conditions consisted of one cycle of denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 40 s. Amplifications were conducted in a Rotor-Gene® 6000 (Corbett Life Science (Pty) Ltd., Mortlake, Australia) and analysed using the software v.2.3.1.

Pathogen DNA was quantified from the root DNA extracts using the same reaction conditions as those used for the standard curve (see section above). The optimal volume of root DNA extract that could be used in each 20 µl qPCR reaction, without resulting in PCR inhibition, was first determined. This was done by monitoring the copy number of the mutated *E. coli* gene, that was spiked into the DNA extraction buffer, in a dilution series of a subset of the root DNA samples. The mutated *E. coli* gene copy numbers were quantified using a previously designed SYBR® Green-based qPCR assay (Daniell *et al.*, 2012). The dilution factor at which the mutated *E. coli* gene was detected for all samples, and at which no further increases in gene copy numbers occurred, was taken as the optimal dilution factor for root DNA extracts. From this, it was established that Pc DNA was best quantified from root DNA extracts that were 10-fold diluted. Therefore, 1 µl of a 10-fold diluted root DNA extract was used in each 20 µl qPCR reaction.

All root DNA samples were analysed in duplicate, and each qPCR run furthermore incorporated one of the standard curve samples (positive control) and a no template control (NTC) (i.e. nuclease-free water). The pathogen root DNA concentration of each unknown sample was calculated by importing the standard curve and extrapolating values. The absolute pathogen DNA quantity (in ng/mg_{DW}) was calculated by using the formula: $\left(\frac{qPCR\ pathogen\ DNA\ concentration \times total\ volume\ of\ extracted\ genomic\ DNA \times dilution\ factor}{mg\ roots\ used\ in\ DNA\ extraction}\right)$ (Moein *et al.*, 2019).

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Phytophthora cinnamomi root baiting quantification

Root baiting quantification of Pc was only conducted for the four sampling months in 2018. For each orchard, for the 4x5 tree group, a subsample of 4 g of roots was taken from each tree and pooled to yield a total of 20 g of roots per group of five trees (Fig. 2). For the 1x20 tree groups, a subsample of 1 g of roots was taken from each tree and pooled to make up a total of 20 g of roots per group of 20 trees (Fig. 2). Samples were individually placed into rectangular plastic containers (4.2 L; 6 cm x 29 cm x 24 cm) and 700 ml of deionised water was added, making sure that the roots were fully submerged. For baiting, 10 lemon leaf discs (1 cm x 1 cm), which had been surface-sterilised using 70% ethanol and dabbed dry with paper towels, were floated on the water surface of each container. A lid was placed onto each root baiting container and the containers were incubated for a 3-day period at room temperature (23-24°C).

After the incubation period, all 10 lemon leaf discs were rinsed with deionised water, briefly dried and then plated onto oomycete-selective PARPH media plates (Kannwischer and Mitchell, 1978), with five leaf discs (each) plated onto two PARPH plates. The PARPH plates were incubated in the dark at 24°C for 3-5 days, after which, leaf disc infection was evaluated by placing the plates upside down onto a light microscope platform and evaluating for hyphal growth using the 100x magnification. Leaf disc infection was considered positive when the characteristic coralloid-type hyphal swellings of Pc were observed from the mycelial outgrowth. *Phytophthora cinnamomi* root colonisation was subsequently calculated as the percentage of infected leaf discs.

Molecular identification of *Phytophthora cinnamomi* from leaf disc baits

In addition to morphological identification, a subset of isolates, emerging from the infected leaf discs, were selected for sequence-based identification. Six isolates were randomly selected from leaf discs yielding characteristic Pc hyphal growth. The six isolates were selected from each sampling month of 2018, yielding a total of 24 isolates. Each isolate was sub-cultured onto potato dextrose agar amended with streptomycin (PDA^{+}) and allowed to grow in the dark at 24°C for ±7 days. Ten mycelial plugs (5 mm diameter) from each isolate were transferred to Petri dishes containing 20 ml of 1:7 diluted pea broth (Chen and Zentmyer, 1970) and incubated in the dark at 24°C for ± 2 weeks. The mycelia were harvested by filtration through sterilised cheesecloths and stored in 2 ml Eppendorf® tubes at -80°C overnight. The stored mycelia were lyophilised and fragmented as described for the small-scale DNA extractions.

Genomic DNA was extracted from the lyophilised mycelia using the same method described for the small-scale DNA extractions. The internal transcribed spacer (ITS1 and ITS2) regions were amplified using ITS4 and ITS6 primers (White *et al.*, 1990). Each PCR reaction consisted of 1 µl genomic DNA, 1x MyTaq™ Mix (Bioline GmbH, Luckenwalde,

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Germany), 0.4 µM each of the ITS 4 and ITS 6 primers and nuclease-free water to a final volume of 25 µl. The PCR amplification conditions consisted of denaturation for 3 min at 95°C, followed by 36 cycles of 95°C for 60 s, 50°C for 30 s and 72°C for 60 s. An extension step of 72˚C for 2 min was performed, followed by storage at 4˚C. Amplifications were performed in a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, USA). The ITS PCR products were then sent to the Central Analytical Facility (CAF, Stellenbosch, South Africa) for sequencing. The ITS sequences were edited manually using BioEdit v7.2.6.1 and were identified through BLAST analysis in the *Phytophthora* database (Park *et al.,* 2013; http://www.phytophthoradb.org/blast.php).

Statistical analysis

The monthly pathogen quantification data (qPCR and root baiting) and soil probe data (soil moisture and temperature) were subjected to analysis of variance (ANOVA) using the GLM (General Linear Models) procedure of SAS statistical software (Version 9.2; SAS Institute Inc., Cary, USA). For the pathogen quantification data, factors used in the ANOVA included year, month and region, whereas orchards were used as block replicates. Due to complex interactions in the aforementioned analysis, ANOVA analysis was also conducted on the monthly pathogen quantities using month and orchard as factors, for each region separately. The soil probe ANOVA analysis was conducted separately for each region, where orchards were considered as independent experiments and years as block replicates of the orchards. Pathogen root quantities obtained from the two sampling strategies (4x5 versus 1x20 tree group) and the two DNA extraction scales (small-scale versus large-scale) were also subjected to ANOVA analysis. Orchards were considered as block replicates for the sampling strategies and months as the subplot factor. Lastly, ANOVA analyses were also conducted on the two quantification methods (qPCR versus root baiting), the two evaluated DNA extraction buffers (PL1 versus CTAB) and the two investigated root age samples (white root tips versus older suberised feeder roots). For all of the data used in ANOVA analyses, the Shapiro-Wilk test was used to test for deviation from normality (Shapiro and Francia, 1972). Some datasets that deviated significantly from normality were transformed in order to improve normality. Fisher's least significant difference (LSD) test was calculated at the 95% confidence level.

Pearson’s correlation analysis and the significance of the correlations were used to investigate correlations between several of the investigated parameters. The analyses were conducted using XLStat (Version 2014; Addinsoft, New York, USA).

The combined effect of the measured parameters (pathogen quantity and/or soil moisture and temperature) was investigated using principal component analysis (PCA) and multifactor analysis (MFA). PCA analysis was conducted on the soil probe data (moisture and temperature) using XLStat (Version 2014; Addinsoft, New York, USA). The average monthly

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values of the six orchards were analysed for each of the sampling years separately (2017 and 2018). MFA analysis was conducted using the pathogen quantification data and soil probe data. In the data analysis, the two variables that were used as blocks consisted of the monthly pathogen quantities (root baiting, root qPCR and root qPCR log-transformed) and the corresponding monthly soil parameters (percentage moisture and 10-14°C, 15-19°C, 20-24°C, 25-29°C and 16-24°C temperature ranges). The MFA analysis was conducted using Statistica (Version 12; Statsoft Inc., Tulsa, USA).

RESULTS

Soil moisture and temperature data

Soil probe data could be successfully recorded for five out of the six orchards. The soil temperature data obtained from the EL orchard, located in the Letaba region, was deemed unreliable since it recorded extremely low temperatures that never reached above 20°C, even in the summer months. The five investigated soil temperature ranges (10-14°C, 15-19°C, 20-24°C, 25-29°C and 16-24°C) and soil moisture levels, varied to different extents across the two regions (Mooketsi and Letaba), over the two years (2017 and 2018) and for the different months (Table 2; Supplementary Tables 1 and 2, Figs. 1-3).

ANOVA analysis showed that there were significant differences between months, when considering the average values of the two years (2017 and 2018), for all of the investigated soil parameters, except for the 10-14°C temperature range in the Mooketsi region ($P = 0.1958$) (Table 3). The post-hoc analysis results of all of the soil parameters are shown in Table 2, but these will not be discussed in extensive detail due to the large number of months and parameters involved. The number of hours at 15-19°C (average of two years) were either zero or very low for some of the months, in both of the production regions (Table 2). In the Letaba region, January, February, March, April and December all had a significantly lower number of hours at 15-19°C, than the other months; January to March had zero hours, while April and December had 47.50 and 3.50, respectively. In Mooketsi, January, February, March, November and December all had a significantly lower number of hours at 15-19°C than the other months; January to March had zero hours and November and December had 14.33 and 1.67 hours, respectively. April also had a relatively low number of hours (41.17 hours) at 15-19°C in Mooketsi, which did not differ significantly from the aforementioned five months (January, February, March, November and December). In Letaba, the months of May, June, July, August and September had a significantly lower number of hours at 20-24°C, than the other months, while for Mooketsi these included the months of June, July and August. In general, for the 16-24°C temperature range, consecutive months did not differ significantly from each other (Table 2).

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According to ANOVA analysis, orchards differed significantly from each other at the 15-19°C temperature range in Mooketsi (P = 0.0366) and the 25-29°C temperature range in Letaba (P = 0.0219) (Table 3). In Mooketsi, the CM orchard had a significantly higher number of hours (282.64 hours) at the 15-19°C temperature range than the FM orchard (226.05 hours). In Letaba, the DL orchard had a significantly higher number of hours (8.55 hours) at 25-29°C than the BL orchard (1.64 hours). The percentage of soil moisture also differed significantly for orchards within the Letaba and Mooketsi regions (P = 0.0003 and < 0.0001, respectively) (Table 3). In Mooketsi, the AM orchard had a significantly higher percentage of soil moisture (53.95%) than the other two orchards (46.98 to 47.28%). In Letaba, the BL orchard had a significantly higher percentage of soil moisture (66.80%) than the DL orchard (64.59%).

The soil temperature and moisture data were further investigated using PCA analysis since this analysis can take the effect of all of the measured parameters into account. The PCA analysis was used to determine whether months could be grouped (warm, moderate and cold) based on their soil parameter values for each of the two production regions (Mooketsi and Letaba) and years (2017 and 2018).

PCA analyses were conducted on the data of each year separately and included both regions (Fig. 3). In 2017, the first two principal components (PC1 and PC2) accounted for 45.02% and 24.49% of the variation in data, respectively, whereas in 2018 this was 41.51% and 27.13%, respectively. The squared cosines of the PCA analyses were used to determine the contribution of the different soil parameters to the position of months on the plots. The 20-24°C temperature range demonstrated the highest quality of representation on PC1 in 2017 and 2018 (squared cosines of 0.838 and 0.764, respectively); i.e. the temperature range contributed most towards the position of the months on the plots. The 15-19°C temperature range was second best in its quality of representation on PC1 in 2017 (squared cosine of 0.772), whereas in 2018 it was second best (squared cosine of 0.507) along with the 10-14°C temperature range (squared cosine of 0.586). The 16-24°C (squared cosines of 0.389 and 0.476) and 25-29°C (squared cosines of 0.141 and 0.055) temperature ranges had a lower quality of representation on PC1 in both years (2017 and 2018, respectively), than the 15-19°C and 20-24°C temperature ranges; however, there was an exception for the 16-24°C temperature range in 2018. The percentage of soil moisture had a relatively small effect and demonstrated the lowest quality of representation on PC1 in 2017 and 2018 (squared cosines of 0.010 and 0.103, respectively). However, the percentage of soil moisture had the highest quality of representation on PC3 in both years (squared cosines of 0.640 and 0.642, respectively). Therefore, the PC1 and PC3 biplots were further investigated (Fig. 3), especially considering the importance of soil moisture in PRR development. The PC1 and PC3 biplots were furthermore considered important since the plots clearly separated the months according to region (Fig. 3), whereas the biplots of PC1 and PC2 did not (data not shown). Considering

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the PC1 and PC3 biplots, the Letaba orchards had a small tendency to have a higher soil moisture content than the Mooketsi orchards (Fig. 3); the Letaba orchards were located on the negative part of PC3 along with the soil moisture vector, whereas the Mooketsi orchards were located on the positive part of PC3. Despite this, all orchards were situated towards the centre of PC3 which indicates that the percentage of soil moisture only had a small effect on the position of months on the plot.

The position of months on the PC1 and PC3 biplots and their location on PC1, was used to subjectively group the months into cold, moderate and warm months. Months that were associated more with the positive part of PC1 were considered as warm months, while those associated with the negative part of PC1 were cold months. The moderate months were located more to the centre of PC1 (either positive or negative part of PC1) (Fig. 3). Since the position of months on the plots were not always similar in 2017 and 2018, the overall classification of months considering both years sometimes resulted in months being classified as (i) cold, (ii) moderate to cold, (iii) moderate, (iv) moderate to warm and (v) warm.

In the Letaba region, the warm months were January, December and only sometimes February, March and April. These months were classified as warm since they were located closer to the end of the 16-24°C and 20-24°C vectors in 2017 and 2018, respectively (Fig. 3). The moderate months included October, November and only sometimes February, March, April, May and September. The moderate months were not associated with any specific temperature range due to their location being more to the centre of the plot (Fig. 3). The cold months included June, July, August, and only sometimes May and September, depending on whether the 2017 or 2018 plots were involved. The cold months were associated with the lowest temperature range (10-14°C) in both years (Fig. 3). Although 2018 seemed warmer than 2017 in Letaba (warm months were associated with the 20-24°C and 16-24°C vectors in 2018 and 2017, respectively), this was not true when the actual data was investigated. This is due to the fact that the two years cannot always be compared directly when they are present on different biplots. To accurately compare the two years, both years would need to be placed onto the same biplot. However, a biplot containing the months from both years could not be interpreted due to the large number of overlapping labels on the plot (data not shown). Based on the actual data, some of the cold months (June and July) had a higher number of accumulated hours at the 10-14°C temperature range in 2018 (184.00 and 365.50 hours, respectively) than in 2017 (63.00 and 67.00 hours, respectively), while some of the warm months (January and February) had less hours at the 25-29°C temperature range in 2018 (5.50 and 13.00 hours, respectively) than in 2017 (23.50 and 48.50 hours, respectively). Furthermore, April and May had more hours at the 15-19˚C temperature range in 2018 (81.00 and 636.00 respectively) than in 2017 (14.00 and 529.50 respectively) (Supplementary Tables 1 and 2). As a result, 2017 was considered warmer than 2018.

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In the Mooketsi region, months could also be subjectively grouped into cold, cold to moderate, moderate, moderate to warm and warm, when the biplots of both years (2017 and 2018) were considered (Fig. 3). The warm months were January, March, April, November, December and only sometimes February, May and October. In 2017, January and February were mainly associated with the 25-29°C vector, whereas the other warm months were associated with the 20-24°C vector. In 2018, all of the warm months (except for one orchard in February) were mainly associated with the 16-24°C vector, suggesting that 2017 was warmer than 2018 (Fig. 3). The moderate months included September and only sometimes February, May, June, July, August and October. The months of June, July and August were sometimes considered as cold months and were associated with the 15-19°C temperature range (Fig. 3). Based on the actual data, it was difficult to determine which year was warmer in Mooketsi, since there were inconsistencies with trends between the hours accumulated at the 20-24°C and 25-29°C temperature ranges for the warm and moderate to warm months (January, February, March and April), however, it appeared as though 2018 was warmer than 2017 when taking all four months into consideration (Supplementary Tables 1 and 2). Nevertheless, for some of the cold to moderate months (June and July) there were more hours accumulated at the 10-14°C temperature range in 2018 (25.67 and 59.00 hours, respectively) than in 2017 (0.00 and 0.00, respectively), suggesting 2018 was cooler than 2017 during winter.

Monthly colonisation patterns of *Phytophthora cinnamomi*

qPCR quantification

To illustrate the trends in Pc root colonisation, the monthly Pc root DNA quantities are shown as line graphs for the six investigated orchards (Figs. 4A and B). The statistical results of the line graph data are shown in Tables 4 to 6.

ANOVA analysis, where the orchards were used as block replicates and not as a factor, showed that there were no significant year x month x region ($P = 0.3841$), month x region ($P = 0.6445$) and year x region ($P = 0.4169$) interactions. There was a significant month x year interaction ($P < 0.0001$). This interaction showed that Pc root DNA quantities were significantly higher in May 2018 than in May 2017, whereas October/November 2018 had significantly lower Pc root DNA quantities than October/November 2017. The months of August and March did not differ significantly in Pc root DNA quantities between the two years. Furthermore, only considering 2017, May 2017 had significantly higher Pc root DNA quantities than March 2017 but did not differ significantly from August and October/November 2017. In 2018, May had significantly higher Pc root DNA quantities than the other three months (March, August and October/November) in the same year (2018). However, the ANOVA analysis further showed that the orchards (block replicates) differed significantly ($P < 0.0181$) for the year x month x

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region and month x region interactions, i.e. the orchards did not behave similarly in the years, months and regions. Therefore, the years (2017 and 2018) and regions (Letaba and Mooketsi) were also investigated separately, using two factor ANOVA analysis (month x orchard).

In the Letaba region, ANOVA analysis showed that there was no significant month x orchard interaction for the Pc root DNA quantities in 2018 (P = 0.3064), but that there was in 2017 (P = 0.0042) (Table 4). Therefore, the average Pc root DNA quantities of the three orchards could be considered for 2018, but not for 2017. In 2017, for orchards DL and EL, the month of May yielded significantly higher Pc root DNA quantities than some of the other months; March and October/November for orchard EL, and August for orchard DL (Table 5). Contrarily, the BL orchard had significantly higher Pc root DNA quantities in August than in May and March, in 2017 (Table 5). In 2018, in all three orchards (averages could be considered), the Pc root DNA quantities differed significantly (P < 0.0001) between the four months (Table 4). May had significantly higher Pc root DNA quantities than the other three months, while August had significantly higher Pc root DNA quantities than October/November, in 2018 (Table 5).

In the Mooketsi region, ANOVA analysis showed that there was no significant month x orchard interaction for Pc root DNA quantities in 2017 (P = 0.1344), but that there was for 2018 (P = 0.0008) (Table 4). In 2017, there were no significant differences in Pc root DNA quantities between the four months (P = 0.2690) (Tables 4 and 6). In 2018, the month x orchard interaction showed that the AM and CM orchards were similar in their Pc root colonisation patterns; both orchards had significantly higher Pc root DNA quantities in May than in the other three months (Table 6). The FM orchard differed from the other two orchards since no monthly patterns were evident in 2018; Pc root DNA quantities did not differ significantly between any of the months. Orchard FM furthermore had significantly lower Pc root DNA quantities than the other two orchards in May (Table 6).

Root baiting quantification

The Pc root colonisation trends, using root baiting analysis in 2018, are shown as line graphs in Fig. 4C for the six investigated orchards. Root baiting analysis was not conducted in 2017. The statistically analysed line graph data from Fig. 4C is shown in Tables 4 to 6. The data was analysed statistically using two factor ANOVA analysis (month x orchard), for the same reasons as specified in the qPCR quantification section.

In the Letaba region, there was a significant orchard x month interaction (P = 0.0147) (Table 4) and, therefore, the data of the orchards were considered separately. All three orchards had a significantly higher percentage of Pc-infected leaf discs in May than in the other three months (Table 5). Furthermore, for two of the orchards (DL and EL), August had a significantly higher percentage of Pc-infected leaf discs than October/November (Table 5).

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In the Mooketsi region, there was also a significant orchard x month interaction (P = 0.0109) for the root baiting data (Table 4). The FM orchard did not exhibit any monthly root colonisation patterns; the percentage of Pc-infected leaf discs did not differ significantly between the four months (Table 6). The remaining two orchards (AM and CM) had significantly higher Pc leaf disc infections in May than in March and October/November. Orchard FM furthermore had a significantly lower percentage of Pc-infected leaf discs in May than the other two orchards (Table 6).

Correlation analyses between soil parameters and *Phytophthora cinnamomi* quantities

Several significant correlations ($P < 0.05$) were observed between the soil temperature ranges and the Pc root quantities (Pc root DNA or percentage Pc-infected leaf discs) when taking both years (2017 and 2018) into consideration (Table 7). However, for the soil moisture data, only one significant negative correlation (r = -0.887; P = 0.045) was found (Table 7). Only correlations that were highly significant ($P \leq 0.009$) will be discussed in this section. Most of these correlations were found between Pc root quantities and temperature ranges in the same month of pathogen quantification, or in three or less months that preceded the month of pathogen quantification. The most significant correlations were found between the February temperature ranges and the percentage of Pc-infected leaf discs obtained in May; a negative correlation with the 25-29°C temperature range (r = -0.999; $P < 0.0001$) and positive correlations with the 16-24°C and 20-24°C temperature ranges (r = 0.998; $P < 0.0001$ and r = 0.974; P = 0.005, respectively). Other highly significant correlations ($P \leq 0.009$) consisted of temperature ranges of a specific month corresponding with its own Pc root quantities: (i) the March 25-29°C temperature range was positively correlated with Pc root DNA quantities obtained in March (r = 0.963, P = 0.008) and (ii) the percentage of Pc-infected leaf discs in May was negatively correlated with the May 20-24°C temperature range (r = -0.965, P = 0.008) and positively correlated with the May 15-19°C temperature range (r = 0.971, P = 0.006). The last two highly significant correlations were difficult to interpret since these correlations were between a specific month's temperature range that was after the month in which Pc was quantified; the April 16-24°C temperature range was negatively correlated with the percentage of Pc-infected leaf discs in March (r = -0.973 and P = 0.005), while the June 20-24°C temperature range was negatively correlated with the percentage of Pc-infected leaf discs obtained in May (r = -0.961; P = 0.009) (Table 7).

The correlation circle, generated with MFA analysis, revealed overall trends in correlations that existed between some of the soil parameters (% moisture and temperature ranges of 10-14°C, 15-19°C, 20-24°C and 25-29°C) and the pathogen quantities (root qPCR 2017 and 2018 and root baiting 2018). The MFA analysis only considered the data of the four months in which Pc was quantified (March, May, August and October/November) for the two years (Fig. 5).

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The correlation circle showed that correlations between pathogen quantities and soil parameters were not strong since the vectors representing the pathogen quantities were not in close proximity to those of the soil parameters. Of the soil parameters, the 15-19°C and 16-24°C temperature ranges were most positively correlated with the pathogen quantities since these vectors were closest to each other and all pointed in the same direction. In contrast, Pc root quantities were negatively correlated with the 20-24°C temperature range due to these two sets of vectors pointing in opposite directions. Pearson's correlation analyses supported the correlation trends of the MFA analysis, as well as the fact that the correlations were weak; there were no significant correlations. The most significant correlations (having the smallest *P*-values) were between the root baiting quantities that were (i) negatively correlated with the 20-24°C temperature range (r = -0.415, P = 0.069), followed by (ii) a positive correlation with the 15-19°C temperature range (r = 0.405, P = 0.077). The second most significant correlations also involved the 15-19°C and 20-24°C temperature ranges, which were positively and negatively correlated with the root qPCR quantities (r = 0.299, P = 0.201 and r = -0.290, P = 0.215, respectively). The contribution of the 16-24 and 25-29°C temperature ranges as well as the percentage of soil moisture to correlations, were smaller than those of the other soil parameters; the ends of the vectors representing these factors did not reach the outer correlation circle. The vectors reaching the outside of the correlation circle represent parameters that had a stronger effect.

Correlation between the root baiting and qPCR quantification data in 2018

The MFA analysis showed that there was a very strong correlation between DNA quantities of the pathogen (qPCR and log-transformed qPCR analysis) and the percentage of Pc-infected leaf discs (root baiting analysis) (Figs. 4 and 5); all of the vectors representing the pathogen quantities were positioned close to each other. This was further supported by highly significant Pearson's correlation values between all three of the parameters (r > 0.600; P < 0.0001).

Comparison of sampling strategies

qPCR quantification

Pearson's correlation analyses showed that the Pc root DNA quantities of the 4x5 and 1x20 tree groups were significantly positively correlated for all three of the investigated sampling months; August 2017 (r = 0.794, P = 0.002), May 2018 (r = 0.932, P < 0.0001) and August 2018 (r = 0.916, P < 0.0001) (Fig. 6A-C). ANOVA analysis furthermore showed that Pc root DNA quantities obtained from the 4x5 tree group did not differ significantly (P > 0.1522) from the Pc root DNA quantities of the 1x20 tree group for two of the sampling months (August 2017 and August 2018) (Table 8). For the third sampling month (May 2018), there was a significant difference (P = 0.0482) between the two sampling strategies, although this was

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almost at 0.05 (Table 8); the 1x20 tree group yielded significantly lower Pc root DNA quantities (0.741 ng/mg_{DW}) than the 4x5 tree group (0.942 ng/mg_{DW}).

Root baiting quantification

Pearson's correlation analyses showed that the percentage of Pc-infected leaf discs, obtained from the 4x5 tree groups, were significantly correlated (r = 0.847, P < 0.0001) with those obtained from the 1x20 tree group when all four of the 2018 sampling months were considered (Fig. 6D). ANOVA analysis furthermore showed that the percentage of Pc-infected leaf discs did not differ significantly between the two sampling strategies (P = 0.8042) (Table 8). However, an important observation was made, in that the 1x20 tree group was less sensitive in detecting the pathogen; in five instances the 1x20 tree group did not detect Pc in samples that were positively detected by the 4x5 tree group, whereas in only one instance the 4x5 tree group did not detect Pc in samples positively detected by the 1x20 tree group (Fig. 6D).

Comparison of DNA extraction scales

Large-scale versus small-scale DNA extraction

Correlation analysis showed that, for August 2017, there was no significant correlation (r = 0.036, P = 0.852) between the two DNA extraction scales. However, for May 2018 and August 2018, the two extraction scales were significantly correlated (r = 0.559 and 0.463; P = 0.001 and 0.010, respectively).

The small-scale DNA extractions yielded significantly higher Pc root DNA quantities (P < 0.0207), than the large-scale DNA extractions, for all of the three investigated sampling months (Table 8). The Pc root quantities for the small-scale DNA extraction were 0.087, 1.430 and 0.150 ng/mg_{DW}, for August 2017, May 2018 and August 2018, respectively. For the large-scale DNA extraction, the Pc root quantities for the corresponding sampling months were 0.033, 0.250 and 0.050 ng/mg_{DW}, respectively.

The variation in the Pc DNA quantities obtained from the two DNA extraction scales between replicates of the same root sample was investigated by calculating the percentage coefficient of variation (%CV). The small-scale DNA extractions yielded less variable Pc root DNA quantities than the large-scale DNA extractions for the 1x20 tree groups for all three sampling months (August 2017, May 2018 and August 2018); the small-scale DNA extractions varied from 67.7% to 104.5%, whereas the large-scale DNA extractions varied from 73.5% to 131.0%. However, for the 4x5 tree groups, for two of the sampling months (August 2017 and August 2018) the small-scale DNA extractions (133.0 and 136.9%, respectively) yielded more variable Pc root DNA quantities than the large-scale DNA extraction (104.1% and 110.3%, respectively).

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Investigating the efficacy of a CTAB buffer in the Nucleospin kit extractions

The CTAB-based buffer, when used in the small-scale Nucleospin® kit DNA extractions, yielded significantly lower (P = 0.008) Pc root DNA quantities than when the Nucleospin® kit's PL1 buffer was used. The DNA extracts of the PL1 buffer yielded an average Pc root DNA quantity of 0.017 ng/mg_{DW}, whereas the CTAB-based buffer yielded significantly lower Pc quantities (0.006 ng/mg_{DW}); approximately 50% less Pc DNA.

Comparison of DNA quantities extracted from different aged roots

There was no significant difference (P = 0.862) between Pc root DNA quantities that were obtained from samples containing only white root tips (0.097 ng/mg_{DW}) and only older suberised feeder roots (0.093 ng/mg_{DW}). The aforementioned Pc DNA quantities were all extracted using small-scale root DNA extractions.

DISCUSSION

The current study was able to identify Pc root colonisation patterns in avocado orchards located in two production regions (Mooketsi and Letaba) in Limpopo, South Africa. The Pc root colonisation patterns that were studied in four sampling months (March, May, August and October/November) over two years (2017 and 2018), showed that colonisation patterns were more evident in 2018 than in 2017. Overall, late autumn (May) was identified as having the highest Pc root colonisation levels, which was likely due to the influence of soil temperatures, tree phenological events and factors that can affect host susceptibility, rather than soil moisture; soil moisture was considered as being only a minor contributing factor. The establishment of an effective orchard sampling strategy and Pc root quantification method were important for accurately determining Pc root colonisation patterns. *Phytophthora cinnamomi* root quantification using qPCR analysis from small-scale root DNA extracts (50 mg roots), was as effective as root baiting quantification in identifying colonisation trends. The evaluation of two orchard sampling strategies consisting of either four groups of five trees each (4x5 tree group) (qPCR and root baiting data) or one group of 20 trees (1x20 tree group) (qPCR data), showed that both sampling strategies were effective in quantifying Pc from avocado roots and establishing seasonal colonisation patterns in avocado orchards.

The identification of late autumn (May) as being the critical period for Pc root colonisation in the Limpopo production regions, is an important finding, although this was not always consistent or evident in both sampling years and in all six orchards. In 2018, the peak in root colonisation levels in May was evident in all three orchards in Letaba (qPCR and root baiting quantification) and in two orchards in Mooketsi (qPCR and/or root baiting quantification). In the third Mooketsi orchard, Pc root quantities did not differ significantly throughout the study, irrespective of the time (months and years) and pathogen quantification method used. In 2017,

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higher Pc root quantities were less evident for May, which was likely due to both regions (to a lesser extent in Mooketsi) having environmental soil conditions that were less conducive to PRR in 2017 than 2018; there were generally warmer soil temperatures in 2017 than in 2018. In 2017, May, but also August, yielded the highest Pc root DNA quantities (qPCR analysis) in two out of the three Letaba orchards, although these differences were not significant in comparison to the other two months (March and October/November). In Mooketsi, no significant differences were evident for the monthly Pc root DNA quantities obtained in 2017.

The peak in Pc root colonisation levels occurring in late autumn (May), was further supported by the use of orchards as block replicates in statistical analyses. This showed that in both years (2017 and 2018), May had significantly higher Pc root DNA quantities than March. Furthermore, in 2018, May had significantly higher pathogen root quantities than the other two months (August and October/November), however, these three months did not differ from each other in 2017. The identification of May as the critical colonisation period (and likely infections) is partially supported by a study conducted by Zentmyer (1981). Zentmyer (1981) showed that Pc root infections in avocado seedlings, planted bimonthly in avocado orchards located in California (USA), were typically highest during the autumn months (September to November). However, contrary to the current study's finding, Zentmyer (1981) also found that equally high Pc root infection levels occurred in late summer (July to September). This may be related to the use of avocado seedlings, in comparison to the avocado orchard trees which were used in the current study, for reasons that will be discussed later on in this section.

Following the peak in Pc root colonisation in late autumn (May), a general decline in Pc root quantities was observed from May to October/November (late spring) in both production regions (Mooketsi and Letaba). This was evident from the fact that in August (late winter), five out of the six orchards (qPCR or root baiting quantification) for 2017 and/or 2018 had significantly lower Pc root quantities than May. August furthermore yielded Pc root quantities that were significantly higher than October/November in four of the orchards (qPCR or root baiting quantification) for 2017 and/or 2018. The low Pc root quantities observed in October/November were also maintained in March. This was due to March having similarly low Pc root quantities to those observed in October/November in five of the orchards (qPCR or root baiting quantification) for 2017 and/or 2018; March and October/November did not differ significantly in Pc root quantities.

The peak in Pc root colonisation observed in late autumn, in the avocado production regions of Limpopo, cannot be explained by a single contributing factor. This was expected since disease development is known to be influenced by complex interactions between several factors (Falcon *et al*., 1984; Downer *et al*., 2002). For example, root flush cannot be the only factor causing the peak in Pc root colonisation since two root flush windows occur in avocado orchards located in Limpopo, South Africa; one from October to January (late spring to early

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summer) and the second from February to April (late summer to late autumn). The abundance of white fleshy feeder roots (i.e. main infection sites) and root exudates produced during root flush windows are important for breaking pathogen dormancy and stimulating the start of the disease cycle. An increase in pathogen activity around root flush windows has already been suggested by Ploetz *et al.* (1992). The importance of root flushes in stimulating PRR development is further supported by the fact that the pathogen relies on chemical stimuli (Khew and Zentmyer, 1973), such as root exudates, to locate the host, and that root exudates are in greater abundance during periods of root proliferation. The breaking of pathogen dormancy (stimulation of pathogen germination) is known to require exogenous chemical stimuli, such as amino and organic acids that are present in root exudates (Mircetech *et al.*, 1968; Mircetech and Zentmyer, 1969; Malajczuk and McComb, 1977). Considering only soil moisture and temperature could also not solely explain the high colonisation levels observed in late autumn since many months were identified as being conducive; similar hours were measured at some of the investigated soil temperature ranges, as well as similar soil moisture levels for several of the months.

Although soil moisture is essential for PRR development (i.e. sporangial production, zoospore release and zoospore dissemination), based on the current study, it seemed to only play a minor role in commercial avocado orchards. Zentmyer and Richards (1952) also suggested that soil moisture only has a small effect on PRR development in irrigated avocado orchards. All of the orchards that were analysed in the current study were irrigated (drip or micro-sprinklers), which can alleviate dry rainfall periods that would otherwise inhibit pathogen development. Furthermore, the orchards from the current study had very good irrigation scheduling, as was evident from the relatively small variation observed in the soil moisture data across the months for both years (46.68 to 52.39% for Mooketsi and 60.65 to 68.99% for Letaba), with production regions also having summer rainfall (November through to March). The fact that significant differences were observed in the percentage of moisture between the months and regions (Mooketsi versus Letaba), is difficult to interpret since none of the other analyses that were conducted in the current study supported the importance of soil moisture. There were no highly significant correlations that were found between the average monthly percentage of soil moisture and Pc quantities. In fact, the only significant correlation that was found included a negative correlation between the soil moisture percentage of September and a pathogen quantity from an unrelated quantification month (March). The MFA analysis also showed that soil moisture was much less correlated with Pc root quantities (qPCR and root baiting quantification) than the different temperature ranges. Furthermore, only a small effect of soil moisture could be seen in the PCA analysis, where the Letaba orchards (higher rainfall region) tended to have higher soil moisture levels than the Mooketsi orchards; however, this was only when PC3 was considered. This fits in with the historical and growers' perception,

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that the Letaba region is more conducive to PRR than Mooketsi (McLeod *et al.*, 2018). The fact that all three Letaba orchards tended to yield higher percentages of Pc-infected leaf discs with the root baiting analysis, further suggests this claim. In addition to soil moisture, soil type also likely contributes to the aforementioned observation. The Letaba orchards have a higher soil clay content (23 to 29%) than the Mooketsi orchards (7 to 19%) and are thus more prone to slower soil drainage. This results in longer periods of soil saturation that are more conducive to Pc root infections and PRR development (Ploetz and Schaffer, 1988; Reeksting *et al.*, 2014a, b).

The high Pc root colonisation levels observed in late autumn can be best explained by factors that affect the host's susceptibility, tree phenological events and also the 15-19°C and 20-24°C soil temperature ranges. The lowering of soil temperatures in late autumn (May) favours Pc growth over the avocado host, making trees more susceptible to root infections (Dann *et al.*, 2013), since avocado trees are typically cold-sensitive (Zentmyer, 1981). In April, and to a greater extent in May, soil temperatures started to accumulate at the 15-19°C temperature range; January to March had zero hours at this temperature range. Another host factor contributing towards the peak in Pc root colonisation is tree stress brought on by the fruit bearing period. Wolstenhome (1981) has previously hypothesised that the late summer and autumn period is the most critical time for Pc-infected avocado trees in South Africa. It is suggested that this is driven by tree stress brought on by the preceding fruit bearing period (from March onwards) (Wolstenholme, 1981). Fruiting likely increases the susceptibility of avocado tree roots to Pc infections since it has a draining effect on the tree's food reserves (strongest sink for photosynthates), also causing a dwarfing effect on the overall tree growth (Wolstenholme, 1981). In addition, the root flush preceding late autumn (February to April) would have further favoured Pc infection and colonisation, as previously discussed. In April and May, soil temperatures (15-19°C versus 20-24°C) were likely more conducive to zoospore production and release from sporangia. *Phytophthora cinnamomi* root quantities that were obtained from the May root baiting analyses were significantly positively correlated with the number of accumulated hours in the 15-19°C temperature range of May, but significantly negatively correlated with the 20-24°C temperature range. This is likely due to the fact that the optimal temperatures for sporangial production occur at 21-30°C (Zentmyer and Marshall, 1959; Byrt and Grant, 1979; Nesbitt *et al.*, 1979; Shearer, 2014), whereas the most efficient production and release of zoospores (main infective agent) have been reported at temperatures of 15-18°C (Khew and Zentmyer, 1973; Hwang *et al.*, 1975; Byrt and Grant, 1979); i.e. each of the two temperature ranges (20-24°C and 15-19°C) are optimal for different reproductive structures (sporangia and zoospores, respectively) and are thus required for effective root infection and colonisation. The highly significant positive correlations of the May pathogen quantities (root baiting analyses) with the 16-24°C and 20-24°C temperature ranges

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of February, and a negative correlation with the 25-29°C temperature range, might be related to the amount of root growth occurring in February, and thus the amount of new infection sites becoming available.

In addition to the importance of the 15-19°C and 20-24°C temperature ranges in understanding the peak in Pc root colonisation, these two temperature ranges were useful for identifying several other aspects relating to temperature differences between months and PRR development. In the PCA analyses, these temperature ranges contributed most towards the position of months on the biplots (higher PC1 squared cosines). In the MFA analyses (comparing overall Pc root quantities with temperature ranges and percentage soil moisture), the two temperature ranges furthermore had the highest overall (all eight quantification months across the two years) correlation with pathogen quantities (qPCR and root baiting quantification). It is, however, important to note that the 20-24°C temperature range consisted of a negative correlation, whereas the 15-19°C temperature range had a positive correlation. The 15-19°C temperature range was also useful for understanding why, in Mooketsi, one orchard did not exhibit any root colonisation patterns and yielded significantly lower Pc root quantities than the other two Mooketsi orchards in May 2018; the orchard had a significantly lower number of hours at 15-19°C. The fact that the lowest Pc root colonisation levels were observed in late summer (March) in 2018, is likely due to the zero hours that were present at the 15-19°C temperature range in both regions, in March and also in the months leading up to March (January and February). The 16-24°C temperature range seemed less important in our analyses, which is likely due to the fact that it is less effective in distinguishing between warm and cold months (based on the PCA squared cosines of PC1). Therefore, it is less effective at differentiating between optimal temperatures for sporangial production (20-24°C) and zoospore production and release (15-19°C), as well as for when the host becomes more susceptible to pathogen infection and colonisation at lower soil temperatures.

The study of Zentmyer (1981) supports the hypothesis of the current study in that an interplay between temperatures of above 15°C and 20°C are important for Pc root infection and colonisation in avocado. Zentmyer (1981) found that in California (USA), most of the months which had the highest Pc root infections, with the exception of November, were characterised by a high number of hours above 15°C (670 to 720 hours) and 20°C (720 to 570 hours). In contrast, the majority of the months which yielded low Pc root infections, had a low number of hours above 15°C (0 to 370 hours) (Zentmyer, 1981), with the exception of May and June. However, although the latter two months had a high number of hours above 15°C (370 and 720 hours), a very low number of hours occurred above 20°C (44 and 62 hours) (Zentmyer, 1981), thus only providing optimal conditions for zoospore production and release and not sporangial production.

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In addition to all of the above-mentioned factors that can influence Pc colonisation, other factors that were not analysed in the current study include management practices and differences between rootstocks and cultivars. An important management practice that was not included as a variable in the current study is mulching. Mulching is known to assist in PRR suppression and can also influence tree vigour (i.e. increase pathogen tolerance) through improved nutrient and water holding capacity of the soil (Wolstenholme and Sheard, 2010; Dann *et al.*, 2013). All of the growers that participated in the current study did not practice mulching in their avocado orchards. It is also likely that root colonisation patterns will differ in mulched orchards due to the effect of mulches on soil temperatures. Gruda (2008) reported that wood chip mulches resulted in the lowering of soil temperatures, and mulches are furthermore known to reduce soil temperature fluctuations (Downer *et al.*, 2002). The latter might negatively affect zoospore production and release in late autumn since these life cycle stages are triggered by a lowering of soil temperatures (Khew and Zentmyer, 1973; Hwang *et al.*, 1975; Byrt and Grant, 1979). Although scion/rootstock combinations would likely have an influence on the Pc root colonisation patterns (i.e. variations in the timing of fruit bearing and root flushes), it was not possible in the current study to conduct statistical analyses on this aspect since three orchards were Maluma-Hass/Duke 7, with other combinations only being represented in one orchard.

In the current study, Pc root quantities were investigated using different quantification methods, including qPCR (small-scale and large-scale DNA extractions) and root baiting analyses. The Pc root DNA quantities obtained through qPCR analysis of small-scale DNA extractions were highly significantly correlated with the percentage of Pc-infected leaf discs obtained through root baiting quantification. This shows that both quantification approaches gave equally reliable indications of Pc root colonisation patterns. The choice in quantification method would thus be dependent on the skills and equipment/facilities available. The large-scale DNA extraction, although also showing correlation with the small-scale DNA extraction, has a drawback in that it resulted in significantly lower Pc root DNA quantities than the small-scale DNA extraction. Furthermore, based on the percentage coefficient of variation estimates, the large-scale DNA extraction method did not consistently result in reduced variability between Pc root DNA quantities obtained from replicates of the same sample. The large-scale DNA extraction is thus not recommended for Pc root quantification. The poor performance of the large-scale DNA extraction is most likely due to the composition of the DNA extraction buffer that was used during extractions; when small-scale DNA extractions were conducted using the Nucleospin® kit's PL1 buffer, significantly higher Pc root DNA quantities were obtained than when the CTAB-based buffer was used. The CTAB-based buffer was used in the large-scale DNA extractions due to cost implications (the kit's extraction buffer is very expensive). Modification of DNA extraction buffers can help to improve the DNA

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quantity and quality of large-scale DNA extractions (Khan *et al.,* 2007) and should thus be investigated in future.

This is the first study that has compared different sampling strategies for quantifying Pc in avocado orchards or any perennial host plant species. It was shown that Pc can be quantified from roots using either four tree groups each containing five trees (4x5 tree group) or one tree group containing 20 trees (1x20 tree group); Pc root quantities were significantly correlated between the two tree groups (qPCR and root baiting quantification). The 1x20 tree group, however, may be slightly less sensitive, since it either sometimes resulted in significantly lower Pc root DNA quantities (qPCR analyses; although the significance level was low [$P = 0.0482$]) or fewer samples had positive Pc detections (root baiting analyses). Whether the 4x5 or 1x20 tree groups are used for seasonal Pc root colonisation studies, is probably not as important as the number of replicates analysed per orchard. In the current study, the seasonal colonisation patterns were assessed using a total of four replicates (one replicate for each group of five trees [4x5 tree group]) per orchard for qPCR and root baiting analyses, which were able to reveal several significant differences in Pc root quantities between the months. Therefore, at least four replicates per orchard should be used. However, due to the high variability in quantification data, which was evident from the percentage coefficient of variation analysis, using more replicates per orchard (either for the 4x5 or 1x20 tree group) might allow for more significant differences to be observed between monthly samplings. This is especially important for periods when low Pc root quantities are present. For example, a larger variation was observed in Pc root DNA quantities between small-scale DNA extraction replicates of the 4x5 tree group for the August 2017 and 2018 sampling months (133.0% and 136.9% CV, respectively) in comparison to the May 2018 sampling month (96.0% CV). This is likely due to the fact that, similar to the nature of distribution of low Pc soil population levels in soil samples (Sena *et al.*, 2018), lower root colonisation levels can lead to an uneven spread of the pathogen within root samples.

A concern with root sampling during months that do not coincide with root flush windows, such as in August, is that pathogen quantification might be inaccurate. For sampling months that were not positioned within a root flush window, a mixture of white root tips and older suberised feeder roots were often required to obtain sufficient roots for sample analysis. Since the white feeder root tips of avocado are the main infection site for Pc (Zentmyer, 1980), this may have influenced the seasonal Pc root colonisation patterns. However, it was shown that samples of two root compositions (white root tips versus older suberised feeder roots) did not differ significantly from one another in Pc root DNA quantities and therefore did not influence the seasonal colonisation trend. This is likely due to Pc infecting the white feeder roots prior to the suberisation of the root tips. As a result, the newly suberised feeder roots still contained

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Pc DNA while still being attached to the tree. Whether root age affected the root baiting results, was not investigated in the current study and thus requires further investigation.

In conclusion, the results from this study will help to improve the precision management of PRR through a better understanding of when critical root colonisation periods occur and thus when PRR control is most important. However, it should be kept in mind that the presented data pertains to the studied orchards and their management practices. Since late autumn was identified as the period with the highest root colonisation levels, it is important that effective management practices, such as phosphonate treatments, are employed in a way which ensures that optimal root phosphite levels are achieved prior to this peak in root colonisation. For example, phosphonate treatments should be applied during the late summer/early autumn root flush window period (February to April). Furthermore, the use of all other integrated management practices, such as irrigation scheduling and inorganic nutrition, should be in place from late summer to late autumn. Knowing when the highest root colonisation levels occur, can also help in assisting to select time points for evaluating the efficacy of management strategies. Quantifications performed in May are likely to reveal greater differences in pathogens quantities as a response to management practices than if quantifications are conducted in March.

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Table 1. Soil characteristics and scion/rootstock combinations of six avocado orchards where the seasonal root colonisation patterns of *Phytophthora cinnamomi* were investigated in two production regions (Mooketsi and Letaba) in the Limpopo province, South Africa. [a]

Orchard	Production region	Scion/Rootstock	pH	Classification	CEC (cmol(+)/kg) [b]	Clay (%)	Silt (%)	Sand (%)	Water holding capacity (mm/m) [c]
AM	Mooketsi	Maluma-Hass/Duke 7	6.9	Loamy sand	5.38	9	8	83	134.85
BL	Letaba	Carmen/Dusa	4.0	Sandy clay loam	5.77	29	6	65	109.70
CM	Mooketsi	Hass/Dusa	5.9	Loamy sand	8.80	7	6	87	129.61
DL	Letaba	Pinkerton/Duke 7	4.8	Sandy clay loam	6.25	23	8	69	110.94
EL	Letaba	Maluma-Hass/Duke 7	4.5	Sandy clay loam	4.76	25	6	69	111.91
FM	Mooketsi	Maluma-Hass/Duke 7	6.5	Sandy Loam	7.59	19	10	71	101.67

[a] Soil sampling was conducted at a depth of 25 cm. Five random soil samples were collected from each orchard and mixed thoroughly. A representative sample of each orchard was sent for soil analysis at Bemlab (Somerset, South Africa).

[b] CEC (cmol(+)/kg) or cation exchange capacity is a key determinant of soil fertility. It refers to the soil's ability to retain and exchange essential cations such as calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^{+}) and ammonium (NH^{4+}).

[c] Water holding capacity (mm/m) is the total amount of water that a soil can hold at field capacity without the loss of water through gravitational forces.

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Table 2. Percentage of soil moisture and accumulated hours at five different soil temperature ranges, in two avocado production regions (Letaba and Mooketsi) situated in the Limpopo province, South Africa, over two years (2017 and 2018) and by different months. [a]

	Moisture (%)		10-14°C		15-19°C		20-24°C		16-24°C		25-29°C	
Month	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**
January	67.549 ab	51.139 ab	0.00 c	0.00 a	0.00 d	0.00 f	720.50 a	609.67 a	714.75 a	609.83 de	14.500 b	123.50 b
February	68.895 a	51.168 ab	0.00 c	0.00 a	0.00 d	0.00 f	623.75 ab	425.50 b	623.75 ab	468.33 f	30.750 a	188.50 a
March	67.722 ab	49.640 bc	0.00 c	0.00 a	0.00 d	0.00 f	666.25 a	642.67 a	666.25 ab	642.67 bcd	8.000 b	41.33 cd
April	67.092 ab	48.128 cd	0.00 c	0.00 a	47.50 d	41.17 ef	530.00 bc	653.50 a	577.50 ab	696.67 abc	0.00 b	8.17 d
May	65.918 bc	47.902 d	0.25 c	0.00 a	582.75 ab	409.33 c	51.25 e	326.33 b	618.50 ab	735.67a	0.00 b	0.00 d
June	63.004 e	48.016 d	123.50 ab	12.83 a	584.00 ab	600.50 b	1.25 e	10.00 c	350.75 cd	549.50 ef	0.00 b	0.00 d
July	63.151 de	48.637 cd	216.25 a	29.50 a	527.50 b	699.67 a	0.00 e	3.50 c	300.00 d	618.83 cde	0.00 b	0.00 d
August	63.982 cde	48.617 cd	38.00 bc	1.50 a	701.50 a	661.83 ab	3.75 e	67.50 c	524.50 bc	708.67 ab	0.00 b	0.50 d
September	63.522 cde	48.891 cd	8.00 bc	0.50 a	544.25 ab	293.00 d	105.75 e	400.67 b	618.50 ab	692.17 abcd	0.00 b	0.67 d
October	65.170 bcde	49.671 bc	0.00 c	0.00 a	495.75 b	123.00 e	248.00 d	620.67 a	740.00 a	743.67 a	0.00 b	0.00 d
November	65.681 bcd	50.885 ab	0.00 c	0.00 a	228.50 c	14.33 f	490.00 c	694.33 a	718.50 a	708.83 ab	0.00 b	11.00 d
December	67.587 ab	52.367 a	0.00 c	0.00 a	3.50 d	1.67 f	733.50 a	683.33 a	737.00 a	685.00 abcd	5.500 b	58.67 c
***P*-value**	0.0004	< 0.0001	0.0141	0.1958	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0003	< 0.0001	0.0035	< 0.0001

[a] Values for months are the average of two years (2017 and 2018), except for November and December where data was only collected in 2017. Values in columns that are followed by the same letter do not differ significantly according to Fisher's least significant difference test ($P > 0.05$).

Table 3. Analysis of variance on the effect of month and orchard on the percentage of soil moisture and number of accumulated hours at different soil temperature ranges in two avocado orchards situated in the Mooketsi production region and three orchards in the Letaba production region.[a]

Source	*P*–values (significance level of the *F*–value)												
	Letaba							Mooketsi					
	Df [b]	% Moisture	10-14°C	15-19°C	20-24°C	16-24°C	25-29°C	Df	% Moisture	10-14°C	15-19°C	20-24°C	16-24°C	25-29°C
Orchard	1	0.0003	0.2760	0.7513	0.1256	0.0873	0.0219	2	< 0.0001	0.3546	0.0366	0.4888	0.3636	0.7456
Orchard (year)	2	0.0496	0.1367	0.5966	0.0167	0.3798	0.2591	3	< 0.0001	0.1462	0.6359	0.2142	0.3903	0.1633
Month	11	0.0004	0.0141	< 0.0001	< 0.0001	0.0003	0.0035	11	< 0.0001	0.1958	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Month x orchard	11	0.9994	0.9153	0.7002	0.6148	0.7648	0.2199	22	0.6573	0.9716	0.1707	0.3267	0.6372	0.3238

[a] Each soil parameter, which included the average percentage soil moisture (% Moisture) and accumulated hours at various soil temperature ranges (10-14°C, 15-19°C, 20-24°C, 16-24°C and 25-29°C), was measured over two years (22 months, January 2017 to October 2018) using data obtained from one soil probe that was positioned in each of the five investigated orchards. Two orchards (BL and DL) were situated in the Letaba region and three orchards (AM, CM and FM) in the Mooketsi region.

[b] Df = degrees of freedom.

Table 4. Analysis of variance on the effect of month and orchard on *Phytophthora cinnamomi* (Pc) quantities in avocado roots that were sampled from six orchards in two production regions (Letaba and Mooketsi) over four different months for two years (2017 and 2018).

		Letaba						Mooketsi					
Source	**Df [a]**	**qPCR 2017 [b]**		**qPCR 2018 [b]**		**Root baiting 2018 [c]**		**qPCR 2017**		**qPCR 2018**		**Root baiting 2018**	
		MS [a]	*P* [a]	MS	*P*	MS	*P*	MS	*P*	MS	*P*	MS	*P*
Orchards	2	2.1771	0.0071	2.2911	0.0253	0.8472	0.0002	1.5700	0.0494	3.0534	0.0016	0.7558	0.0818
Orchards (block)	9	1.1207	0.7162	3.4838	0.2252	0.4333	0.2769	1.3740	0.7405	5.606	0.0068	0.9391	0.6535
Month	3	1.4564	0.0676	22.6508	< 0.0001	15.4588	< 0.0001	0.9866	0.2609	13.4411	< 0.0001	2.0222	0.0075
Month x orchard	6	4.5718	0.0042	2.0552	0.3064	0.72440	0.0147	2.5321	0.1344	6.1064	0.0008	2.8854	0.0109

[a] Df = degrees of freedom, MS = mean square, *P* = significance level of the *F*-value.

[b] Quantitative real-time PCR (qPCR) quantification of Pc was conducted by extracting DNA from root samples, followed by qPCR analysis using a Pc-specific probe-based assay on the root DNA extracts.

[c] Root baiting quantification was conducted by baiting roots with lemon leaf discs, followed by plating out the baits onto an oomycete-selective medium and determining the percentage of leaf discs infected.

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Table 5. *Phytophthora cinnamomi* (Pc) root colonisation of avocado roots in the Letaba production region in four months over two growing seasons (2017 and 2018) as assessed using two different quantification methods (qPCR analyses and root baiting).[a]

Orchards/average orchards	March	May	August	October/November
Root qPCR 2017 [b]				
BL	0.0000 f (-2.0000)	0.0569 cdef (-1.4744)	0.2042 ab (-0.7932)	0.0501 abcde (-1.2266)
DL	0.1049 abcd (-0.9847)	0.1337 abc (-0.8614)	0.0512 def (-1.5141)	0.2460 a (-0.6638)
EL	0.0134 ef (-1.7988)	0.1032 abcd (-1.1245)	0.0601 bcde (-1.3219)	0.0138 ef (-1.7963)
Root qPCR 2018 [b]				
Average three orchards	0.0540 bc (-1.4828)	1.9452 a (0.0232)	0.1039 b (-1.2853)	0.0189 c (-1.7696)
Root baiting 2018 [c]				
BL	0.00 e (0.00)	100.00 a (1.57)	5.00 de (0.16)	2.50 e (0.08)
DL	2.50 e (0.08)	95.00 a (1.41)	20.00 cd (0.44)	2.50 e (0.08)
EL	2.50 e (0.08)	100.00 a (1.57)	50.00 b (0.85)	25.00 c (0.52)

[a] For each quantification method and year separately (quantitative real-time PCR (qPCR) 2017, qPCR 2018 and root baiting 2018), values in columns and rows that are followed by the same letters do not differ significantly according to Fisher's least significant difference test ($P \geq 0.05$).

[b] qPCR quantification was conducted in each orchard using avocado root samples from four groups of trees (each group containing five trees). Values are the average of the four tree groups (one replicate each). The actual Pc DNA quantities (ng/mg_{DW}) are shown followed by the Log(x + 0.01) transformed values, which were used for post-hoc analysis, in brackets. There was no significant orchard x month interaction ($P = 0.3046$) in 2018, therefore, the averages of the three orchards were analysed. In 2017, the month x orchard interaction was significant ($P = 0.0042$).

[c] Root baiting quantification was conducted in each orchard using avocado root samples from four groups of trees (each group containing five trees). Values are the average of the four tree groups (one replicate each). Post-hoc analysis was conducted on arcsine transformed values, which are shown, followed by the actual percentage of infected leaf discs in brackets. There was a significant month x orchard interaction ($P = 0.0147$).

Table 6. *Phytophthora cinnamomi* (Pc) root colonisation of avocado roots in the Mooketsi production region in four months over two growing seasons (2017 and 2018) as assessed using two different quantification methods (qPCR analyses and root baiting). [a]

Orchards/average orchards	March	May	August	October/November
Root qPCR 2017 [b]				
Average three orchards	0.03944 a (-1.5945)	0.09795 a (-1.1534)	0.10519 a (-1.2097)	0.10329 a (-1.2289)
Root qPCR 2018 [b]				
AM	0.0445 cd (-1.5062)	0.0079 ab (-0.0372)	0.0366 cd (-1.4285)	0.0558 c (-1.3231)
CM	0.1487 c (-1.3021)	2.9591 a (0.3895)	0.4531 b (-0.3766)	0.0000 d (-2.0000)
FM	0.0861 cd (-1.4310)	0.2477 c (-1.1761)	0.0398 cd (-1.4123)	0.0283 cd (-1.7275)
Root baiting 2018 [c]				
AM	0.00 d (0.00)	55.00 ab (0.84)	2.50 d (0.08)	0.00 d (0.00)
CM	15.00 cd (0.28)	65.00 a (1.07)	37.50 abc (0.64)	0.00 d (0.00)
FM	7.50 cd (0.24)	0.00 d (0.00)	22.50 bcd (0.36)	20.00 bcd (0.33)

[a] For each quantification method and year separately (quantitative real-time PCR (qPCR) 2017, qPCR 2018 and root baiting 2018), values in columns and rows that are followed by the same letters do not differ significantly according to Fisher's least significant difference test ($P \geq 0.05$).

[b] qPCR quantification was conducted in each orchard using avocado root samples from four groups of trees (each group containing five trees). Values are the average of the four tree groups (one replicate each). The actual Pc DNA quantities (ng/mg_{DW}) are shown followed by the Log(x + 0.01) transformed values, which were used for post-hoc analysis, in brackets. In 2017, there was no significant orchard x month interaction ($P = 0.1344$), therefore, the data of the three orchards were combined. In 2018 there was a significant orchard x month interaction ($P = 0.0008$).

[c] Root baiting quantification was conducted in each orchard using avocado root samples from four groups of trees (each group containing five trees). Values are the average of the four tree groups (one replicate each). Post-hoc analysis was conducted on arcsine transformed values, which are shown, followed by the actual percentage infected leaf discs in brackets. There was a significant month x orchard interaction ($P = 0.0109$).

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Table 7. Pearson's correlation analyses between *Phytophthora cinnamomi* root quantities, determined using root baiting and qPCR analyses in four different months (March, May, August and October/November), and the monthly soil moisture percentages and accumulated hours at five soil temperature ranges calculated over two years (22 months, January 2017 to October 2018) in five avocado orchards.[a]

Month	Soil temperature (°C) / moisture (%)	March root qPCR	March root baiting	May root baiting	August root qPCR	October/ November root baiting
February	16-24°C	NS	NS	0.998 **(< 0.0001)**	NS	NS
	20-24°C	NS	NS	0.974 **(0.005)**	NS	-0.915 (0.030)
	25-29°C	NS	NS	-0.999 **(< 0.0001)**	NS	NS
March	25-29°C	0.963 **(0.008)**	NS	NS	NS	NS
April	15-19°C	NS	NS	0.935 (0.020)	NS	NS
	16-24°C	NS	-0.973 **(0.005)**	NS	NS	NS
	25-29°C	0.911 (0.032)	NS	NS	0.952 (0.013)	NS
May	15-19°C	NS	NS	0.971 **(0.006)**	NS	NS
	20-24°C	NS	NS	-0.965 **(0.008)**	NS	NS
June	20-24°C	NS	NS	-0.961 **(0.009)**	NS	NS
July	16-24°C	NS	NS	-0.919 (0.027)	NS	NS
	20-24°C	0.911 (0.032)	NS	NS	0.952 (0.013)	NS
August	15-19°C	-0.901 (0.037)	NS	NS	NS	NS
	20-24°C	0.886 (0.045)	NS	NS	NS	NS
	25-29°C	0.911 (0.032)	NS	NS	0.952 (0.013)	NS
September	25-29°C	0.911 (0.032)	NS	NS	0.952 (0.013)	NS
	Moisture	-0.887 (0.045)	NS	NS	NS	NS
October	15-19°C	NS	NS	0.901 (0.037)	NS	NS
	20-24°C	NS	NS	-0.901 (0.037)	NS	NS
November	15-19°C	NS	NS	0.901 (0.037)	NS	NS

[a] The Pearson's correlation value (*r*-value) is shown followed by the *P*-value (significance level of the *F*-value) in brackets. NS = non-significant ($P \geq 0.05$). *P*-values that were highly significant (≤ 0.009) are in bold.

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Table 8. Analysis of variance on the effect of sampling strategy and DNA extraction scale on *Phytophthora cinnamomi* DNA quantities obtained from avocado orchard tree roots over three sampling months (August 2017, May 2018 and August 2018).

		August 2017		May 2018		August 2018	
Source [a]	**Df** [b]	**MS** [b]	***P*** [b]	**MS**	***P***	**MS**	***P***
Orchard	5	0.00424056	0.0300	2.18166192	0.0002	0.05049194	0.0008
Sampling strategy	1	0.00184686	0.1522	0.24331530	0.0482	0.00435004	0.1529
Sampling strategy (Orchard)= Error(a)	5	0.00064830		0.03598488		0.00153344	
DNA extraction scale	1	0.01766785	0.0129	8.27414229	0.0025	0.06469856	0.0207
Sampling strategy x DNA extraction scale	1	0.00150444	0.3988	0.90500313	0.2158	0.00128325	0.7072
Error(b)	10	0.00193674		0.51818647		0.00858755	

[a] Sources of variance include the two sampling strategies (4x5 and 1x20 tree groups) and two DNA extraction scales (small-scale and large-scale) that were used for pathogen quantification.

[b] Df = degrees of freedom, MS = mean square, *P* = significance level of the *F*-value.

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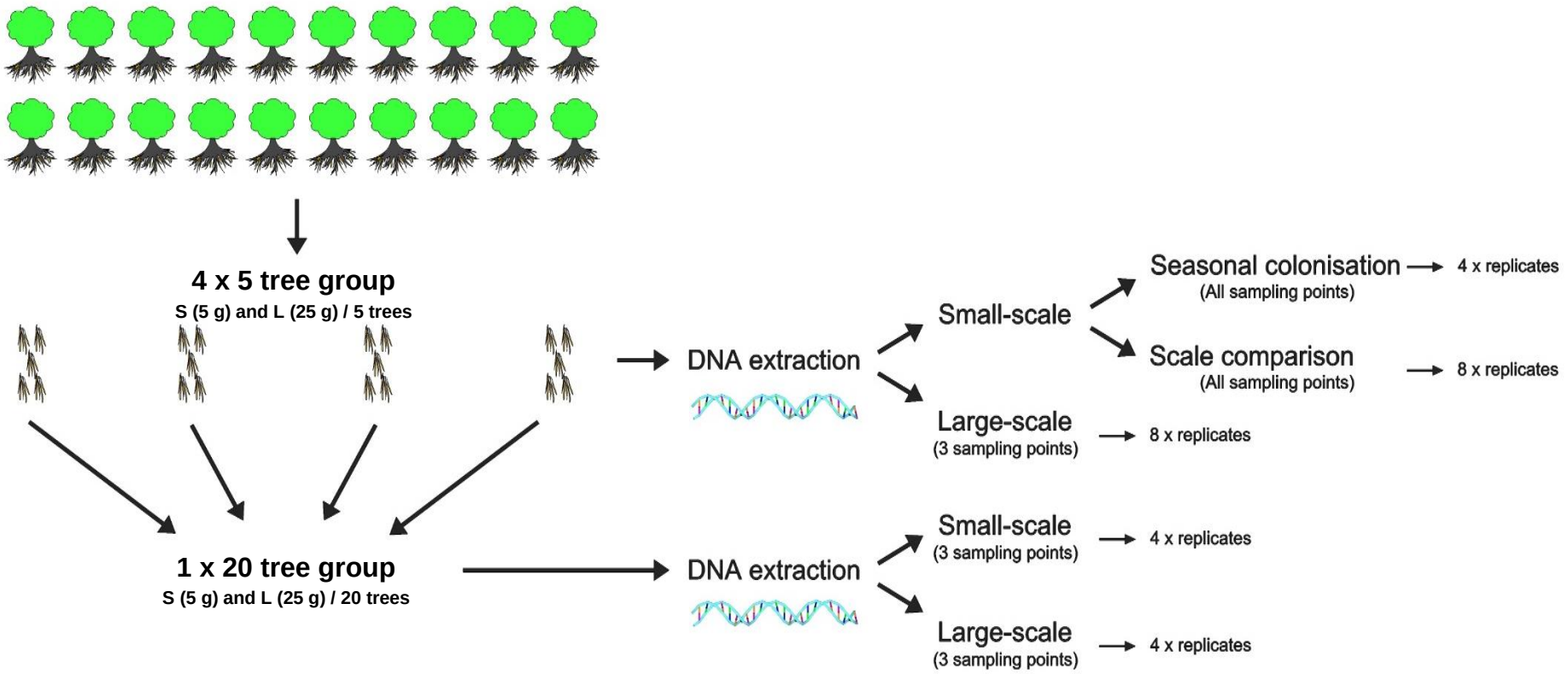

Figure 1. A schematic diagram illustrating the two orchard sampling strategies (4x5 versus 1x20 tree group) and two DNA extraction scales (small-scale [S] versus large-scale [L]) that were investigated for qPCR quantification of *Phytophthora cinnamomi* (Pc) from avocado tree roots in each of the six orchards. The "3 sampling points" refers to samplings conducted in August 2017, May 2018 and August 2018. The "all sampling points" refers to a total sampling of eight months (March 2017, May 2017, August 2017, November 2017, March 2018, May 2018, August 2018 and November 2018). The 4x5 tree group consisted of four groups of five pooled trees' roots, and 1x20 tree group consisted of one group of 20 pooled trees' roots. The number of replicates on the diagram is the total number of replicates that were used for each method in the statistical analysis.

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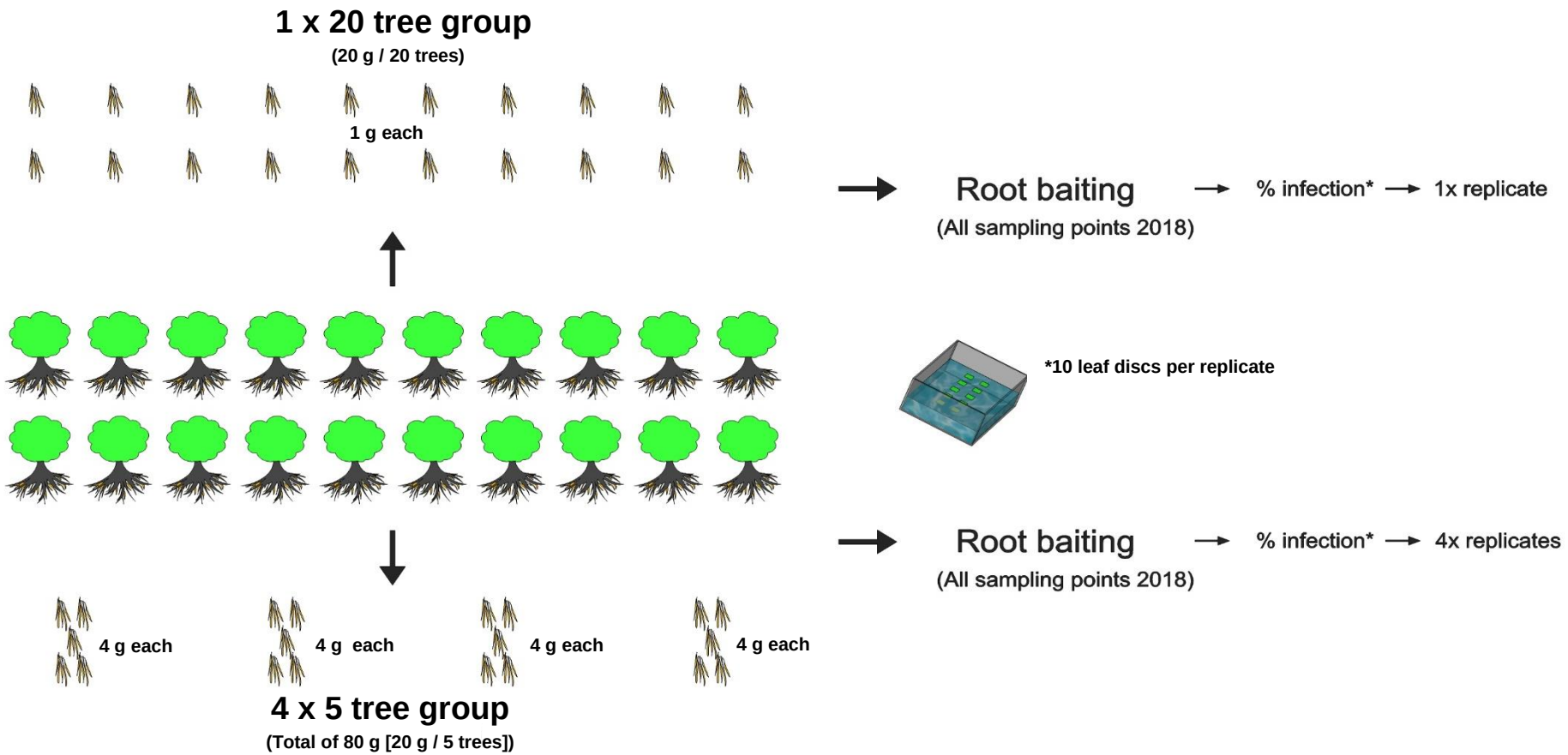

Figure 2. A schematic diagram illustrating the two orchard sampling strategies (4x5 versus 1x20 tree group) that were used for the root baiting quantification of *Phytophthora cinnamomi* (Pc) from avocado tree roots in six avocado orchards, for all of the sampling months in 2018 (March, May, August and October/November). The 4x5 tree group consisted of four groups of five pooled trees' roots. The 1x20 tree group consisted of one group of 20 pooled trees' roots. In each orchard, a total of one replicate and four replicates were thus analysed for the 1x20 and 4x5 tree groups, respectively. The percentage of Pc-infected leaf discs (*) were calculated from 10 leaf discs per replicate. The number of replicates on the diagram indicate the total number of replicates that were used for each method in the statistical analysis.

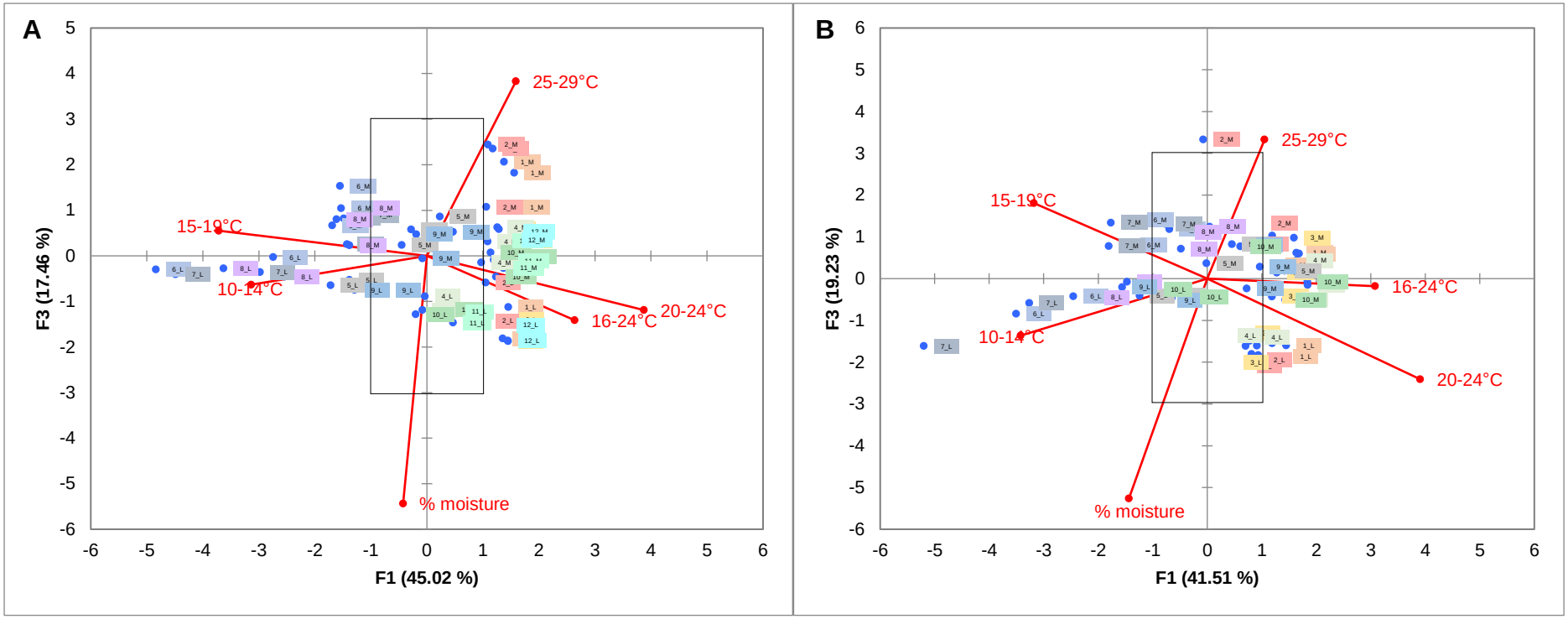

Figure 3. Plot of the first and third principal components (PC1 and PC3) of five avocado orchards from two production regions (Mooketsi and Letaba), according to their accumulated hours at specific soil temperature ranges (15-19°C, 16-24°C, 20-24°C and 25-29°C) and soil moisture levels (%) recorded for the different months in (A) 2017 and (B) 2018 using soil probe data. The soil probe data was recorded over two years (22 months, January 2017 to October 2018). Numbers on the plots indicate the different months: [1] January [2] February [3] March [4] April [5] May [6] June [7] July [8] August [9] September [10] October [11] November [12] December; in 2018, the soil probe data of November and December were not recorded and thus do no feature on the plot (B). The letters "L" and "M" following the months, indicate the production regions, Limpopo and Mooketsi, respectively. The rectangles encompass the months that were subjectively classified as having moderate soil moisture and temperature. The percentage of variation accounted for by each principal component is indicated in brackets.

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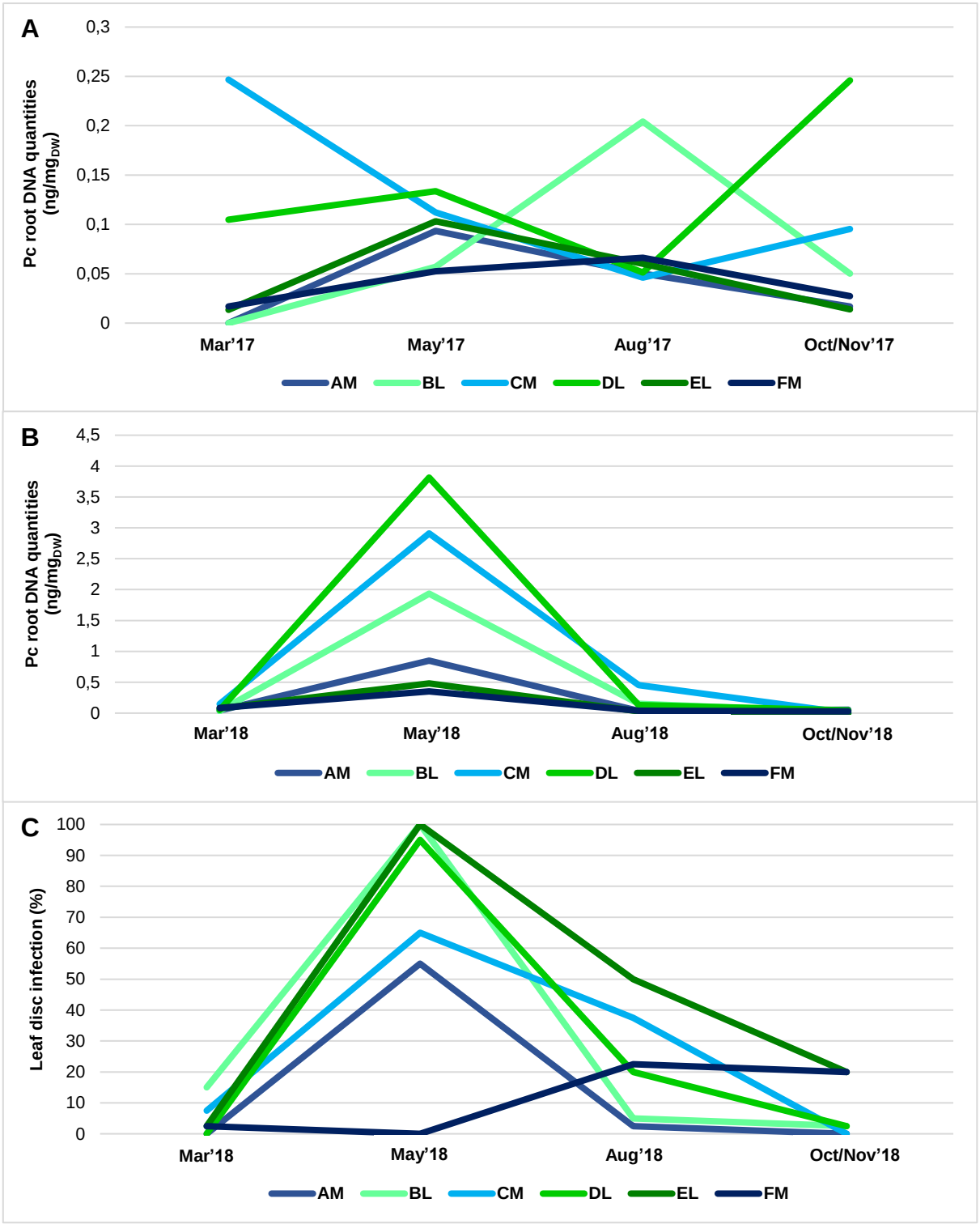

Figure 4. Seasonal root colonisation patterns of *Phytophthora cinnamomi* (Pc) in six asymptomatic avocado orchards over four sampling months, determined by: (A and B) Pc quantitative real-time PCR (qPCR) quantification for 2017 (A) and 2018 (B), and (C) Pc root baiting quantification in 2018. Three of the orchards were located in the Mooketsi (blue-shaded lines) region and three in the Letaba (green-shaded lines) region. For each quantification method, each sampling month represents the average of four groups of trees (each group consisting of the roots of five pooled trees) per orchard and one replicate per group.

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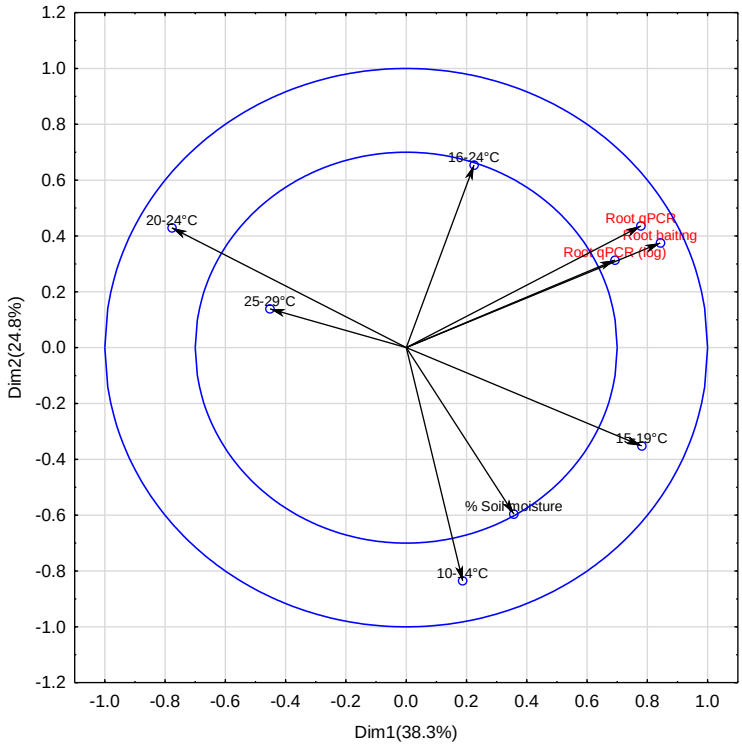

Figure 5. Multifactor analysis (MFA) correlation circle depicting correlations between soil probe temperature data (black) and *Phytophthora cinnamomi* (Pc) quantification data (red), which were obtained in five avocado orchards. The Pc root baiting quantification data was obtained by baiting the roots with lemon leaf discs, followed by plating out the baits onto oomycete-selective media. In addition, quantitative real-time PCR (qPCR) analyses of root DNA extracts using a Pc-specific qPCR assay was conducted. The qPCR data were also log-transformed for the analysis. The Pc quantification data were obtained over two growing seasons (2017 and 2018), for four months in each season. The soil moisture and temperature data were obtained from a soil probe that logged moisture and temperatures on an hourly basis at a 20 cm soil depth. The hourly data was summarised into five soil temperature ranges (10-14°C, 15-19°C, 20-24°C, 16-24°C and 25-29°C) on a monthly basis, and the average monthly percentage of soil moisture (% Soil moisture), for the same eight months during which Pc quantification data were obtained.

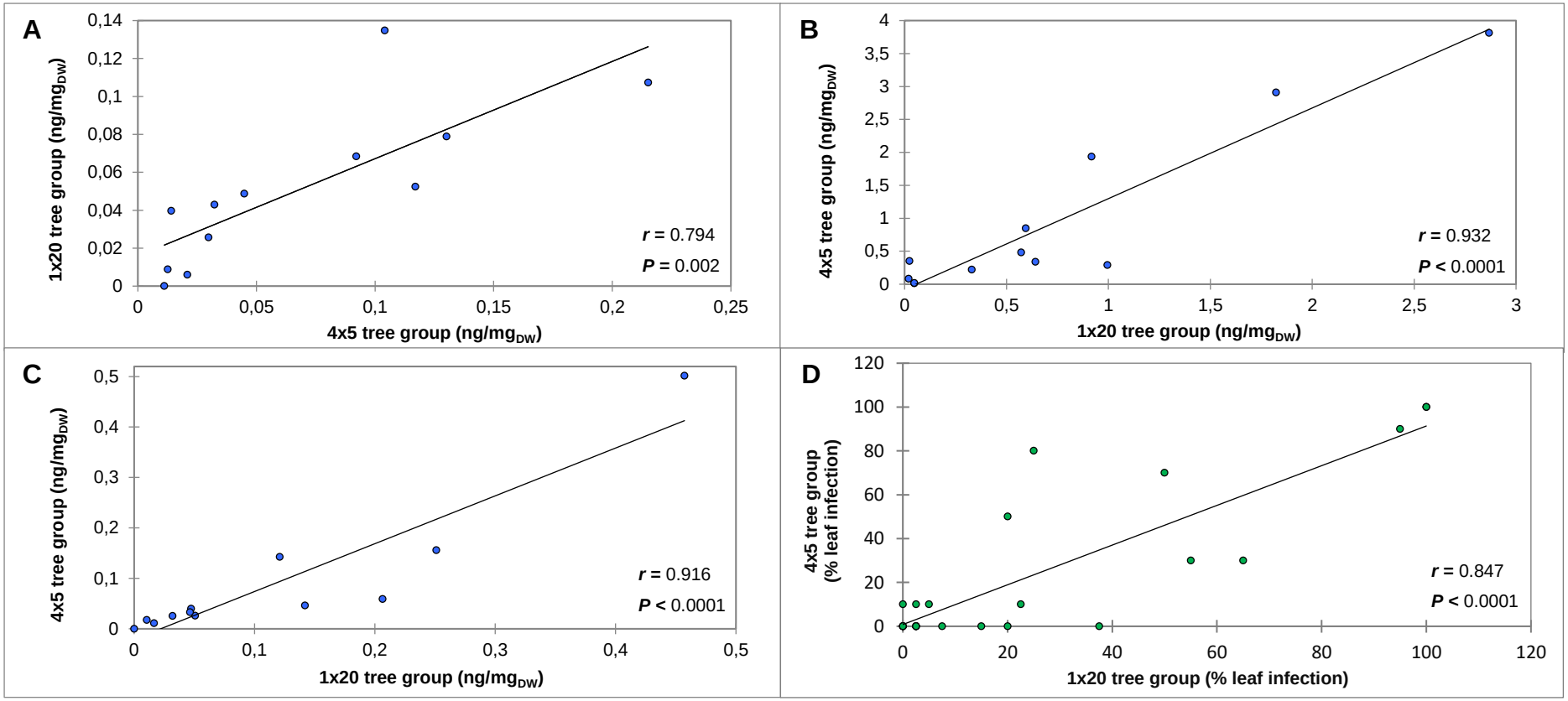

Figure 6. Scatterplot graphs of Pearson's correlation analyses between *Phytophthora cinnamomi* (Pc) root quantities obtained using two sampling strategies consisting of a 4x5 tree group (four groups of five pooled trees) versus a 1x20 tree group (one group of 20 pooled trees) in six avocado orchards. The Pc root quantities were determined in different months and using different quantification methods: (A) quantitative real-time PCR (qPCR) analysis in August 2017, (B) qPCR analysis in May 2018, (C) qPCR analysis in August 2018 and (D) root baiting quantification from four months (March, May, August and October) in 2018. For the root qPCR analyses, in each orchard, the 4x5 tree group represents the average of eight replicates (two replicates per group) and the 1x20 tree group represents the average of four replicates (four replicates per group). For the root baiting quantification, the 4x5 tree group and the 1x20 tree group are the average of four and one replicates, respectively, for each orchard.

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CHAPTER 3

Assessing the efficacy of management strategies against avocado root rot using molecular quantification techniques

ABSTRACT

Phosphonates and rootstock tolerance are two key Phytophthora root rot (PRR) management strategies that are employed by avocado producers. It is thus important to develop an effective *Phytophthora cinnamomi* (Pc) root and soil quantification approach to be able to accurately evaluate the efficacy of these strategies. Assessments conducted in four avocado orchards situated in two production regions (Mooketsi and Letaba) in Limpopo, South Africa, showed that under the trial conditions set out by the current study, quantitative real-time PCR (qPCR) analyses were ineffective for evaluating the efficacy of phosphonate treatments (in three orchards) and rootstock tolerance (in one orchard). The Ras-related *Ypt*1 protein gene qPCR assay was employed for all quantifications since it was shown to be superior in terms of sensitivity, efficiency and linearity in comparison to the published internal transcribed spacer (ITS) and *atp9-nad9* mitochondrial gene region, PCR and qPCR assays, respectively. The *Ypt*1 assay was developed into a multiplex qPCR assay to try and improve Pc quantification accuracy from rhizosphere soil, however, it still failed to reveal differences in the efficacy of phosphonate treatments. The preventative phosphonate trunk injection dosage (0.3 g a.i./m^2) for two phosphonate treatments (1x versus 2x trunk injections) were deemed ineffective in suppressing the pathogen, for both of the evaluated sampling months (May and October 2018), since no significant differences in Pc soil and root DNA concentrations were observed between the treatments. A significant negative correlation was, however, found between the root phosphite and Pc root DNA concentrations, suggesting the importance of root phosphite concentrations in Pc suppression. The 2x trunk injection treatment consistently yielded significantly higher root phosphite concentrations than the untreated control, whereas the 1x trunk injection treatment did not. The potentially more PRR-tolerant R0.06 rootstock tended to yield higher Pc root DNA concentrations than Dusa® in November 2017, however, this was not the case for the two other sampling months (March and May 2018). Two orchards had significantly higher Pc soil or root DNA concentrations in May than in October (2018). This study suggests that in order to observe the effect of management strategies on Pc root DNA concentrations, a larger number of sample replicates must be used in orchard trials. The quantification of Pc from soil will also require better DNA extraction methods and sampling strategies.

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INTRODUCTION

Following the discovery of Phytophthora root rot (PRR) on avocado by Tucker (1928) and Wager (1931, 1942), various studies have focused on finding effective preventative and curative disease management practices. Although some effective management practices have been identified, there are none that can control the disease on their own. This has led to the development of an integrated management strategy, otherwise known as the 'Pegg wheel'. The 'Pegg wheel' covers six management principles including soil selection, irrigation management, chemical control, inorganic nutrition, organic amendments and tolerant rootstocks (Wolstenholme and Sheard, 2010).

In avocado production worldwide, chemical control of PRR has been dominated by the use of phosphonates since the discovery of phosphonate trunk injections by Darvas *et al.* (1984) (Dann *et al.*, 2013; McLeod *et al.*, 2018). In South Africa, in a preventative management strategy, avocado producers typically apply one phosphonate trunk injection twice a year in accordance with the root flush windows (McLeod *et al.*, 2018). However, this application strategy has become problematic due to the strict maximum fruit residue level (MRL) regulations imposed by the European Union (EU) in 2014, for phosphonate products used on avocado (McLeod *et al.*, 2018). Many producers have been unable to maintain EU standards despite following phosphonate label recommendations, thus leading to market access problems. The late spring/early summer phosphonate application window is likely the main contributor to high fruit residues due to the small developing fruit acting as a strong sink for phosphite (McLeod *et al.*, 2018). Therefore, several growers in South Africa no longer apply trunk injections in summer and rather conduct one trunk injection during the fall application window using only the preventative dosage (0.3 g a.i./m^2 canopy) (personal communication J.P.B. Wessels, ProCrop, South Africa). However, it is unknown how this changed management strategy will affect PRR management in the long-term.

Phosphite (the breakdown product of phosphonates) plant tissue concentrations, have been found in some, but not all, host-pathogen systems to be indicative of pathogen or disease suppression. Van der Merwe and Kotzé (1994) were able to negatively correlate phosphite root concentrations with observed *Phytophthora cinnamomi* (Pc) root colonisation levels in avocado roots when phosphite levels were less than 9.5 µg/g_{FW}. Contrarily, in Australia, avocado growers use different critical root phosphite concentrations of 25-30 µg/g_{FW}, which are considered sufficient for Pc suppression in avocado (Giblin *et al.*, 2007). Although the two aforementioned studies are not peer-reviewed, it is the only information available for the Pc-avocado system. One peer-reviewed study has been conducted on avocado, involving the *Phytophthora citricola*-avocado system, where it was found that 21 µg/g_{FW} was sufficient in suppressing avocado trunk cankers caused by the pathogen (El-Hamalawi *et al.*, 1995). In different Australian native plant species, an even wider range of critical phosphite

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concentrations have been reported for Pc suppression in plant stems. Significant negative linear correlations were furthermore reported between phosphite stem concentrations and the inhibition of lesion development for approximately one-third of the investigated plant species (Shearer *et al.*, 2012). The variability between the aforementioned studies illustrates that phosphite tissue concentrations are important in pathogen suppression, but that the concentrations at which phosphite causes pathogen suppression in different host-pathogen systems still requires evaluation.

Rootstock resistance is considered an essential preventative management strategy for PRR control. However, despite several decades of intensive investigations into the identification of PRR-resistant avocado rootstocks (Zentmyer and Thorn, 1956; Zentmyer *et al.*, 1963; Botha *et al.*, 1989; Menge *et al.*, 1992; Kremer-Köhne and Duvenhage, 2000; Smith *et al.*, 2011), only tolerant rootstocks have been discovered thus far. In South Africa, the first devastating effect of planting PRR-susceptible rootstocks became evident when the susceptible rootstock Edranol was first introduced in the 1950s (Kremer-Köhne and Köhne, 2007). Subsequently, Westfalia Technological Services (WTS) at Westfalia Fruit, one of the largest avocado producers in South Africa, has invested heavily into PRR-tolerance selection and breeding programs. Two prominent rootstocks that have been discovered by the program include the Dusa® rootstock (also known as R0.09) and more recently the rootstock R0.06 (Engelbrecht and Van den Berg, 2013), which has potential superiority over Dusa® in terms of PRR tolerance (Van Rooyen, 2017). While the Dusa® rootstock has dominated nursery sales in South Africa since its commercialisation in 2001 (Wolstenholme, 2003; Retief, 2011), rootstock R0.06 is not yet commercially available.

Limited information is available regarding the extent of Pc colonisation in the R0.06 and Dusa® rootstocks, since studies have mainly focused on their host defence response inductions during pathogen attack (Engelbrecht and Van den Berg, 2013; Van den Berg *et al.*, 2018). *Phytophthora cinnamomi* root infection and colonisation have only been investigated in Dusa®, where it was shown that less Pc root colonisation occurred in Dusa® in comparison to the susceptible R0.12 rootstock (Engelbrecht *et al.*, 2013). This observation was made using the artificial inoculation of avocado seedlings under glasshouse conditions, followed by an assessment of Pc root colonisation through quantitative real-time PCR (qPCR) analyses, using a Pc-specific nested qPCR assay targeting the *Lpv* putative storage protein gene (Engelbrecht *et al.*, 2013). For the R0.06 rootstock, the extent of root colonisation has not been reported yet, however, a reduced and delayed zoospore germination rate was more evident for R0.06 when compared to the moderately tolerant R0.10 and susceptible R0.12 rootstocks (Van den Berg *et al.*, 2018).

The efficacy of management strategies against PRR may be better understood through investigations into pathogen DNA concentrations within host root tissues and rhizosphere soil.

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These molecular-based studies would require efficient Pc-specific qPCR assays, to ensure accurate and reliable pathogen quantification. Various studies have aimed to develop Pc-specific conventional PCR and qPCR assays targeting different gene regions. In addition to the *Lpv* gene which has been used in the Pc-avocado system, three other gene regions have been targeted. These include the Ras-related *Ypt*1 protein gene (Schena *et al.*, 2008; Trzewik *et al.*, 2016), the *atp9-nad9* mitochondrial gene region (Bilodeau *et al.*, 2014; Miles *et al.*, 2017) and the internal transcribed spacer (ITS) region (Williams *et al.*, 2009; Langrell *et al.*, 2011; Kunadiya *et al.*, 2017). Kunadiya *et al.* (2017) re-evaluated the Pc-specificity of most of the aforementioned published assays, including the conventional PCR and nested qPCR assays targeting the *Lpv* gene (Kong e*t al.*, 2003; Engelbrecht *et al.*, 2013). The species-specificity of each of the assays was evaluated by testing 10 closely related *Phytophthora* spp. from clade 7 (Kunadiya *et al*., 2017); the phylogenetic *Phytophthora* clade which includes Pc (Cooke *et al.*, 2000; Yang *et al.*, 2017). Most of the published Pc-specific assays did not include species from clade 7 which are closely related to Pc (Kunadiya *et al.*, 2017). Kunadiya *et al.* (2017) found that, of the evaluated conventional PCR assays, only the assay of Schena *et al.* (2008) targeting the *Ypt*1 gene, and the Langrell *et al.* (2011) assay targeting the ITS region, were Pc-specific. Of the evaluated qPCR-related assays, an isothermal amplification- (recombinase polymerase amplification) based assay of Miles *et al.* (2015) targeting the *atp9-nad9* region, was Pc-specific (Kunadiya *et al.*, 2017). Kunadiya *et al.* (2017) did not evaluate the Pc-specific qPCR assay of Bilodeau *et al.* (2014), which was later also employed by Miles *et al.* (2017). The qPCR assay of Bilodeau *et al.* (2014) also targets the *atp9-nad9* region and was evaluated for specificity by investigating closely related species from clade 7, such as *P. parvispora*, thus confirming the assay's Pc-specificity (Miles *et al*., 2017).

The studies of Bilodeau *et al.* (2014) and Miles *et al.* (2017), using the *atp9-nad9* region, were focused on developing qPCR assays that could be used during quarantine screening of plant samples for invasive and destructive *Phytophthora* spp. Consequently, the qPCR assay has not been evaluated for its quantitative potential. Since the *atp9-nad9* region is a mitochondrial gene region, copy numbers may vary between morphological structures (zoospores, chlamydospores and oospores), as well as in cells of different physiological status (Miles *et al.*, 2017), thus the assay's quantitative ability may be limited.

Shortly after the studies of Kunadiya *et al.* (2017) and Miles *et al.* (2017), a Pc-specific probe-based qPCR assay targeting the *Ypt*1 gene was developed by Masikane (2017). The assay was shown to be Pc-specific since closely related species, such as *P. parvispora* and *P. niederhauserii,* were included in specificity testing (Masikane, 2017). Furthermore, the assay was recently used to successfully study the seasonal colonisation patterns of Pc in avocado orchards in South Africa (Chapter 2). The *Ypt*1 qPCR assay has a limit of quantification of 700 fg, which is less sensitive than that reported for the *atp9-nad9* qPCR

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assay (100 fg) (Bilodeau *et al.*, 2014; Miles *et al.*, 2017). As such, the *atp9-nad9* qPCR assay could have a greater potential in accurately detecting and quantifying Pc soil population levels, which are inherently very low under field conditions (Hendrix and Kuhlman, 1965; Eden *et al.*, 2000).

Another challenge facing qPCR quantification of soil DNA is the variability in PCR inhibitors and the efficacy of DNA extractions between different soil samples (Daniell *et al.*, 2012; Wang *et al.*, 2017). Therefore, it is important that an artificial internal DNA standard is used when conducting a qPCR analysis of soil microbes. Daniell *et al.* (2012) showed that the use of an internal foreign reference gene in soil DNA extractions can demonstrate both DNA loss during extraction and PCR inhibitory effects. The internal foreign reference gene used by Daniell *et al.* (2012) consisted of a SYBR® Green-based assay targeting a mutated *Escherichia coli* gene, which allows for the relative DNA quantification of soil microbes. The relative quantification conducted by Daniell *et al.* (2012) requires that two separate qPCR assays must be performed on each soil DNA sample; one for the targeted microbe and another for the foreign gene. Subsequently, probe-based qPCR assays targeting the internal foreign reference gene have been developed, where co-amplification of the targeted microbe and the foreign gene is possible within the same reaction; the probe of each assay is labelled with a different coloured fluorescent dye (Fall *et al.*, 2015). This multiplex probe-based assay is preferable since it limits the cost and labour associated with relative quantification of pathogens from soil samples.

The first aim of this study was to determine whether qPCR quantification of Pc from root tissue and rhizosphere soil could differentiate between the efficacy of two phosphonate treatments (1x versus 2x trunk injections). The efficacy of the phosphonate trunk injection treatments was also assessed by measuring root phosphite concentrations. Secondly, three Pc-specific qPCR assays (Langrell *et al.* 2011; Bilodeau *et al.*, 2014; Masikane, 2017) were evaluated for their sensitivity, efficiency and linearity, to ensure optimal quantification of Pc from the soil. The assay which performed the best was then incorporated into a multiplex probe-based assay for the relative quantification of Pc from rhizosphere soil. The third aim of this study was to compare Pc DNA concentrations from the roots of two rootstocks (Dusa® and R0.06) ranging in their PRR tolerance, under orchard conditions.

MATERIALS AND METHODS

Optimisation and evaluation of three *Phytophthora cinnamomi*-specific qPCR assays

Three Pc-specific qPCR assays targeting three different gene regions were evaluated for their sensitivity, efficiency and linearity: (i) Ras-related *Ypt*1 protein gene (Masikane, 2017), (ii) internal transcribed spacer (ITS) region (Langrell *et al.*, 2011), and (iii) the *atp9-nad9* mitochondrial gene region (Bilodeau *et al.*, 2014; Miles *et al.*, 2017). The sensitivity of a qPCR

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assay depends on its limit of quantification (LOQ) which represents the lowest pathogen DNA concentration (within samples) that can be determined with acceptable precision and accuracy, according to the stated conditions of the assay. The efficiency of a qPCR assay refers to the rate at which the polymerase enzyme converts the reaction reagents into amplicons; low reaction efficiencies (less than 0.85) can indicate problems with the assay, such as poor primer design, which can affect the accuracy of quantification. A standard curve was constructed for each qPCR assay using genomic DNA extracted from the STEU-8674 Pc isolate, as described in Chapter 2. The genomic DNA used in the *Ypt*1 and ITS standard curves consisted of eight five-fold dilutions with a concentration range of 54.74 ng/µl to 0.0007 ng/µl. For the *atp9-nad9* assay, the standard curve consisted of seven four-fold dilutions with a concentration range of 54.74 ng/µl to 0.013 ng/µl.

The qPCR reactions for all three Pc-specific assays consisted of a total volume of 20 µl, which included 2 µl of genomic DNA. The concentration of the primers and probes used for the ITS SYBR® Green-based assay and two probe-based assays (*Ypt*1 and *atp9-nad9*) are shown in Table 1. All primers and probes were synthesised by Inqaba biotec™ (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa). For the *Ypt*1 assay, a 1x SensiFast™ Probe mix (Bioline GmbH, Luckenwalde, Germany) was used, while the ITS and *atp9-nad9* assays used a 1x SYBR® SensiFast mix (Bioline GmbH, Luckenwalde, Germany) and 1x PerfeCTa qPCR ToughMix® (Quanta BioSciences Inc., Gaithersburg, USA), respectively. In addition, the *atp9-nad9* assay was the only assay which included 6 mM $MgCl_2$ in each reaction. The amplification conditions for the *Ypt*1 assay, consisted of one cycle of denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 40 s. The ITS assay amplification conditions consisted of one cycle of denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. For the *atp9-nad9* assay, amplification conditions consisted of denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 57°C for 90 s. A second annealing temperature of 60°C and a 1x SensiFast™ Probe mix (Bioline GmbH, Luckenwalde, Germany), were used in an attempt to further optimise the *atp9-nad9* assay. All amplifications were conducted in a Rotor-Gene® 6000 (Corbett Life Science (Pty) Ltd., Mortlake, Australia) and analysed using the software v.2.3.1.

Phosphonate trials

Orchard selection and trial layout

The trials were conducted in three asymptomatic orchards (without obvious aboveground symptoms of PRR decline) that were located in two production regions (Mooketsi and Letaba) in Limpopo, South Africa. Two of the orchards (BL and EL) were situated in Letaba, and one (FM) was situated in Mooketsi. The scion/rootstock combinations included Carmen/Dusa®

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(BL) and Maluma-Hass/Duke 7 (EL and FM). None of the orchards were mulched. The soil types and irrigation systems used in each orchard has previously been described (Chapter 2).

Three treatments were evaluated: (i) untreated control, (ii) 1x trunk injection and (iii) 2x trunk injections. A total of eight trees (single replicates) were selected for each treatment at each orchard (i.e. 24 trees per orchard). The trial had a completely randomised design wherein, for each orchard, eight untreated control trees were selected randomly within a one-hectare area. One replicate from each of the two phosphonate treatments was positioned on either side of an untreated control tree. Trees from all three orchards received their last phosphonate trunk injection in April 2017. For the 1x trunk injection treatment, trees were injected in April 2018; a post-harvest treatment that was carried out after the summer foliar flush had hardened off. For the 2x trunk injection treatment, trees were injected in November 2017 (after the spring foliar flush had hardened off) and in April 2018 (after the summer foliar flush had hardened off). Each trunk injection was applied at a preventative dosage of 0.3 g a.i./m^2 (Avoguard® 500 SL; Nulandis, Kempton, South Africa) and conducted as described by Darvas *et al.* (1984), according to the registered label recommendation.

Root and soil sampling

Roots and soil were sampled over two months (May 2018 and October 2018), which were 4 and 23 weeks, respectively, after the April 2018 injections. From each tree, a total of approximately 20 g of white feeder roots (May) or a mixture of white and suberised feeder roots (October) was collected. The different root compositions for the two sampling months is due to the October sampling month being positioned outside of a root flush window; therefore, less availability of white feeder roots. The roots were washed free from soil, and 10 g of roots from each tree was stored in 15 ml Falcon tubes at -80°C for DNA extraction and qPCR analysis. The remaining 10 g of roots (wet weight) were placed into brown paper bags and dried for ±4 days at 40°C for phosphite quantification.

From each tree, approximately 50 g of rhizosphere soil was sampled. The rhizosphere soil was obtained from soil that was loosely adhering to the feeder roots. The rhizosphere soil was shaken off the feeder roots and stored in a plastic bag at 4°C. A subsample of 30 g of soil (wet weight) was placed into brown paper bags and dried at 40°C for ±4 days. The dried soil samples were passed through a 4 mm sieve and stored in 50 ml Falcon tubes at -80°C for DNA extraction and qPCR analysis.

Root DNA extraction and qPCR analysis

Two root DNA extraction replicates were conducted on the root samples of each tree. Each root sample was lyophilised individually in 15ml Falcon tubes using a condenser vacuum (VirTis®; SP Scientific, Warminster, USA) and fragmented with a sterile scalpel. The

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fragmented roots were transferred into 50 ml Falcon tubes containing 6 g of glass beads (2 mm diameter) and shaken at 30 Hz for 5 min using a Retsch® MM400 mixer mill (Retsch GmbH, Haan, Germany). A subsample of 50 mg of roots was transferred into a 2 ml Eppendorf® tube, whereafter DNA was extracted using the Nucleospin® PLANT II kit (Macherey-Nagel GmbH and Co., Düren, Germany) according to the manufacturer's protocol; a few slight modifications were made as described for the small-scale DNA extraction method in Chapter 2.

Phytophthora cinnamomi was quantified from the extracted root DNA samples using the *Ypt*1 qPCR assay (Masikane, 2017). The same reaction and amplification conditions were used as described above for the *Ypt*1 standard curve, except that 1 µl of a 1:10 dilution of the root DNA extract was used instead of 2 µl genomic Pc DNA, and each extracted root DNA sample was analysed in duplicate instead of triplicate.

Soil DNA extraction and multiplex qPCR analysis

Two soil DNA extraction replicates were performed on the rhizosphere soil of each tree. Each 50 ml Falcon tube, containing approximately 15 g of oven-dried soil from one tree, was shaken manually for ±10 s. A 500 mg subsample was placed into a 2 ml Eppendorf® tube with 0.5 g of glass beads (2 mm diameter) and shaken with a Retsch® MM400 mixer mill (Retsch GmbH, Haan, Germany) at 30 Hz for 5 min. A 250 mg subsample was measured from each tree replicate and placed into the Nucleospin® Type A Bead Tubes from the NucleoSpin® Soil kit (Macherey-Nagel GmbH and Co., KG, Düren, Germany), which was used to perform the soil DNA extractions. Soil DNA extractions were conducted according to the manufacturer's instructions, with a few modifications as described below.

Prior to starting the soil DNA extraction kit protocol, the required amount of SL1 DNA extraction buffer was spiked with a plasmid containing the exogenous internal positive control (EIPC) DNA fragment (Fall *et al.*, 2015) to a final concentration of 1.2 x 10^3 copies/µl. The EIPC DNA fragment was synthesised into the pBluescript II SK plasmid by Inqaba biotec™ (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa). The plasmid was transformed into *E. coli* cells using standard procedures (Sambrook *et al.*, 1989), and extracted from the *E. coli* cell pellets using the QIAGEN® Plasmid Midi Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions with one exception: in the final step, the plasmid DNA pellet was re-dissolved using nuclease-free water instead of TE water. Prior to spiking the DNA extraction buffer, the plasmid was linearised using the restriction enzyme *Eco*R I (Fermentas, Waltham, USA) and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega Co., Madison, USA) kit.

The Nucleospin® Type A Bead Tubes containing the 250 mg of pulverised soil, 150 µl of SX Enhancer buffer and 700 µl of the plasmid spiked SL1 DNA extraction buffer, was placed

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horizontally into MN Bead Tube Holders and vortexed for 5 min using the VX-200 Vortex Mixer (Labnet International Inc., Edison, USA) at full speed (3400 rpm). After the vortex step, the extraction tubes were centrifuged for 2 min, and the supernatant was transferred into new 2 ml Eppendorf® tubes, as recommended by the manufacturer. For the final step, DNA was eluted from the DNA extraction column using one 30 µl aliquot of elution buffer (SE) and stored at -20°C.

A probe-based multiplex assay was optimised that co-amplified the EIPC plasmid DNA fragment and the pathogen's *Ypt*1 gene within one qPCR reaction. The *Ypt*1 qPCR assay was selected since it was the most sensitive in detecting Pc and had the greatest efficiency and linearity out of the three assays (see Result section). Firstly, a standard curve was developed using the *Ypt*1 assay, whereby each standard was spiked with a constant EIPC plasmid DNA concentration (8000 copies) to confirm that co-amplification would not affect the performance of the assay. Subsequently, an unspiked *Ypt*1 standard curve was developed. A separate EIPC plasmid standard curve was developed using the same amplification conditions as the *Ypt*1 assay. The *Ypt*1 standard curves were developed using the same Pc genomic DNA dilution series as described for the Pc standard curve above. The EIPC plasmid standard curve was developed using the linearised extracted EIPC plasmid DNA at a concentration range of $1x10^6$ copies/µl to 12.8 copies/µl using eight five-fold dilutions. The EIPC primers and probes (Table 1) were synthesised by Inqaba biotec™ (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa). For both qPCR standard curve assays, each 25 µl qPCR reaction consisted of 2 µl genomic DNA (Pc or EIPC plasmid), 1x SensiFast™ Probe mix (Bioline GmbH, Luckenwalde, Germany), primer and probe concentrations as indicated in Table 1 and 4.8 mM of $MgCl_2$. All amplifications were conducted in a Rotor-Gene® 6000 (Corbett Life Science (Pty) Ltd., Mortlake, Australia) and analysed using the software v.2.3.1. The *Ypt*1 standard curve signal was acquired using cycling Green A, while the EIPC plasmid standard curve was acquired using cycling Yellow A. All standard curve samples were analysed in triplicate, including a no template control (NTC) (i.e. nuclease-free water).

Phytophthora cinnamomi was quantified from the soil DNA extract using the same reaction mixture and amplification conditions that were used to develop the multiplex *Ypt*1/EIPC plasmid standard curves. A 2 µl volume of soil DNA was used in each 25 µl qPCR reaction. The optimal volume of soil DNA extract that could be used in each 25 µl qPCR reaction, without resulting in PCR inhibition, was first determined. This was done by monitoring the copy number of the EIPC plasmid DNA, which was spiked into the DNA extraction buffer, in a dilution series of a subset of the soil DNA samples. The multiplex *Ypt*1/EIPC plasmid qPCR assay was conducted on the sample dilution series. The dilution factor at which the EIPC plasmid DNA was detected for all samples, and at which no further increases in gene copy numbers occurred, was taken as the optimal dilution factor for soil DNA extracts. Since

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there was no difference in EIPC copy numbers between the diluted and 2 µl undiluted samples, 2 µl of undiluted soil DNA extract was used in each 25 µl qPCR reaction.

All soil DNA samples were analysed in duplicate. *Phytophthora cinnamomi* soil DNA concentrations, of each soil DNA sample, were calculated by importing the unspiked *Ypt*1 standard curve. The EIPC plasmid DNA concentrations of each soil DNA sample were calculated by importing the EIPC plasmid standard curve and extrapolating values. The relative pathogen DNA concentration (in ng/mg_{DW}) was calculated by using the formula: $\left(\frac{\left(\frac{pathogen\ DNA\ concentration\ of\ sample}{EIPC\ gene\ copy\ number\ of\ sample}\right) \times EIPC\ gene\ copy\ number\ used\ for\ spiking}{mg\ soil\ used\ in\ DNA\ extraction}\right)$ (Moein *et al.*, 2019).

Root phosphite extraction and quantification

The dried root samples were pulverised using an electric IKA basic analytical mill R (IKAR - Werke GmbH and Co. KG, Staufen, Germany). A subsample of 250 mg of pulverised roots was taken from each sample and transferred into individual 50 ml Falcon tubes. Twenty millilitres of extraction water, spiked with an internal control containing Triethyl phosphate® (TEP) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) to a final concentration of 0.05 µg/ml, was added to each 50 ml Falcon tube. The extraction mixture was incubated overnight on a rotary shaker incubator (3082U; Labcon, Midrand, South Africa) at 23°C and at a frequency of 160 rpm. Following the overnight incubation, samples were centrifuged (Centrifuge 5810R; Eppendorf, Hamburg, Germany), using a swinging bucket rotor head, for 10 min at 4000 rcf (g) and 23˚C. Five millilitres of the resultant supernatant was transferred into 15 ml Falcon tubes and vortexed briefly. One millilitre of the supernatant was passed through an AcroPrep® advance 0.2 µm 96 well plates (1 ml) containing a Supor® short tip Natural PP base membrane (PALL Co., Midrand, South Africa), using the Pall multi-well plate vacuum manifold along with a Biopointe® 2.2 ml 96-well square well V-bottom plate (BioPointe Scientific, Claremonte, USA) fitted to the bottom of the filtrate plate, in which the filtrate was collected. For each sample, 700 µl of the plate filtrate was passed through a 10K Nanosep® centrifugal device (PALL Co., Midrand, South Africa) by centrifugation (Microcentrifuge 5424; Eppendorf, Hamburg, Germany) at 14000 rcf (g) for 20 min. Two hundred microlitres of the filtrate were pipetted into vials for LC-MS/MS phosphite quantification.

A phosphite standard curve was prepared for each LC-MS/MS run, as described by McLeod *et al.* (2018). All standard curve samples, and positive control phosphite root samples, were spiked with the TEP internal control to a final concentration of 0.05 µg/ml. Positive controls that were included in each run were the phosphite concentrations 1.5 and 7 µg/ml, as well as phosphonate fungicides Phosguard® 400 SL (Nulandis, Kempton, South Africa) and Brilliant® SL (Arysta LifeScience South Africa (Pty) Ltd., La Lucia Ridge, South Africa) at the concentrations 2 µg/ml and 1.5 µg/ml, respectively. The positive controls were also spiked

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with TEP to a final concentration of 0.05 µg/ml. All phosphite root extracts (one replicate per sample), were analysed by the Central Analytical Facility Mass Spectrophotometry division at Stellenbosch University (CAF, Stellenbosch, South Africa) using LC-MS/MS analyses as previously described (McLeod *et al.*, 2018). The recovery rate, based on spiking a few of the untreated control samples with phosphite to final concentrations of 0.25 µg/ml and 1 µg/ml, showed that the average recovery rate of the method was 55%. Therefore, a conversion factor of 1.82 was applied to the root phosphite concentrations of all samples.

Rootstock trials

Orchard selection and trial layout

The rootstock trial was conducted in one orchard (GL) which has a known high PRR disease pressure and where phosphonates are not applied. The orchard is located in the Letaba production region of Limpopo, South Africa, at Westfalia Fruit. The orchard is irrigated by use of micro-sprinklers and is annually mulched with wood chips. The trial contained eight different rootstocks, all grafted with Hass®, which were replicated five times each in a completely randomised block design. Each block replicate consisted of five trees. One or two trees were randomly selected from each of the highly tolerant Dusa® and R0.06 rootstock block replicates, thus resulting in a total selection of eight trees per rootstock (Dusa® and R0.06). At the time of sampling, the trees had a disease severity score of 0 or 1 based on the Ciba-Geigy tree health rating scale. The Ciba-Geigy scale ranges from 0 (healthy tree) to 10 (dead tree) (Mavuso and Willis, 2009).

Root sampling

Roots were sampled from each tree over three sampling months (November 2017, March 2018 and May 2018). Approximately 50 g of roots, including a mixture of white and suberised feeder roots, were sampled from four sides of each tree at a depth of 0 to 20 cm. The roots, from which loosely adhering soil was shaken, were stored at 4°C until analysed. The roots from each tree were washed free of soil, using tap water, followed by air drying on paper towels for ±10 min at 24°C. A subsample of 10 g of roots was taken from each tree, stored in 15 ml Falcon tubes and lyophilised for qPCR analysis as described in the “Root DNA extraction and qPCR analysis” section of the phosphonate trials.

Root DNA extraction and qPCR analysis

DNA was extracted from 50 mg of lyophilised roots and Pc was quantified from the root DNA extracts using the *Ypt*1 qPCR assay of Masikane (2017) as described in the “Root DNA extraction and qPCR analysis” section of the phosphonate trials.

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Statistical analysis

For the phosphonate trial data, analyses of variance (ANOVA) was performed on the root phosphite concentrations and Pc DNA concentrations (roots and soil), using the GLM (General Linear Models) Procedure of SAS statistical software (Version 9.4; SAS Institute Inc., Cary, USA). For the rootstock trial data, ANOVA was performed on the Pc root DNA concentrations. Deviations from normality in data sets were tested with the Shapiro-Wilk test from normality (Shapiro and Francia, 1972). Some datasets that deviated significantly from normality were transformed in order to improve normality. Fisher's least significant difference (LSD) test was calculated at the 95% confidence level to compare means for significant effects (Otto, 1998). Pearson's correlation analyses and the significance of correlations were conducted on the root phosphite concentrations and Pc DNA concentrations (root and soil) using XLStat (Version 2014; Addinsoft, New York, USA).

RESULTS

Optimisation and evaluation of three *Phytophthora cinnamomi*-specific qPCR assays

The three Pc-specific qPCR assays differed from each other in their sensitivity, efficiency and linearity (Table 1). The *Ypt*1 assay performed the best with regards to sensitivity (limit of quantification [LOQ] = 700 fg), efficiency (0.91) and linearity (R^2 = 0.997) compared to the optimised *atp9-nad9* assay using the PerfeCTa qPCR Toughmix® (LOQ = 15 pg; E = 0.86 and R^2 = 0.981, respectively) (Table 1). The *atp9-nad9* assay yielded even lower efficiencies (< 0.85) when the SensiFast™ Probe mix was used at an annealing temperature of 57 or 60˚C. The ITS assay, although being sensitive (700 fg) and having acceptable linearity (R^2 = 0.996), had a very low efficiency (0.80) (Table 1).

For the quantification of Pc DNA from soil samples, a multiplex qPCR reaction was used where the *Ypt*1 gene and the EIPC plasmid DNA were co-amplified. Co-amplification of the two genes only resulted in a slightly lower efficiency (0.89), but similar linearity (R^2 = 0.997) for the *Ypt*1 assay, than when the *Ypt*1 gene was not co-amplified with the EIPC plasmid DNA.

Phosphonate trials

Phytophthora cinnamomi root qPCR quantification

Levene's test for homogeneity showed that there was a significant difference (P = 0.0008) in variance between the data of the three orchards, for the Log(x + 0.01) transformed Pc root DNA concentrations. Therefore, a weighted analysis was conducted.

ANOVA analysis showed that the Pc root DNA concentrations did not differ significantly between the treatments (P = 0.3361), but that there was a significant month x orchard interaction (P = 0.0008) (Table 2). For the BL orchard, the sampling month of May yielded significantly higher Pc root DNA concentrations than October. In the other two orchards (EL

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and FM), there were no significant differences in the monthly Pc root DNA concentrations (Table 3).

Phytophthora cinnamomi soil qPCR quantification

Levene's test for homogeneity showed that there was no significant difference (P = 0.3337) in variance between the data of the three orchards, for the Log(x + 0.00001) transformed Pc soil DNA concentrations (Table 2). Therefore, a weighted analysis was not required.

ANOVA analysis showed that the only meaningful, although not significant, interaction or factor for the Pc soil DNA concentrations was the month x orchard interaction (P = 0.0537) (Table 2). For the EL orchard, the May sampling month had a significantly higher Pc soil DNA concentration than October (Table 3). Similar to the Pc root DNA concentrations, the three treatments did not differ significantly from each other in Pc soil DNA concentrations (P = 0.4422) (Table 2).

Root phosphite quantification

Levene's test for homogeneity showed that there was a significant difference (P = 0.0011) in variance between the data of the three orchards, for the Log(x + 1) transformed root phosphite concentration data. Therefore, a weighted analysis was conducted.

ANOVA analysis showed that there was a significant orchard x treatment x month interaction for the root phosphite concentrations (P = 0.0492) (Table 2). This interaction was thus investigated further (Table 4). For all three orchards and for both sampling months (May and October), the 2x trunk injection treatment yielded significantly higher root phosphite concentrations (19.164 to 57.109 µg/g_{FW}) than the untreated control (1.363 to 30.545 µg/g_{FW}) (Table 4). In contrast, the 1x trunk injection treatment, for two of the orchards (EL and FM), did not differ significantly from the untreated control at either sampling month. In the third orchard (BL), this was only true for the May sampling month and not for October. The root phosphite concentrations of the 2x trunk injection treatment did not differ significantly from the 1x trunk injection treatment (19.709 to 50.309 µg/g_{FW}) for either of the sampling months in two of the orchards (EL and FM). However, for the BL orchard, for both sampling months, the 2x trunk injection treatment yielded significantly higher root phosphite concentrations than the 1x trunk injection treatment (Table 4).

Correlation analyses between Phytophthora cinnamomi quantities and root phosphite concentrations

Only two significant correlations were observed between the three parameters (root phosphite, Pc root DNA and Pc soil DNA) that were investigated in the phosphonate trials. Firstly, a highly significant negative (r = -0.348; P = 0.003) correlation was found between the root phosphite

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concentrations in May, and the May Pc root DNA concentrations, indicating that as root phosphite increased, the Pc DNA in roots decreased. However, no significant correlation existed between the root phosphite concentrations in October, and the October Pc root DNA concentrations (r = -0.071; P = 0.556). Secondly, a highly significant positive (r = 0.632; P < 0.0001) correlation was observed between the root phosphite concentrations of the two sampling months, demonstrating that the trends in root phosphite concentrations amongst the three treatments remained the same regardless of the sampling month (May or October). No significant correlations were found between the Pc root DNA and Pc soil DNA concentrations for either sampling month; May (r = -0.034; P = 0.780) and October (r = 0.129; P = 0.280). There were likewise no other significant (P ≥ 0.100) correlations between the three investigated parameters when compared in all possible combinations.

Rootstock trials

Phytophthora cinnamomi root qPCR quantification

The May 2018 sampling month yielded little to no Pc root DNA concentrations for either rootstock (Dusa® [0.00917 ng/mg_{DW}] and R0.06 [0 ng/mg_{DW}]), and many replicates furthermore contained no Pc root DNA. Consequently, there was no significant difference (P = 0.3506) in Pc root DNA concentrations between the two rootstocks in May. There was also no significant difference (P = 0.3995) between the Pc root DNA concentrations obtained from the Dusa® (0.08325 ng/mg_{DW}) and R0.06 (0.05410 ng/mg_{DW}) rootstocks in the March 2018 sampling month. However, in the November 2017 sampling month, there was a meaningful difference (P = 0.0793) between Pc root DNA concentrations at the 90% confidence level. There was a tendency for the Dusa® (0.49580 ng/mg_{DW}) rootstock to yield higher Pc root DNA concentrations than the R0.06 (0.14003 ng/mg_{DW}) rootstock.

DISCUSSION

The current study showed that qPCR quantification of Pc in avocado roots and rhizosphere soil, using the *Ypt*1 and multiplex *Ypt*1/EIPC assays, respectively, were ineffective in revealing the efficacy of management strategies. The management strategies that were investigated included phosphonate trunk injections (1x versus 2x trunk injections) and PRR-tolerant rootstocks (Dusa® versus R0.06). To improve the qPCR quantification of Pc DNA from soil samples, two additional published qPCR assays targeting the *atp9-nad9* and ITS regions were also investigated. This was deemed important, since qPCR assays with higher sensitivity may yield less variable results and also help in reducing false-negative detections during pathogen DNA quantification. However, neither of the two assays (*atp9-nad9* or ITS) were more useful than the *Ypt*1 assay; the *atp9-nad9* assay was less sensitive than the *Ypt*1 assay, whereas the ITS assay could not be used quantitatively due to its efficiency being too low.

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The *Ypt*1 assay was used for all of the Pc DNA quantifications from the root (*Ypt*1) and soil (multiplex *Ypt*1/EIPC) samples since it had the highest sensitivity (700 fg) and an acceptable efficiency, in comparison to the *atp9-nad9* assay. The *Ypt*1 assay yielded efficiencies (0.91[singleplex] and 0.89[multiplex]) that were lower than that recommended for an optimised qPCR assay (0.95 to 1.05). However, qPCR assay efficiencies higher than 0.85 are still considered acceptable (Bustin and Huggett, 2017), provided that the data are interpreted critically. qPCR assays with efficiencies less than 0.85 are not recommended since this can negatively affect the accuracy of quantifications (Bustin and Huggett, 2017). The efficiency of the *Ypt*1 assay was also deemed acceptable for quantification purposes since the assay has already successfully been used to investigate Pc root colonisation patterns (Chapter 2), and the efficacy of foliar phosphonate treatments in other studies (Masikane 2017, 2019). The optimised *atp9-nad9* assay had a low sensitivity (15 pg) and relatively low efficiency (0.86) in the current study. This was unexpected since two previous studies reported a much higher sensitivity (100 ag to 100 fg) and better efficiency (1.03 to 1.12) (Bilodeau *et al.*, 2014; Miles *et al.*, 2017), which included multiple laboratory evaluations (Miles *et al.*, 2017). The different results obtained in the current study might be due to the use of a different qPCR machine (Rotor-Gene® 6000 machine) than which was used by Miles *et al.* (2017) (Bio-Rad® CFX 96 and Applied Biosystems® ABI vii A7). Furthermore, standard curves in the work of Miles *et al.* (2017) were developed using only four standards, instead of the minimum required number of five standards (Bustin and Huggett, 2017). This may have affected the efficiency and accuracy, but not the sensitivity of the assay.

The ITS assay yielded an efficiency of 0.80, which is unacceptable for quantification purposes. The low reaction efficiency produced by the ITS assay may be due to poor primer design and/or the large size of the DNA fragment (Taylor *et al.*, 2010). The latter can be expected since the assay was not designed as a qPCR assay but as a conventional PCR assay that yields a large 686 bp PCR fragment (Langrell *et al.*, 2011), which is suboptimal for qPCR assays. A Pc-specific qPCR assay that was not evaluated in the current study, is the isothermal assay of Miles *et al.* (2015) that also targets the *atp9-nad9* region. This assay was shown to be a Pc-specific qPCR assay by Kunadiya *et al.* (2017). However, the assay was not evaluated in the current study since Miles *et al.* (2015) only published *Phytophthora* genus-specific primers and not a Pc-specific probe. The Pc-specific probe cited by Kunadiya (2017) as being that of Miles *et al.* (2015) was, in fact, the probe that was first published by Bilodeau *et al.* (2014).

Based on the Pc DNA concentrations obtained from roots that were sampled in two different months (May and October), preventative phosphonate trunk injections (0.3 g a.i./m^2) were deemed ineffective in suppressing the pathogen. *Phytophthora cinnamomi* root DNA concentrations from trees that received 1x trunk injection (after the summer foliar flush had

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hardened off) or 2x trunk injections (after the summer and spring foliar flushes had hardened off), did not differ significantly from the untreated control. This was surprising since the 2x trunk injection treatment yielded significantly higher root phosphite concentrations than the untreated control and would thus have been expected to reduce Pc root colonisation. The importance of root phosphite concentrations as being indicative of Pc suppression was also suggested by the significant negative correlation that was found between the root phosphite concentrations and Pc root DNA concentrations for the month of May. The fact that the root phosphite concentrations in October were not significantly correlated with the October Pc root DNA concentrations, is likely due to the higher and less variable Pc root DNA concentrations that were obtained in May in comparison to October. Van der Merwe and Kotzé (1994) also previously reported that root phosphite was important for Pc suppression in avocado since a negative correlation existed between root phosphite concentrations and pathogen suppression (i.e. reduced colonisation levels) in a glasshouse study. The 2x trunk injection treatment (0.3 g a.i./m^2) was furthermore expected to be effective since it is registered in South Africa (Fighter® and Avoguard®) for the control of PRR. In contrast, the 1x trunk injection treatment was not expected to be effective (reduce Pc DNA concentrations) since it was not applied according to label recommendations and it also generally yielded root phosphite concentrations that did not differ significantly from the untreated control. Contrary to the current study, Ali *et al.* (1999, 2000) were able to associate foliar phosphonate spray dosages with significant reductions in Pc root colonisation levels in native plant species in Australia under glasshouse conditions, when pathogen isolation studies were used. Furthermore, Darvas *et al.* (1984) reported that phosphonate trunk injections in avocado orchards resulted in a significant reduction in the percentage of Pc-infected roots, when roots were directly plated onto general media. However, although a similar number of single tree replicates (10) were used to that of the current study (8), sampling was done on a monthly basis for 12 months (Darvas *et al.*, 1984), instead of the two months that were selected in the current study. This suggests that the use of two sampling months in the current study may have limited the ability to observe Pc suppression in avocado roots by phosphite. The aforementioned studies, however, were all conducted on diseased trees, as opposed to the current study where asymptomatic trees were investigated. It is thus possible that in the current study, the colonisation levels were too low for root phosphite concentrations to have a significant effect.

A few other aspects may have led to the Pc root DNA concentrations not being indicative of PRR control for the phosphonate trunk injection treatments. Irrespective of the mode of action of phosphite *in planta*, which can be direct (toxicity) or indirect (host defence response) (Jackson *et al.*, 2000; Massoud *et al.*, 2012), it can be expected that there will be either hindered growth and/or death of the pathogen inside of the roots. If the latter is true, qPCR quantification may give false-positive results, since it quantifies both dead and viable pathogen

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structures. Furthermore, the effect of phosphite on Pc suppression may not only be related to root colonisation, but also to the pathogen's ability to reproduce asexually. In a previous study which focused on the Pc-*Banskia* and Pc-*Eucalyptus marginata* systems, it was reported that phosphonates can result in a significant reduction in the number of Pc zoospores released from infected seedling stems (Wilkinson *et al*., 2001). Furthermore, Masikane (2017) found that root baiting quantification was better than root qPCR analyses for assessing the efficacy of phosphonate foliar sprays (Masikane, 2017). Root baiting quantification is beneficial in that it ensures that only viable pathogen structures are detected.

The effect of phosphonate treatments on Pc soil population levels was investigated to determine whether the treatments can reduce soil inoculum build-up. Since a high variability in PCR inhibitors and the efficacy of DNA extractions can occur between different soil samples (Daniell *et al.*, 2012; Wang *et al.*, 2017), a multiplex qPCR assay was developed to allow for the relative quantification of Pc from soil samples; relative quantification can correct for potential DNA loss during extractions and can reduce false-negative detections. However, in the current study, the quantification of Pc from soil samples using the multiplex *Ypt*1/EIPC qPCR assay was unsuccessful since very low Pc DNA concentrations were detected, including samples taken from the untreated controls. This might have contributed to the lack of significant differences observed between the Pc soil DNA concentrations quantified from the 2x trunk injection treatment and the untreated control. The difficulty in quantifying Pc from soil is likely due to the erratic nature of Pc distribution within soil (Pryce *et al.*, 2002), its naturally low occurring soil population levels (Hendrix and Kuhlman, 1965; Eden *et al.*, 2000) as well as the small quantities of soil that can be analysed using commercial soil DNA extraction kits (Sena *et al.*, 2018). The poor performance of Pc soil DNA extractions has likewise been reported in another study (Sena *et al.*, 2018). Sena *et al.* (2018) were unable to detect Pc soil propagules using DNA-based methods, despite positive detections being reported for soil baiting culture methods. However, the inability to detect Pc soil propagules in the study of Sena *et al.* (2018), in comparison to the current study, is likely due to their use of bulk soil samples rather than rhizosphere soil. Rhizosphere soil is expected to have higher soil population levels than bulk soil and thus would provide a better approach for investigating soil inoculum.

The roots of the Dusa® rootstock tended to yield higher Pc root DNA concentrations than the R0.06 rootstock in November 2017. This can be expected, since a reduction in Pc root colonisation has previously been associated with PRR-tolerant rootstocks (Engelbrecht *et al.*, 2013), and it has been suggested that R0.06 is more PRR-tolerant than Dusa® (Van Rooyen, 2017). Data from the current study thus supports the theory that the R0.06 rootstock may have a greater PRR tolerance than Dusa®. However, lower Pc root colonisation levels do not always mean that the rootstock has a greater PRR tolerance since tolerant rootstocks can also have

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a high root regenerative ability and, thus, can compensate for the root damage caused by Pc (Kellam and Coffey, 1985). The lower Pc root colonisation levels associated with the R0.06 rootstock may be due to a reduction and/or delay in zoospore germination (i.e. decreased pathogen infection rate). In addition, during early stages of infection, strong activation of β-1,3-glucanase and the deposition of impermeable callose at the site of host plant cell penetration can occur in this rootstock (Van den Berg *et al.*, 2018).

Comparisons between a wide range of PRR-susceptible to PRR-tolerant rootstocks was outside the scope of the current study but would be useful, along with a different quantification method, to evaluate rootstocks in future investigations. Although different rootstocks (Dusa® and Duke 7) were used in the phosphonate trials of the current study, it was difficult to compare these rootstocks directly since variations between soil factors of each orchard occurred, making some orchards more conducive to Pc root infection and colonisation than others; the higher clay content observed in the BL orchard may have led to longer periods of soil saturation and thus created more favourable conditions for the higher Pc root colonisation levels shown for this orchard. The use of a root baiting quantification approach may provide an interesting alternative to qPCR quantification for comparing host-pathogen interactions since only viable inoculum can be detected; e.g. root baiting may illustrate differences in the reproductive capacity of the pathogen due to the varied host defence responses amongst rootstocks.

Although qPCR quantification of Pc from roots and soil were unsuccessful in revealing the effect of management strategies (phosphonates or rootstock tolerance) in the current study, important observations were made regarding the effect of sampling time on Pc DNA concentrations. In the phosphonate trials, the Pc soil and root DNA concentrations obtained in one of the orchards (EL and BL, respectively) was significantly higher in May than in October. This supports a previous study conducted in un-mulched orchards, which observed a peak in Pc root colonisation in May (Chapter 2). This peak in Pc root colonisation could also potentially coincide with higher Pc soil DNA concentrations, as was partially observed in the current study. As a result, May could also potentially be a critical sampling month for soil quantification analyses. Contrarily, in the rootstock trials, November 2017 yielded higher Pc root DNA concentrations than May 2018 and thus does not support the theory that a peak in Pc root colonisation occurs in May. This may be due to the annual mulch applications conducted in the rootstock trial orchard, lowering the soil temperatures to less favourable conditions for the pathogen during the critical root colonisation period; wood chip mulches have previously been reported to lower soil temperatures by Gruda (2008). Alternatively, the fact that mulches reduce soil temperature fluctuations (Downer *et al.*, 2002), might negatively affect zoospore release in late autumn (April to May) since zoospore release is triggered by a

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lowering of soil temperatures (Khew and Zentmyer, 1973; Hwang *et al.*, 1975; Byrt and Grant, 1979).

In conclusion, qPCR analyses of avocado root and rhizosphere soil samples were not useful for assessing the efficacy of management strategies. From a disease management perspective, investigating soil inoculum is important for orchard replant situations and further work would thus be required to investigate useful soil quantification techniques. From the current study, it was noticeable that the Pc DNA concentrations were highly variable in both the root and soil samples. Therefore, it might be useful to, in future, use a trial design where a larger number of trees are assessed within replicates. The trial design of the current study consisted of eight single tree replicates, which might not be representative of Pc root colonisation levels amongst trees in avocado orchards. Sampling more trees per treatment may help to reduce variability and thus increase the likelihood of obtaining significant differences between phosphonate treatments. For example, Masikane (2019) was able to observe significant reductions in Pc root DNA concentrations by phosphonate treatments in asymptomatic orchards, when a sampling strategy consisting of six replicates, each containing four trees, was used.

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Table 1. Primers, probes, amplification conditions and quantitative real-time PCR (qPCR) quality parameters of three *Phytophthora cinnamomi*-specific qPCR assays. [a]

Target gene	Primer or probe	Final concentration in reaction (mM)	Sequence (5' – 3')	Annealing temp. (°C)	Annealing time (s)	Limit of quantification [b]	Efficiency (%) [c]	Linearity (R^2)	Reference
*Ypt*1	Pcy3F	320	TGCCCCCATTCAACAGACGC	60	40	700 fg	0.91	0.997	Masikane (2017)
	Pc5R	320	CAGCACCATATATTTGTTCAGTCAG						
	PcP5	160	FAM-AGCTTCCAACAGGCGAATAGGACC-BQH1						
EIPC	EIPC100F	240	AGGCTAGCTAGGACCGATCAATAGG	60	40	12 copies	0.98	0.998	Fall *et al.* (2015)
	EIPC100R	240	AGTGCTTCGTTACGAAAGTGACCTTA						
	EIPC100P	80	HEX-CCTATGCGTTCCGAGGTGACGACCTTGCC-BQH1						
ITS	PciF2	300	GGAACTGAGCTAGTAGCCTC	64	30	700 fg	0.80	0.996	Langrell *et al.* (2011)
	PciR2	300	CAATTGAGATGCCACCACAA						
atp9-nad9	PhyG_ATP9_2FTail	900	AATAAATCATAACCTTCTTTACAACAAGAATTAATG	57	90	15 pg	0.86	0.981	Bilodeau *et al.* (2014)
	PhyG_R6Tail	900	AATAAATCATAAATACATAATTCATTTTTATA						
	Pcinn_nad9sp_probe 1	150	FAM-AAGAAATATTTAGTTTATTAATATATAATATAACT-BQH1						

[a] *Ypt*1 = Ras-related *Ypt*1 protein gene, EIPC = an exogenous internal positive control plasmid DNA fragment, ITS = internal transcribed spacer region, *atp9-nad9* = mitochondrial encoded *atp9-nad9* gene region.

[b] The sensitivity of a qPCR assay depends on its limit of quantification (LOQ) which represents the lowest pathogen DNA concentration (within samples) that can be determined with acceptable precision and accuracy, according to the stated conditions of the assay.

[c] The efficiency of a qPCR assay refers to the rate at which the polymerase enzyme converts the reaction reagents into amplicons; low reaction efficiencies (less than 0.85) can indicate problems with the assay, such as poor primer design, which can affect the accuracy of quantification.

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Table 2. Analysis of variance on the effect of different phosphonate treatments on root phosphite concentrations and *Phytophthora cinnamomi* (Pc) root and soil DNA concentrations in three avocado orchards that were measured in two different months (May and October 2018).[a]

		Phosphite concentration		Pc root DNA concentration		Pc soil DNA concentration	
Source	**Df**	**MS**	***P*-value**	**MS**	***P*-value**	**MS**	***P*-value**
Orchard	2	26.7962	< 0.0001	4.5659	0.0207	0.6711	0.6414
Treatment	2	14.6750	< 0.0001	1.2280	0.3361	1.2405	0.4422
Treatment x orchard	4	0.6497	0.6252	0.5391	0.7451	0.9942	0.6203
Month	1	13.8150	< 0.0001	1.6886	0.1420	3.4361	0.1303
Treatment x month	2	0.2823	0.5291	0.7066	0.4018	0.5033	0.7101
Month x orchard	2	0.0650	0.8627	6.0915	0.0008	4.4831	0.0537
Treatment x month x orchard	4	1.1104	0.0492	0.8154	0.3801	0.8281	0.6881

[a] The ANOVA analyses were conducted on Log(x + 1), Log(x + 0.01) and Log(x + 0.00001) transformed data for the root phosphite, Pc root DNA and Pc soil DNA concentrations, respectively. Df = degrees of freedom, MS = mean square, *P*-value = significance level of the *F*-value.

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Table 3. The effect of month on the *Phytophthora cinnamomi* (Pc) root and soil DNA concentrations in three avocado orchards.[a]

	Pc root DNA concentration (ng/mg_{DW})		Pc soil DNA concentration (pg/mg_{DW})	
Orchard	**May**	**October**	**May**	**October**
BL	0.1394 a	0.0199 b	0.0023 ab	0.0363 ab
EL	0.0408 b	0.0199 b	0.0225 a	0.0002 b
FM	0.0165 b	0.0235 b	0.0117 ab	0.0002 b

[a] For the Pc root and soil DNA concentration values separately, values in columns and rows followed by the same letter do not differ significantly according to Fisher's least significant difference test ($P \geq 0.05$). Post-hoc analyses were conducted on Log(x + 0.01) and Log(x + 0.00001) transformed data for the Pc root and soil DNA concentrations, respectively. The actual Pc root DNA concentrations **(**ng/mg_{DW}) and Pc soil DNA concentrations **(**pg/mg_{DW}) are shown. Values are the average of eight replicates per treatment, with each replicate consisting of one tree.

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Table 4. The effect of phosphonate treatments on root phosphite concentrations over two sampling months (May and October) in three avocado orchards.[a]

	Orchard BL		Orchard EL		Orchard FM	
Treatment [b]	**May**	**October**	**May**	**October**	**May**	**October**
Control	8.836 gh	1.363 i	19.055 def	12.255 fgh	30.545 bcd	21.218 def
1x trunk injection	14.000 h	12.472 h	32.218 bcd	19.709 def	50.309 ab	22.400 cde
2x trunk injection	21.382 de	19.164 efg	41.145 abc	28.291 cde	57.109 a	41.200 abc

[a] Values in columns and rows followed by the same letter do not differ significantly according to Fisher's least significant difference test ($P \geq 0.05$). Post-hoc analysis was conducted on Log(x + 1) transformed root phosphite data. The actual root phosphite concentrations ($\mu g/g_{FW}$ roots) are shown. Values represent the average of eight replicates per treatment, with each replicate consisting of one tree.

[b] The 1x trunk injection treatment consisted of the application of one phosphonate trunk injection in April 2018 (after the summer foliar flush had hardened off), whereas the 2x trunk injection treatment consisted of phosphonate trunk injections being applied in November 2017 (after the spring foliar flush had hardened off) and in April 2018. The trunk injections were all applied at the preventative dosage of 0.3 g a.i./m^2.

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APPENDIX A

Supplementary Table 1. Percentage of soil moisture and accumulated hours at five different soil temperature ranges from two avocado production regions (Letaba and Mooketsi) in the Limpopo province in South Africa, for different months in 2017. [a]

	Moisture (%)		10-14°C		15-19°C		20-24°C		16-24°C		25-29°C	
Month	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**
January	68.225	51.502	0.00	0.00	0.00	0.00	719.50	603.00	708.00	603.33	23.50	133.33
February	68.804	51.370	0.00	0.00	0.00	0.00	623.50	522.67	623.50	522.67	48.50	141.67
March	67.206	48.711	0.00	0.00	0.00	0.00	736.50	684.67	736.50	684.67	7.50	15.33
April	66.939	47.516	0.00	0.00	14.00	54.00	454.00	629.67	468.00	687.67	0.00	2.00
May	64.795	46.681	0.50	0.00	529.50	454.00	37.50	274.00	536.00	728.00	0.00	0.00
June	60.653	47.149	63.00	0.00	634.50	562.67	0.00	0.00	378.50	523.00	0.00	0.00
July	61.535	48.955	67.00	0.00	676.50	744.00	0.00	0.00	447.50	692.67	0.00	0.00
August	61.511	48.422	45.50	3.00	695.00	709.00	2.00	6.67	498.50	682.67	0.00	0.00
September	63.113	48.969	1.50	0.00	598.50	362.33	106.00	348.67	695.50	711.00	0.00	0.00
October	65.209	50.234	0.00	0.00	377.50	69.67	365.50	674.00	743.00	743.67	0.00	0.00
November	65.681	50.885	0.00	0.00	228.50	14.33	490.00	694.33	718.50	708.67	0.00	11.00
December	67.587	52.367	0.00	0.00	3.50	1.67	733.50	683.33	737.00	685.00	5.50	58.67

[a] Values represent the average for one-year (2017) of each month (January to December) for the soil parameters: (i) percentage of soil moisture (%) and (ii) hours accumulated at five different soil temperature ranges (10-14°C, 15-19°C, 20-24°C, 16-24°C and 25-29°C).

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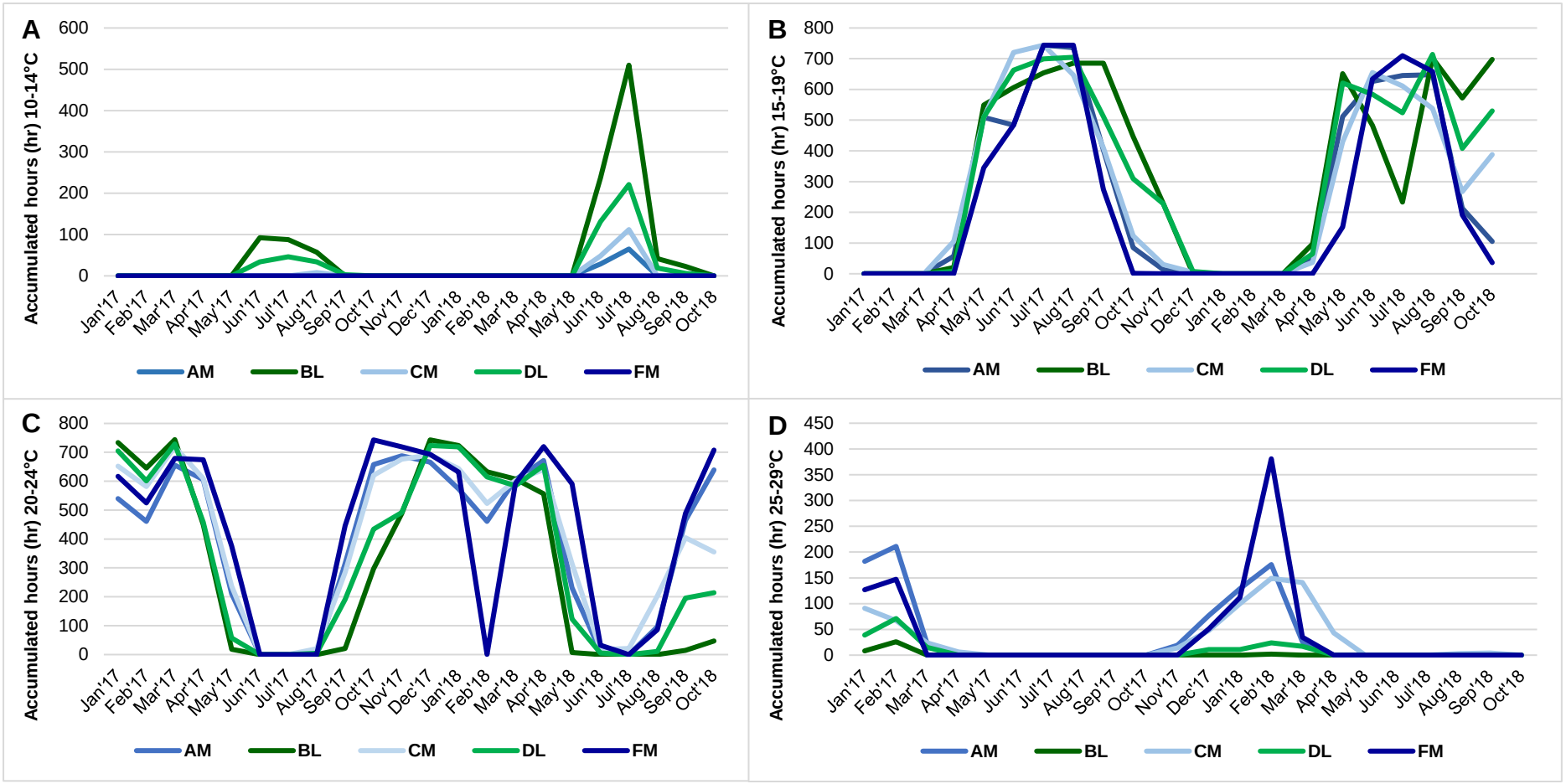
Supplementary Table 2. Percentage of soil moisture and accumulated hours at five different soil temperature ranges from two avocado production regions (Letaba and Mooketsi) in the Limpopo province in South Africa, for different months in 2018. [a]

	Moisture (%)		10-14°C		15-19°C		20-24°C		16-24°C		25-29°C	
Month	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**	**Letaba**	**Mooketsi**
January	66.873	50.775	0.00	0.00	0.00	0.00	721.50	616.33	721.50	616.33	5.50	113.67
February	68.986	50.966	0.00	0.00	0.00	0.00	624.00	328.33	624.00	414.00	13.00	235.33
March	68.239	50.570	0.00	0.00	0.00	0.00	596.00	600.67	596.00	600.67	8.50	67.33
April	67.244	48.741	0.00	0.00	81.00	28.33	606.00	677.33	687.00	705.67	0.00	14.33
May	67.042	49.123	0.00	0.00	636.00	364.67	65.00	378.67	701.00	743.33	0.00	0.00
June	65.355	48.884	184.00	25.67	533.50	638.33	2.50	20.00	323.00	576.00	0.00	0.00
July	64.766	48.319	365.50	59.00	378.50	655.33	0.00	7.00	152.50	545.00	0.00	0.00
August	66.454	48.812	30.50	0.00	708.00	614.67	5.50	128.33	550.50	735.00	0.00	1.00
September	63.931	48.812	14.50	1.00	490.00	223.67	105.50	452.67	541.50	673.33	0.00	1.33
October	65.131	49.108	0.00	0.00	614.00	176.33	130.50	567.33	737.00	743.67	0.00	0.00

[a] Values represent the average for one-year (2018) of each month (January to October) for the soil parameters: (i) percentage of soil moisture (%) and (ii) hours accumulated at five different soil temperature ranges (10-14°C, 15-19°C, 20-24°C, 16-24°C and 25-29°C). November and December are excluded from the table since soil probe data was not collected for these months in 2018.



Supplementary Figure 1. Monthly accumulated hours at four soil temperature ranges (A) 10-14°C (B) 15-19°C (C) 20-24°C and (D) 25-29°C measured over two years (2017 and 2018) using soil probes at a depth of 0-20 cm. One soil probe was positioned in each of the three orchards located in the Mooketsi region (blue-marked line) and each of the two orchards located in the Letaba region (green-marked lines). The temperature ranges were selected according to information published by Zentmyer (1981).

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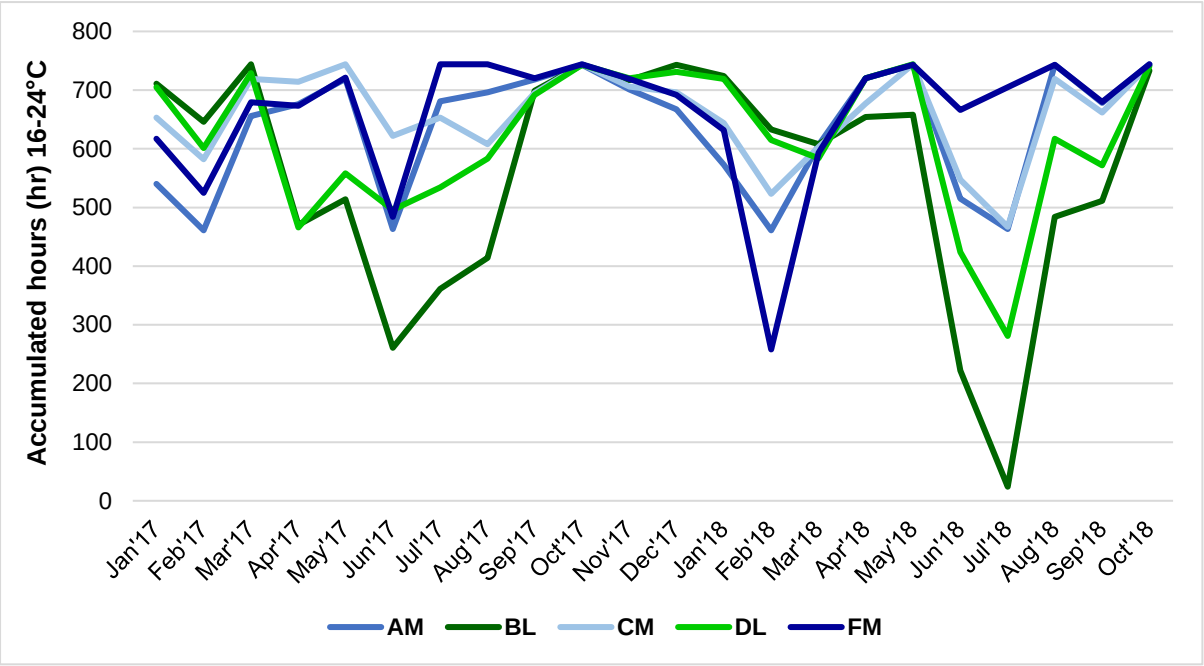
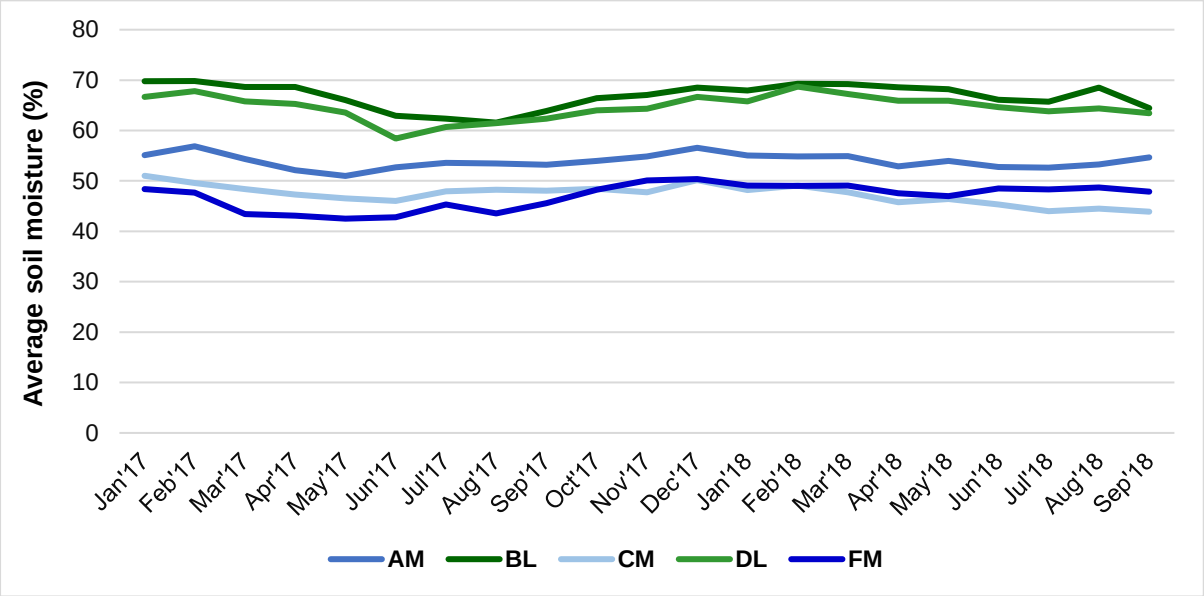


Supplementary Figure 2. Monthly accumulated hours at the soil temperature range of 16-24°C measured over two years (2017 and 2018) using soil probes at a depth of 0-20 cm. One soil probe was positioned in each of the three orchards located in the Mooketsi region (blue-marked lines) and each of the two orchards located in the Letaba region (green-marked lines). The soil temperature range was selected according to information published by Burgess *et al.* (2017).



Supplementary Figure 3. Monthly measurements of the average percentage of soil moisture (%) recorded over two years (2017 and 2018) using soil probes at a depth of 0-20 cm. One soil probe was positioned in each of the three orchards located in the Mooketsi region (blue-marked lines) and each of the two orchards located in the Letaba region (green-marked lines).