QUANTIFICATION OF APPLE REPLANT PATHOGENS FROM ROOTS, AND THEIR OCCURRENCE IN IRRIGATION WATER AND NURSERY TREES

by

SHAHROOZ MOEIN

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Supervisor: Prof. Adele McLeod

Co-supervisor: Prof. Mark Mazzola

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SUMMARY

Apple replant disease (ARD), an economically important disease of apple, occurs when young apple trees are planted on soil previously cultivated to apple or closely related species. Soil is considered as the main inoculum source of pathogens causing ARD, which include some *Rhizoctonia* spp., *Cylindrocarpon*-like spp., parasitic nematodes (*Pratylenchus* spp.) and oomycetes (*Pythium, Phytopythium* and *Phytophthora*). However, additional inoculum sources might be nursery trees and irrigation water. Investigations into inoculum sources, and effective disease management strategies, require knowledge on relationships between pathogen quantification techniques and the extent of plant damage.

The relationship between quantification techniques [percent root infection, and pathogen DNA biomass (absolute and relative)] and disease in apple seedlings were investigated for the ARD pathogens *Pythium sylvaticum*, *Pythium irregulare*, *Pythium ultimum*, *Phytopythium vexans* and *Phytophthora cactorum* in glasshouse trials. Quantification data from natural *P. irregulare* nursery infections were also investigated. In glasshouse trials, the percent root infection and pathogen DNA biomass quantities (absolute and relative) were good predictors of apple seedling growth reductions for *P. sylvaticum, Pht. vexans* and *P. ultimum,* when targeting the fine feeder root system. Significant and high correlations were also found for these pathogens between pathogen DNA biomass and percent infected roots. This, however, was not true for *Ph. cactorum* and *P. irregulare.* For *P. irregulare* only a low, but significant correlation was found between percent root infection and seedling stunting. In the nursery where *P. irregulare* was investigated, good and significant correlations were found between percent root infection and pathogen DNA biomass (absolute and relative).

Apple nursery trees and irrigation water (only oomycetes) were investigated as ARD inoculum sources over two seasons in the Western Cape province of South Africa. Nursery trees were a large inoculum source of *P. irregulare, Cylindrocarpon*-like spp. and *Pratylenchus* spp., and to a lesser extent of *P. ultimum. Pythium sylvaticum* was rarely detected, whereas *Ph. cactorum* and *Pht. vexans* were absent in nursery trees. The nurseries differed in the occurrence of trees infected with specific pathogens, but none were free of ARD pathogens. Almost all trees were infected with *P. irregulare* (95%) and *Cylindrocarpon*-like species (100%). *Pythium ultimum* was present in 60% of nurseries and 41% of trees. An average of 35% of trees were infested with *Pratylenchus* spp., with some trees (6-22%) having unacceptably high infestation levels. Irrigation water analyses in dams, and at the exit point of irrigation lines over a 5 month period in each of two seasons revealed the presence of a few ARD pathogens. Only *P. irregulare* was considered as important and was detected in each of the sampled months in 31 to 82% of the orchard water samples, and the pathogen also

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occurred in dam water. *Pythium ultimum, Phytopythium litorale* and *Pythium* spp. complex B2A were rarely detected in orchard water.

The study demonstrated that percentage root infections and pathogen DNA biomass quantity (absolute and relative) can provide a good prediction of apple seedling growth reduction for *P. sylvaticum, Pht. vexans* and *P. ultimum,* but not *Ph. cactorum* and *P. irregulare*. Oomycete pathogen DNA biomass in roots can be quantified equally well using relative or absolute qPCR amplification. Knowledge was also gained on the colonization pattern of the root system by different oomycete pathogens. Apple nursery and irrigation water of apple orchards were shown to be inoculum sources of apple replant pathogens by using two approaches, conventional isolation and qPCR assay. qPCR significantly improved detection of ARD pathogens relative to conventional isolations. For nurseries, rootstock management strategies will be required. The biological significance of ARD oomycete pathogens in irrigation water requires further investigation.

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OPSOMMING

Appel herplant siekte (AHS), ‘n ekonomies belangrike siekte van appels, kom voor wanneer jong appelboompies geplant word op grond waarop daar voorheen appels of naby-verwante spesies geplant was. Grond word gesien as die hoof inokulumbron van patogene wat AHS veroorsaak, wat sommige *Rhizoctonia* spp., *Cylindrocarpon*-agtige spp., parasitiese nematodes (*Pratylenchus* spp.) en oömisete (*Pythium, Phytopythium* en *Phytophthora*) insluit. Addisionele inokulumbronne kan egter kwekerybome en besproeiingswater wees. Ondersoeke na inokulumbronne, en effektiewe siektebestuurstrategieë, vereis kennis oor die verhoudings tussen patogeen kwantifiseringstegnieke en die mate van plantskade.

Die verhouding tussen kwantifiseringstegnieke [persentasie wortel-infeksie, en patogeen DNS biomassa (absoluut en relatief)] en siekte in appelsaailinge, is vir die AHS patogene *Pythium sylvaticum*, *Pythium irregulare*, *Pythium ultimum*, *Phytopythium vexans* en *Phytophthora cactorum* in glashuisproewe ondersoek. Kwantifiseringsdata vanaf natuurlike *P. irregulare* kwekery-infeksies is ook ondersoek. In glashuisproewe was die persentasie wortel-infeksie en patogeen DNS biomassa hoeveelhede (absoluut en relatief) goeie voorspellers van appelsaailinggroei vermindering vir *P. sylvaticum, Pht. vexans* en *P. ultimum,* wanneer die fyn voedingswortelsisteem aangeval word. Betekenisvolle en hoë korrelasies is ook vir hierdie patogene gevind tussen patogeen DNS biomassa en persentasie geïnfekteerde wortels. Dit was egter nie waar vir *Ph. cactorum* en *P. irregulare* nie. Vir *P. irregulare* is slegs ‘n lae, maar betekenisvolle korrelasie tussen wortel-infeksie en saailing verdwerging waargeneem. In die kwekery waar *P. irregulare* ondersoek is, is goeie en betekenisvolle korrelasies tussen persentasie wortel-infeksie en patogeen DNS biomassa (absoluut en relatief) waargeneem.

Appel kwekerybome en besproeiingswater (slegs oömisete) is as AHS inokulumbronne, oor twee seisoene in die Wes-Kaap Provinsie van Suid-Afrika, ondersoek. Kwekerybome was ‘n groot inokulumbron van *P. irregulare, Cylindrocarpon*-agtige spp. en *Pratylenchus* spp., en tot ‘n minder mate van *P. ultimum. Pythium sylvaticum* is bykans nooit waargeneem nie, terwyl *Ph. cactorum* en *Pht. vexans* afwesig in kwekerybome was. Die kwekerye het verskil in die voorkoms van bome geïnfekteer met spesifieke patogene, maar geen een was vry van AHS patogene nie. Amper alle bome was geïnfekteer met *P. irregulare* (95%) en *Cylindrocarpon*-agtige spesies (100%). *Pythium ultimum* was teenwoordig in 60% van die kwekerye en 41% van die bome. ‘n Gemiddeld van 35% van die bome was geïnfesteer met *Pratylenchus* spp., met sommige bome (6-22%) wat onaanvaarbare hoë infestasie-vlakke gehad het. Besproeiingswater analises in damme, en by die uitgangspunte van besproeiingslyne oor ‘n 5 maand periode, in elk van twee seisoene, het op die teenwoordigheid van ‘n paar ARD

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patogene gedui. Slegs *P. irregulare* is as belangrik beskou en is in elk van die monsterneem maande in 31 tot 82% van die boordwatermonsters waargeneem, en die patogeen het ook in damwater voorgekom. *Pythium ultimum, Phytopythium litorale* en *Pythium* spp. kompleks B2A is selde in boordwater waargeneem.

Die studie het getoon dat persentasie wortel-infeksies en patogeen DNS biomassa hoeveelheid (absoluut en relatief) ‘n goeie aanduiding kan verskaf vir appelsaailing groeivermindering vir *P. sylvaticum, Pht. vexans* en *P. ultimum,* maar nie vir *Ph. cactorum* en *P. irregulare* nie. Oömiseet patogeen DNS biomassa in wortels kan ewegoed gekwantifiseer word deur die gebruik van relatiewe en absolute qPCR amplifisering. Kennis is ook opgedoen oor die kolonisasie-patroon van die wortelsisteem deur verskillende oömiseet patogene. Appelkwekerye en besproeiingswater van appelboorde is aangedui as inokulumbronne van appel herplant patogene deur die gebruik van twee benaderings, konvensionele isolasie en qPCR toetse. qPCR het waarneming van AHS patogene betekenisvol verbeter relatief tot konvensionele isolasies. Onderstam bestuurstrategieë sal in kwekerye toegepas moet word. Die biologiese belang van AHS oömiseet patogene in besproeiingswater benodig verdere ondersoek.

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CHAPTER 1

Etiology and management of apple replant disease, and methods for investigating inoculum sources

INTRODUCTION

The apple tree *Malus domestica* belongs to the family Rosaceae, and is cultivated worldwide. *Malus sieversii* is its wild ancestor, and the centre of origin of both *Malus* spp. is in Central Asia. This deciduous fruit tree can be small if grafted onto rootstocks or large if grown from seed. There are more than 7,500 different known apple cultivars world-wide that display a variety of different characteristics (Gibson, 2008).

In 2012, about 63 million tons of apples were produced world-wide. Half of this total were produced by China, followed by the United States which was the second largest producer delivering about 6% of the total world-wide apple production. Third place belongs to Turkey, whereas Italy, India, Poland and Iran are among the other large apple producing countries in the world (FAOSTAT, 2012). South Africa is about the sixteenth largest apple producing region in the world. In 2014, about 792549 tons of apples were produced on 22925 hectares in South Africa. The Ceres area is the largest production region (6724 ha), followed by the Groenland region (5819 ha), Langkloof East (4191 ha) and Villiersdorp (3707 ha) (HORTGRO, 2014).

Apple replant Disease (ARD) is characterized by the poor growth of young apple trees that are replanted onto orchard soil previously cultivated with apple trees or closely related species. The disease is of great economic importance world-wide exhibiting the capacity to significantly limit apple production (Mazzola, 1998). Symptoms of ARD include growth reductions (stunting), a reduction in yield and vigor of trees (Traquair, 1984), small root systems and decayed or discolored roots, shortened internodes and rosetted leaves (Savory, 1966; Traquair, 1984). Trees affected by ARD will start bearing fruit 2 to 3 years after healthy trees, thus negatively affecting the economic value of the orchard throughout its lifespan (Mazzola, 1998). The severity of ARD differs in orchards (Hoestra, 1968; Mazzola, 1988). Other terminologies that have been used to describe ARD, include soil sickness (Utkhede and Li, 1987), replant specific sickness (Hoestra and Oostenbrink, 1962), continuous cropping obstacle, replant problem (Koch, 1955), and rejection component (McKenry, 1999) and replant disorder (Eayre *et al*., 2000).

Biological agents have been identified as the main factors causing ARD, since soil fumigation results in significant growth increases of apple trees in fumigated soils compared to non-fumigated soils (Covey *et al*., 1979; Abawi, 1981; Gur *et al*., 1991). The same effect

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has also been shown by applying soil pasteurization (Hoestra, 1968, Jaffee et al., 1982a). Abiotic factors such as imbalanced nutrients, soil acidity, soil structure and drainage, site deterioration and lack or excess of moisture and phytotoxic metabolites have been suggested as being involved in apple replant disorder. However, these factors singly cannot cause apple replant disease symptoms (Proebsting and Gilmore, 1971; Patrick, 1955; Rowe and Catlin, 1971; Abawi, 1981; Mizutani *et al*., 1987; Gur and Cohen, 1989; Utkhede and Smith, 1993), but could enhance ARD severity.

Multiple biological agents contribute to ARD development, and these can vary from orchard to orchard. The main groups of agents involved include nematodes, fungi and oomycetes. *Pratylenchus penetrans* is the main nematode involved in most countries, but in some countries such as South Africa other species are also involved. Among the fungi *Rhizoctonia* (multi- and binucleate spp.) and *Cylindrocarpon*-like spp. are important, with some multinucleate *Rhizoctonia* being the most virulent. Several oomycetes belonging to the genera *Phytophthora* and *Pythium* have been shown to form part of the ARD pathogen complex, with *Phytophthora cactorum* being among the most virulent and common species along with a few *Pythium* spp. (Tewoldemedhin *et al.*, 2011a, b; Mazzola and Manici, 2012).

In ARD, the soil is considered as being the main source of pathogen inoculum. However, since nursery material can often be contaminated with pathogens in many crop systems (Morgan *et al*., 2002), this material may also serve as an inoculum source at the start of the season. This is especially important in South Africa, since nursery trees are continuously produced from the same mother-layer blocks, which could accumulate pathogens over time. Furthermore, the rootstocks derived from the mother-layer blocks are planted in open fields, which can sometimes also harbour soilborne pathogens and pests. Another external inoculum source of inoculum could be irrigation water, since oomycete pathogens are commonly recovered from such sources (Yamak *et al*., 2002; Porter and Johnson, 2004).

This literature review will summarize the current knowledge concerning the biological causal agents of ARD. This will be followed by a summary of methods used to manage the disease and isolation and baiting techniques, molecular identification and quantification of ARD pathogens. Information is also provided on the occurrence of oomycetes, *Rhizoctonia* and *Pratylenchus* in irrigation and surface water. The review is finalized by a conclusion section that also provides a rationale for research conducted in this thesis.

BIOLOGICAL CAUSAL AGENTS OF ARD

Oomycetes

Depending on which classification schemes are used, the genera *Pythium* and *Phytophthora* can belong to the Kingdom Chromista or Stramenopila and the class or sub phylum Oomycota

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(Alexopoulos *et al*., 1996; Kirk *et al*., 2008). The genus *Pythium* is highly diverse and consists of more than 120 species (Dick, 1990). Within this genus, some species has recently been assigned to a new genus, *Phytopythium* (de Cock *et al*., 2014). There are some significant differences, but also some similarities, in the biology of oomycetes compared to fungi, and they are thus often called fungal-like. For example, chitin is the main compound of which true fungal cell walls are comprised, but the cell walls of oomycetes are composed of glucans and cellulosic compounds (Erwin and Ribeiro, 1996; Hardham, 2005).

The genus *Phytophthora* has been described as one of the most economically important pathogen groups in agriculture and forestry, and consists of more than 100 species (Erwin and Ribeiro, 1996; Brasier 2008). The first species, *Phytophthora infestans,* was described and published by de Bary (1876). This genus contains numerous species of global significance, such as *Ph. infestans,* the causal agent of the Irish potato famine, *Phytophthora ramorum*, the causal agent of sudden oak death, and *Phytophthora cinnamomi*, the causal agent of Eucalypt dieback. Members of this genus can cause root rots, crown rots, fruit rots, pod rots, foliar blights and diebacks of a wide array of hosts representing many plant families (Erwin and Ribeiro, 1996). The host specificity of various species of *Phytophthora* can range from broad to very narrow (Erwin and Ribeiro, 1996). Host range varies from host-specific species as in *Phytophthora idaei*, a pathogen of *Rubus*, to a very broad host range with thousands of host species as in *Ph. cinnamomi* (Erwin and Ribeiro, 1996). As a genus, *Phytophthora* is usually associated with colonizing living plant tissue and most species are thus plant pathogens. However, *Phytophthora gonapodyides* has been reported to colonize living and dead leaf tissue in streams, implying saprobic behaviour (Erwin and Ribeiro, 1996; Hansen and Delatour, 1999; Drenth *et al*., 2006; Jung *et al*., 2011).

Several studies in the USA have reported a couple of *Phytophthora* spp. as agents of ARD in apple orchards, including *Phytophtora drechsleri, Ph. cinnamomi, Phytophthora parasitica, Phytophthora cambivora* and *Ph. cactorum* (Julis *et al*., 1978, 1979; Matheron *et al*., 1988; Mazzola, 1998). Utkhede *et al*. (1992a) conducted pathogenicity studies in sterilized replant soil with three species of *Phytophthora*. He reported that *Ph. cactorum* and *Ph. cinnamomi* significantly reduced the growth of apples trees, but that *Ph. cambivora* was the most virulent of the three species on young apple trees (5 months old) and killed all of the inoculated apple trees. Recent research in Washington State also reported *Ph. cambivora* associated with ARD in this region (Mazzola and Brown, 2010). In South Africa, only *Ph. cactorum* has been identified in ARD orchards, with pathogenicity testing showing that *Ph. cactorum* are highly virulent towards apple seedlings (Tewoldemedhin *et al*., 2011b). Aside from its involvement in ARD, *Phytophthora* is known to also cause other symptoms on mature apple trees including root, collar and crown rot (Erwin and Ribeiro, 1996). *Phytophthora cactorum* has most frequently been identified as the main species causing these symptoms,

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including in South Africa (Van der Scheer, 1971; Van der Merwe and Mathee, 1973; Julis *et al*., 1979; Mircetich and Matheron, 1983; Helton *et al*., 1984; Zondo *et al*., 2007). In South Africa, using an *in vitro* assay, it was shown that the two rootstocks MM106 and MM109 were significantly more susceptible to infection by this pathogen during summer than in winter. The MM106 rootstock was also shown to be more resistant than MM109 from July to January (Zondo *et al*., 2007).

A large number of *Pythium* species are known to be parasitic on a wide range of crops causing various disease symptoms (Cook *et al*., 2007). Species in this genus differ in host range with some having a wide range of host plants, for example *Pythium irregulare* and *Pythium ultimum* (Van der plaats-Niterink, 1981; Levesque and De Cock, 2004), whereas *Pythium sulcatum* is a pathogen of just a few species (Van der plaats-Niterink, 1981). Several species within the genus are non-pathogenic, and several species have also shown potential as biocontrol agents in suppressing pathogenic *Pythium* species (Vallance *et al*., 2009; Bahramisharif *et al*., 2013) The new genus *Phytopythium* has recently been described and consists of about 15 species. The first indications for the presence of this new genus was by Lévesque and de Cock (2004) who provided evidence that *Pythium* phylogenetic clade K belongs to a different genus. Subsequently, De Cock *et al.* (2014) officially described clade K as the genus *Phytopythium.* Morphological comparison of this genus with *Pythium* and *Phytophthora* showed that *Phytopythium* forms morphological structures with characteristics that is between *Pythium* and *Phytophthora*. *Phytopythium* has unique lobate antheridia, papillate and internally proliferating sporangia. Evidence of the distinct nature of *Phytopythium* as a genus is further provided by phylogenetic sequence data (de Cock *et al*., 2014).

Several *Pythium* species have been associated with ARD but only a few of them are highly virulent toward apple trees or seedlings, with some even enhancing the growth of apple seedlings (Mazzola *et al*., 2002b). In England, *Pythium sylvaticum* and *Pythium intermedium* were shown to cause growth reductions of apple seedlings, which were comparable to growth reductions of seedlings planted in non-fumigated ARD soil (Sewell, 1981). More than 17 *Pythium* species were associated with ARD in apple orchards in Washington State, with the most prominent species being *P. ultimum*, *Pythium heterothallicum, P. irregulare, P. intermedium* and *P. sylvaticum* (Mazzola, 1998; Mazzola *et al*., 2002a, b, 2009). In New York and Canada, *P. irregulare* was isolated from several ARD soils (Jaffe *et al*., 1982a; Braun, 1991, 1995). Manici *et al*. (2003) could only isolate *P. intermedium* from ARD soils in Italy. Several other studies have reported *P. irregulare* (Swell, 1981; Jeffers *et al*., 1982; Braun, 1991, 1995)*, P. sylvaticum* (Mulder, 1969; Sewell, 1981), *P. intermedium* (Sewell, 1981) and *P. ultimum* (Utkhede *et al*., 1992a) as being highly virulent toward apple seedlings. In the Western Cape region of South Africa, Van Schoor *et al*. (2009) reported that the genus *Pythium* was associated with ARD, but the specific species were not investigated. In a

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subsequent study in South Africa, Tewoldemedhin *et al*. (2011b) identified nine *Pythium* species associated with ARD including *P. irregulare*, *Phytopythium vexans*, *P. heterothallicum, Pythium dissotocum, Phytopythium litorale, Pythium attrantheridium, Pythium folliculosum, P. sylvaticum* and *Pythium minus*. *Pythium minus* and one isolate of *P. attrantheridium* were the only non-pathogenic isolates identified. *Pythium irregulare* and *Pht. vexans* had the widest distribution in South Africa. Pathogenicity assays indicated that *P. irregulare*, and *P. sylvaticum* were highly virulent toward apple seedlings, and this was also the first report of *Pht. vexans* being highly virulent on apple. Some of the other species such as *P. folliculosum, P. heterothallicum* and *P. dissotocum* were moderately virulent, whereas *Pht. litolare* was weekly virulent. One isolate of *P. attrantheridium* was non-pathogenic and the other was weekly pathogenic (Tewoldemedhin *et al*., 2011b). Additionally, in South Africa Mcleod *et al.* (2009) reported *Pythium helicandrum* as being associated with apple in South Africa, but pathogenicity studies were not conducted.

The new genus *Phytopythium* has recently been described and consists approximately 15 species. However, description of this genus has not been completed. Lévesque and de Cock (2004) described *Pythium* clade K, but molecular work provided evidence that *Pythium* clade K belongs to the genus *Phytopythium*. Morphological comparison of this genus with two other genera *Pythium* and *Phytophthora* showed *Phytopythium* based on morphological structures is between the *Pythium* and *Phytophthora* genera. *Phytopythium* has unique lobate antheridia, papillate and internally proliferating sporangia. Among ARD pathogens, highly virulent *Phytopythium vexans* and weekly virulent *Phytopythium litorale* have subsequently been placed in the genus (de Cock *et al*., 2014).

Pythium and *Phytophthora* have a sexual and asexual life cycle. An important overwintering structure in *Pythium* and *Phytophthora* is sexually formed thick-walled oospores, although some *Pythium* species form hyphal swellings and some *Phytophthora* species form chlamydospores that also play an important role in survival. Chlamydospores are rarely found in *Pythium* (Van der Plaats-Niterink, 1981). The formation of oospores may require the presence of corresponding compatible mating types in which case the species is referred to as a heterothallic species. On the other hand, oospores may be formed in single culture in the absence of a corresponding compatible mating type and the species is then called homothallic (Van der Plaats-Niterink, 1981; Judelson, 2009). Sporangia and zoospores that are important for the proliferation and dispersal of oomycetes, are the asexual structures (Van der Plaats-Niterink, 1981). However, some *Pythium* species like *P. ultimum var. ultimum* and *P. heterothallicum* produce sporangia, but not zoospores (Van der Plaats-Niterink, 1981). It has been reported that these species sometimes produce sporangia that germinate directly and produce vegetative hyphae (Aragaki *et al*., 1967; Erwin and Ribeiro, 1996) that can infect the host tissue (Hill *et al*., 1998). Sporangial shape is somewhat distinctive for each *Phytophthora*

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species, but can range from spherical, subspherical, ovoid, obovoid, ellipsoid, limoniform, pyriform, obpyriform, turbinate, to obturbinate (Erwin and Ribeiro, 1996).

The zoospores of *Pythium* and *Phytophthora* have some similarities and differences in their mode of production and behaviour. The zoospores of *Phytophthora* cleave inside the sporangium and are then released into the vesicle, from which they quickly disperse. This is not the case with the closely related genus *Pythium*, where the zoospores cleave inside the discharge vesicle after the protoplast is released from the sporangium (Gisi *et al*., 1979). In the genus *Phytophthora*, a single sporangium release up to 50 reniform, heterokont zoospores, which are capable of swimming for hours (Thomson and Allen, 1976). The primary role of these motile spores is short distance dispersal. Plant root exudates released from wounds or natural openings create chemical and electrical gradients in the soil solution, which acts as an attractant for a swimming zoospore (Islam and Tahara, 2001; Ivors, 2012). When zoospores make contact with their host, encystment occurs and flagella are shed or absorbed (Hardham, 2007). The encystment triggers the excretion of an anti-desiccation material and an adhesive protein (Hardham, 2007). The zoospore secretes the adhesive material that affixes it to the host rapidly so that adhesion is completed within 1 to 2 minutes after host contact (Hardham and Gubler, 1990). Once encystment and attachment are completed, the germ tube emerges for penetration of the host and also secretes adhesive substances to ensure close contact with the host (Hohl, 1991; Hardham, 2007). During unfavourable conditions, zoospores can also encyst by secreting a wall and shedding their flagella (Hardham, 2007). Encystment also occurs naturally when zoospores collide with soil particles and can be induced by agitating a suspension of zoospores (Erwin and Ribeiro, 1996). Depending on the *Phytophthora* sp., penetration may either be inter- or intra-cellular (Hardham, 2007). In some species, the production of appressoria has been observed for penetration (Hardham, 2007).

The importance of different propagules in the disease cycle of *Pythium* and *Phytophthora* differs depending on the mechanism of dispersion. If dispersal is through free water, zoospores play an important role in proliferation of *Pythium* and *Phytophthora.* However, irrespective of the mechanism of dispersal, zoospores will always be important in most species since they are required to swim and locate roots (Hickman and Ho, 1966; Martin and Loper, 1999; Broders *et al.*, 2009). However, some *Pythium* species like *P. ultimum* var. *ultimum* and *P. heterothallicum* do not produce zoospores and zoospores are also rarely found in *P. irregulare* (Van der Plaats-Niterink, 1981). If dispersal is through irrigation water, zoospores account for 95% of propagules of *Phytophthora* recovered from irrigation water (Carlile, 1983), since they have the ability to swim, while other propagules (e.g. mycelium, chlamydospores, etc.) tend to sink to the bottom of water (Thomson and Allen, 1974). In fields and orchards, movement of infested soil, infested nursery material and infested dust particles,

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all containing mycelia, chlamydospores or oospores, are other methods of dispersal of *Pythium* and *Phytophthora* propagules (Marais, 1980; Weste and Marks, 1987).

Soil temperature and soil type are important factors in the interaction of oomycetes with their plant hosts, and it also affects the severity of infection caused by oomycete species. For example *Pythium aphanidermatum* and *Pythium myriotylum* are known to cause serious damage in warmer areas, whereas *P. irregulare* and *P. ultimum* are more prevalent and virulent at lower temperatures (Thomson *et al*., 1971; Ingram and Cook, 1990). The high water holding capacity of clay soils creates favourable conditions for the dispersal of zoospores, and high disease severities have been reported in these soil types (Workneh *et al*., 1999; Martin and Loper, 1999).

Plant parasitic nematodes

Several parasitic organisms are able to reduce the yield potential of agricultural crops. Of these parasites, nematodes cause serious damage to most crops. Producers often have problems identifying damage caused by nematodes since the symptoms caused by nematodes are very similar to other pathogens and abiotic factors (Windham and Edwards, 1999; Castillo and Vovlas, 2007). Plant parasitic nematodes consist of about 4,100 known species world-wide (Decraemer and Hunt, 2006). Most nematodes are transparent, microscopic and vermiform with un-segmented and bilaterally symmetrical bodies (Ferris and Ferris, 1998; Windham and Edwards, 1999; Decraemer and Hunt, 2006). The nematode's body is like a tube within a tube, the cuticle is the outer tube and the reproductive system is the inner one. Different genera of nematodes in their adult stage can be sexually dimorphic (Decraemer and Hunt, 2006).

There are six stages in a nematode's life cycle including the embryo, four juveniles and the adult stage (Decraemerand Hunt, 2006). The nematode's eggs can be found in root tissue or soil (Ferris and Ferris, 1998). Depending on environmental conditions and nematode species, the life cycle is completed within 2 to 6 weeks (Windham and Edwards, 1999; Castillo and Vovlas, 2007; Agrios, 2008; Khan, 2008). There are two kinds of reproduction mechanisms within nematode species. One mechanism occurs between mating partners and the other one is parthenogenetically, which means that reproduction does not require fertilization of the female and only female offspring are produced (Windham and Edwards, 1999). Several factors including biotic and abiotic factors, such as soil texture, pH, temperature, parasites, predators and other soil organisms, influence the different stages of a nematode's life cycle (Edmunds and Mai, 1966; Walker, 1969; Norton, *et al*., 1971; Brodie, 1976; Thomas, 1978; Norton and Niblack, 1991; Sikora, 1992; Decraemer and Hunt, 2006; Castillo and Vovlas, 2007; Agrios, 2008; Bilgrami, *et al*., 2008; Khan, 2008). A range of

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temperatures between 16-32 °C is ideal for the activity of most nematodes (Windham and Edwards, 1999). However, depending on the environment, life stage and species, the optimum temperature is variable (Windham and Edwards, 1999; Robinson and Perry, 2006).

Plant parasitic nematodes can feed on different parts of the plant and differ in their feeding behaviour. While certain species may feed on leaves or stems, the preponderance of plant parasitic nematodes feed on the root system and these species have the most significant economic impact in agriculture, e.g. *Meloidogyne* spp., *Heterodera* spp. and *Pratylenchus* spp. (Sasser and Freckman, 1987). These nematodes, which are known as endoparasitic nematodes can penetrate the root system and feed on the inside of the root. Penetration and movement of endoparasitic nematodes inside the root system may cause significant physical damage and diminish the yield of the host plant (Zunke, 1990b; Hussey and Williamson, 1998). The endoparasitic nematodes also cause root wounds that serve as suitable entrances for secondary pathogens, which can increase host damage and in some cases death of the host plant (Krall, 1978; Windham and Edwards, 1999; Duncan and Moens, 2006). Ectoparasitic nematodes feed through their stylet on the external root system (Ferris and Ferris, 1998; Decraemer and Hunt, 2006; Agrios, 2008). The feeding behavior of *Pratylenchus penetrans* is known as being ectoparasitic (Zunke, 1990a), but endoparasitic feeding behaviour has also been reported for *Pratylenchus* spp. (Sasser and Freckman, 1987; Jones *et al*., 2013). Nematodes are attracted by plant root exudates (Krall, 1978; Tsai and Van Gundy, 1990), and once the nematode finds the host root it will find a suitable place for feeding on the root (Zunke, 1990a; Khan, 2008). Ectoparasitic nematodes are usually migratory nematodes that feed on different parts of host roots and can move on the root system. Endoparasitic nematodes are usually sedentary nematodes, but can also be migratory nematodes, which search for a good source of nutrients and then feed on it for their whole life span. Therefore, these nematodes do not move to other places on the host but stay at the initially selected feeding site for their whole life time. (Decraemer and Hunt, 2006). A large group of plant parasitic nematodes is classified as obligate parasites. These obligate parasites need to live in the host to feed (Windham and Edwards, 1999; Khan, 2008).

Not all nematodes are parasitic, and most are actually free living nematodes that feed on fungi and bacteria, and contribute to about 27% of the readily available nitrogen in the soil (Ekschmitt *et al*., 1999). These nematodes also have an important role in promoting rhizosphere colonization of useful rhizobacteria (Kimpinski and Sturz, 1996; Knox *et al*., 2003). In organically grown tomato, a negative correlation has been observed between plant parasitic and free-living nematodes. For this reason in sustainable agriculture, the main aims must be to increase the population of free-living nematodes and decrease the population of plant parasitic nematodes (Nahar *et al*., 2006).

There are several reports on plant parasitic nematodes associated with apple replant

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disease worldwide, including South Africa (Hoestra and Oostenbrink, 1962; Mai and Abawi, 1978; Utkhede *et al*., 1992b; Dullahide *et al*., 1994; Van Schoor *et al*., 2009). The root lesion nematode *Pratylenchus,* is the most important and main genus of plant parasitic nematode involved with ARD in Australia, Canada, South Africa and the United States (Parker and Mai, 1956; Mai *et al*., 1957; Mai and Parker 1957; 1967; 1970; Parker, 1966; Dunn and Mai, 1972; Mai and Abawi, 1978; Jaffee *et al*., 1982a,b; Merwin and Stiles, 1989; Utkhede *et al*., 1992a,b; Dullahide *et al*., 1994; Van Schoor *et al*., 2009; Tewoldemedhin *et al*., 2011b). The genus *Pratylenchus* has more than 70 species, for example *Pratylenchus hexincisus, Pratylenchus thorni, Pr. penetrans* and *Pratylenchus scribneri* (Castillo and Vovlas, 2007). *Pratylenchus penetrans* is a migratory nematode based on its feeding habits, and is considered as an endoparasitic nematode. This nematode causes serious damage and has a negative economic impact on apple orchards (McElroy, 1972). In New York, *P. penetrans* was identified in all orchards expressing symptoms of ARD. In 2009, Van Schoor reported that in South Africa, *Xiphinema* was more prevalent than *Pratylenchus* spp. in ARD orchards (Van Schoor *et al*., 2009), but the role of *Xiphinema* in ARD is unknown. However, in a different study in South Africa, *Xiphinema* was less prevelant than *Pratylenchus* species*. Pratylenchus penetrans* was found in one ARD orchard containing high numbers of nematodes, but other species of *Pratylenchus* were also identified including *Pr. scribneri* and *Pr. delattrei* that occurred at very low levels, each also in only one orchard. In one of the ARD orchards a combination of *Pr. scribneri, Paratrichodorus porosus* and *Xiphinema taylori* were found at low levels (Tewoldemedhin *et al*., 2011b).

Cylindrocarpon-like fungi

Genera with *Cylindrocarpon*-like asexual morphs, which were previously linked to the sexual genus *Neonectria,* has a world-wide distribution (Brayford, 1993; Lombard *et al*., 2014). The group of *Cylindrocarpon*-like fungi include species and isolates that range from weak pathogens to pathogens with an important economic impact (Mantiri *et al*., 2001). Black foot of grapevine is a well-known economically important disease that is caused by *Cylindrocarpon*-like fungi (Brayford, 1993; Booth, 1966; Halleen *et al*., 2004; 2006). Other important diseases that are caused by *Cylindrocarpon*-like fungi include apple replant disease (Tewoldemedhin *et al*. 2011c), root rot on ginseng (Seifert *et al*., 2003) and beech cankers (Castlebury *et al*., 2006).

The taxonomy of genera with *Cylindrocarpon*-like anamorphs has changed substantially since the first introduction of the genus *Neonectria* by Wollenweber in 1913 (Mantiri *et al*., 2001; Lombard *et al*., 2014). *Cylindrocarpon*-like asexual morphs were informally divided into four groups by Booth in 1966 based on the absence or presence of chlamydospores and microconidia, whereas the sexual genus *Neonectria* was informally

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divided into five groups (Booth, 1959; Samuels and Brayford, 1994). Several phylogenetic studies have subsequently shown that *Cylindrocarpon/Neonectria* are paraphyletic (Samuels and Brayford, 1994; Mantiri *et al*., 2001; Brayford *et al*., 2004; Halleen *et al*., 2004; 2006; Castlebury *et al*., 2006). Between 2004 and 2011, four new genera were introduced within *Cylindrocarpon/Neonectria*, which included *Campylocarpon, Thelonectria*, *Ilyonectria* and *Rugonectria* (Halleen *et al*., 2004; Chaverri *et al*., 2011). Recently the multi-gene study and morphological comparisons conducted by Lombard *et al.* (2014) showed that *Ilyonectria* is represented by more than one genus, and the new genus *Dactylonectria* was described containing 10 new species (Lombard *et al*., 2014). Lombard *et al*. (2014) further showed that *Cylindrodendrum* is not a synonym of *Cylindrocarpon/Neonectria* as suggested by Chaverri *et al*. (2011). The previous *Cylindrocarpon/Neonectria* complex thus now consists of a total of seven genera that have Cylindrocarpon-like asexual morphs (although some species are sterile), i.e. *Neonectria*, *Thelonectria*, *Ilyonectria*, *Rugonectria*, *Campylocarpon*, *Cylindrodendrum* and *Dactylonectria*. Within the Nectriaceae family, these genera belong to phylogenectic clades IV and VI that contain several important plant pathogens (Lombard *et al*., 2015). Morphologically, it is almost impossible to distinguish between some of these genera and species, and therefore DNA sequence data are crucial for the identification of the genera (Lombard *et al*., 2014). Traditionally sequence data of the ß-tubulin region was used for identification of isolates with *Cylindrocarpon*-like anamorphs, however Lombard *et al*., (2015) suggested that the RNA polymerase II largest subunit (*rpb1*) and RNA polymerase II second largest subunit (*rpb2*) regions might be better. The suitability of these two regions as barcodes for species within these genera must, however, still be determined for each genus (Lombard *et al*., 2015).

In South Africa, ten apple orchards were investigated for the presence of *Cylindrocarpon*-like species and four species including '*Cylindrocarpon' liriodendri*, '*Cylindrocarpon' pauciseptatum*, '*Cylindrocarpon' destructans* and '*Cylindrocarpon' macrodidymum* were found to be associated with the roots of apple trees. '*Cylindrocarpon' macrodidymum* was isolated most frequently and also had the widest distribution. It was the first report of this species occurring on apple trees world-wide (Tewoldemedhin *et al*., 2011c). These species were subsequently placed in the genus *Dactylonectria* (Lombard *et al*., 2014) and further segregated into five species *(Dactylonectria torresensis, Dactylonectria macrodydima, Dactylonectria novozelandia, Dactylonectria alcacerensis* and *Dactylonectria estremocensis*) (Cabral *et al*., 2012; Lombard *et al*., 2014). Therefore, the species status of the initially identified '*C'. macrodidyma* isolates in South Africa is uncertain due to the new taxonomic system. The ten isolates of '*C'. macrodidymum* from apple trees in South Africa varied in virulence, since nine isolates had low virulence and one isolate was highly virulent (Tewoldemedhin *et al*., 2011c). The second most abundant *Cylindrocarpon*-like species

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identified on apple in South Africa, which also had a wide distribution, *was 'Cylindrocarpon' destructans.* '*Cylindrocarpon' destructans* has been re-classified as fitting into the *Ilyonectria radicola* complex, which consists of more than 12 species (Chaverri *et al*., 2011; Cabral *et al.*, 2012), but its taxonomy remains in flux. Pathogenicity assays with the South African '*C'. destructans* isolates showed that the isolates can cause a reduction in plant biomass and root rot. Two out of the ten evaluated isolates were highly virulent and the others had low virulence toward apple seedlings (Tewoldemedhin *et al*., 2011c).

Tewoldemedhin *et al*. (2011c) also reported '*C'. pauciseptatum,* currently known as *Dactylonectria pauciseptatum* (Lombard *et al*., 2014), for the first time from apple orchards world-wide. This was also the first report of this species on any crop in South Africa. Three isolates were tested in pathogenicity trials, with one isolate being non-pathogenic and two others exhibiting low virulence towards apple seedlings (Tewoldemedhin *et al*., 2011c). Isolation of '*C'. liriodendri,* now known as *Ilyonectria liriodendri* (Chaverri *et al*., 2011; Lombardi *et al*., 2014), was also a first report from apple orchards world-wide. Pathogenicity testing of five *I. liriodendri* isolates showed that one isolate was highly virulent towards apple seedlings, whereas the other four isolates had low virulence (Tewoldemedhin *et al*., 2011c).

The *Ilyonectria radicola* complex (syn. *Cylindrocarpon destructans*) is the predominant species group reported as being associated with apples world-wide, along with isolated reports of two other species (Jaffee *et al*., 1982b; Merwin and Stiles, 1989; Braun, 1991; 1995; Mazzola, 1998). Apart from apple, the *I. radicola* complex is also associated with a large host crop range including apricot, conifer, pear, peach and ginseng (Traquair and White, 1992; Hamelin *et al*., 1996; Seifert *et al*., 2003). Similar to Tewoldemedhin *et al*. (2011c) other studies have also found that the virulence range of this species complex varies towards apple (Dullahide *et al*., 1994; Mazzola, 1998; Manici *et al*., 2003; Tewoldemedhin *et al*., 2011c). Other species that have been associated with ARD and have been reported as pathogenic towards apple seedlings include '*Cylindrocarpon' heteronema* Berk. and BR and '*Cylindocarpon' lucidum*. Due to taxonomic changes these species have been transferred to *Neonectria ditissima* and *Thelonectria lucidum* respectively (Chaverri *et al*., 2011). *Thelonectria lucidum* was shown to cause stunting and black lesions on apple feeder roots (Jaffee *et al*., 1982b; Braun, 1991; 1995).

The presence of fungi with *Cylindrocarpon*-like anamorphs may have an important role in increasing the severity of ARD, since the root rot lesions caused by these pathogens could favour the entrance of other pathogens (Tewoldemedhin *et al*., 2011c). It was first shown by Braun (1991, 1995) that increased stunting of apple was observed when *Pythium* spp. was co-inoculated with *T. lucidum*. Subsequently, Tewoldemedhin *et al*. (2011) also showed that *P. irregulare* and *'C'. macrodidymum* acted synergistically, causing more severe growth reductions on apple seedlings than either species alone.

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Rhizoctonia

The fungal pathogenic genus *Rhizoctonia* has several host plants world-wide, and the damage caused by this group of pathogens is economically important (Sneh *et al*., 1966; Anderson, 1982; Adams, 1988). The genus consists of multinucleate, binucleate, and uninucleate groups, which are identified using fluorescence microscopy following the staining of nuclei with the fluorochrome, acridine orange. *Rhizoctonia* isolates are also further subdivided into anastomosis groups (Yamamoto and Uchida, 1982). In the genomic era, sequence data of the internal transcribed spacer (ITS) region is often used for determining the nuclear status and anastomose groups within this genus (Gonzalez *et al*., 2001).

The multinucleate species, *Rhizoctonia solani,* is an important species that has more than 250 host plant species including perennial and annual, forest and agricultural crops (Mazzola, 1997). *Rhizoctonia solani* is able to cause different symptoms such as damping-off of young seedlings and root- and crown rot of plants at various growth stages (Ogoshi, 1987; Sneh *et al*., 1991; Carling and Sumner, 1992). Among tree fruit crops, apple, avocado and peach have been reported as being susceptible to *R. solani* (Hine, 1961; Mircetich and Zentmyer, 1964; Burr *et al*., 1978). On avocado, *R. solani* has been reported to cause growth reductions, and brown lesions on young roots (Mircetich and Zentmyer, 1964). On peach, *R. solani* can cause reduction in root weight (Hine, 1961). On apple, *R. solani* was first recognized as an important pathogen in 1978 (Burr *et al*., 1978), which was later confirmed by a few other studies (Farr *et al*., 1989, Mazzola, 1997; Tewoldemedhin *et al*., 2011b). Burr *et al*. (1978) and Farr *et al*. (1989) both reported that *R. solani* was pathogenic towards apple, causing intense root rot with brown lesions.

Mazzola (1997) was the first to investigate the *Rhizoctonia* anastomosis groups affecting apple trees. *Rhizoctonia solani* AG-5 and AG-6 were identified as the main anastomosis groups from apple trees in Washington State, and caused severe stunting. The isolates were even highly virulent pathogens when inoculated onto apples trees that were 27 weeks old, causing severe root rot and finally death of trees. *Rhizoctonia solani* AG-6 was also obtained from apple trees in Italy, where isolates belonging to this AG differed in virulence from low to moderately virulent towards apple seedlings (Manici *et al*., 2003). In South Africa, *R. solani* AG-5 and AG-6 have not been found associated with ARD (Tewoldemedhin *et al*., 2011b). *Rhizoctonia* solani AG-6 has been reported as a pathogen that causes crater disease in South Africa (Meyer *et al*., 1998).

Several binucleate *Rhizoctonia* anastomosis groups have been associated with ARD. Mazzola (1997) recovered five binucleate *Rhizoctonia* groups including AG-G, AG-A, AG-I, AG-J and AG-Q from apple orchard soil and apple roots. Most of these isolates were non-pathogenic, and only some isolates within AG-A, AG-I and AG-Q were pathogenic (Mazzola,

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1997). Mazzola *et al*. (2015) subsequently isolated AG-G from apples in Washington State and pathogenicity studies showed that the isolate were highly virulent towards apple seedlings. In another study, Tewoldemedhin *et al*. (2011b) recovered seven binucleate *Rhizoctonia* groups including AG-A, AG-L, AG-I, AG-F, AG-K, AG-G and AG-R from apple orchards in South Africa. Of these, AG-A was isolated most frequently from orchards. Pathogenicity tests showed that most of the isolates were non-pathogenic, and that only one isolate of AG-I and one isolate of AG-F had low virulence. This was also the first report that AG-F, AG-L, AG-K and AG-R were associated with apple trees in the world (Tewoldemedhin *et al*., 2011b).

DETECTION AND QUANTIFICATION OF ARD PLANT PATHOGENS

Several approaches exist for the detection and quantification of the main groups of ARD pathogens, i.e. fungi, oomycetes and the nematode *Pratylenchus.* Oomycetes and certain fungi can be isolated from water, soil and plant material using baiting techniques that make use of pear fruit, leaf disk, seeds or seedlings baiting (Mark and Mitchell 1970; Eye *et al*., 1976), whereas almost all soilborne fungi and oomycetes can also be isolated using soil dilutions and root isolations (Mazzola *et al*., 2002a,b). For *Pratylenchus,* a few general techniques used for nematode extraction and quantification can be used, such as pie-pan modifications of the Baermann funnel technique (Jaffee *et al*., 1981; Mazzola, 1998; S Yao *et al*., 2006). The aforementioned techniques are all conventional approaches that have been used for decades, whereas molecular methods based on pathogen DNA detection and quantification have become increasingly important in the past two decades (Mazzola and Zhao, 2010; Tewoldemedhin et al., 2011a,b).

Baiting technique

Baiting techniques can be used for determining if inoculum is present, but it will not be quantitative. It can therefore be used for qualitative analyses or detection of water-borne pathogens and the presence of plant pathogens in roots and soil (Klotz *et al*., 1959; McIntosh, 1966; Gill, 1970; Thomson, 1972; Clausz, 1974; Thomson and Allen, 1974; Taylor, 1977; Middleton, 1985; Wilson *et al*., 1998; Oudemans, 1999). The technique is frequently used for oomycetes, but less often so for fungi. One example of an ARD fungal pathogen that can be isolated using baiting is *Rhizoctonia* (Mazzola, 1997).

Depending on the specific oomycete pathogen that requires isolation, different leaf disks like avocado, potato, lemon (Grimm and Alexander, 1973), pine needles (Dance *et al*., 1975), carnation petals (Ponchet *et al*., 1972) and soybean leaf disks (Schmitthenner and Canady, 1983) can be used. The disks are used for baiting by floating the disk on a soil slurry

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of sampled soil (Ostrofsky *et al*., 1977). In some instances, roots can also be mixed with water and baited with the leaf disks (Jung *et al*., 1999). Aside from leaf disk baiting, Gill (1970) used rooted cuttings of various plant species as bait flotations in an irrigation pond, which allowed the recovery of *P. myriotylum* and an unidentified *Pythium* isolate. Asparagus seedlings (Falloon, 1982), *Persea indica* seedlings (Zentmyer *et al*., 1960; Zentmyer, 1980), lupine seedlings (Chee and Newhook, 1965), alfalfa seedlings (Mark and Mitchell, 1970), soybean seedlings (Eye *et al*., 1976) and apple seedlings (McIntosh, 1966) are others examples of floating baiting methods.

Pear fruit baiting methods have been used for isolating *Phytophthora* species since these pathogens are very difficult to isolate (Mircetich and Matheron, 1976; Greenhalgh, 1978). The method was used to isolate *Phytophthora* species from irrigation canal water that serviced the apple production areas of Washington State (Yamak *et al*., 2002). For several years this was the only method of isolation. Other types of fruits have also been used for baiting including apple fruit (Tucker, 1931; Campbell, 1949; Luo *et al*., 1988), unripe cacao pods (Turner, 1965; Chee and foong, 1968), avocado fruit (Zentmyer *et al*., 1960; Zentmyer, 1980) and lemon fruit (Klotz and Fawcett, 1939; Klotz and DeWolfe 1958).

A few baiting techniques have been reported for Rhizoctonia. Mazzola (1997) in an ARD study used apple fruit for baiting *Rhizoctonia* from orchard soil. In another ARD study, white clover was used to bait *Rhizoctonia* (Ridgway *et al*., 2008). Paulitz and Schroeder (2005) developed a quantitative toothpick method for studying *Rhizoctonia* soil populations in wheat and barley fields. Shokes and McCarter (1979) used beet seeds to bait *Rhizoctonia* from ponds.

Root isolations

This method is very common for isolating a wide range of soil-borne pathogens. For studying ARD pathogens, it is important to use the fine root system for isolation of fungi and oomycetes, since the main effect of ARD pathogens are on the fibrous root system, which results in the destruction of the root hairs (Mazzola, 1998). Emmett *et al*., (2014) quantified the amount of ARD pathogens (*P. sylvaticum, Cylindrocarpon* spp. and *P. irregulare*) from different branching orders of apple seedling roots grown in ARD soil. They found that the pathogens primarily colonized the first and second order roots. They also investigated two rootstock genotypes (G.210 and M.26) and found that disease development in ARD is based mostly on fine-root tip attraction.

Since ARD pathogens affect the fine root system, the processing of roots prior to isolation is important. Unlike most pathogen isolations from plant tissue, surface sterilization of ARD roots should not be conducted. The roots are only washed under tap water and dried in a laminar flow prior to plating (Jaffee *et al*., 1982; Mazzola, 1998). However, several ARD

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studies have used surface sterilization of the roots, which might have affected the detection of the pathogens (Jaffee *et al*., 1982; Utkhede and Li, 1987). Utkhede and Li (1987) used surface sterilization of the fine roots by rinsing the fine roots with distilled water, followed by dipping in 10% NaOCl for 3 min and final wash step in distilled water for 4 min. Tewoldemedhin *et al*. (2011b) used a 5s dip in 99% ethanol in their ARD study.

Several synthetic media have been used in ARD studies for isolating fungi and oomycetes. These include water agar (Jaffee *et al*., 1982a; Mazzola, 1998; Mazzola and Gu, 2000; Tewoldemedhin *et al*., 2011b), corn meal agar (Jaffee *et al*., 1982a; Utkhede and Li, 1987) and potato dextrose agar (Utkhede and Li, 1987). *Pythium* semi-selective media (PSSM) has been used in several ARD studies (Mazzola *et al*., 2001; Mazzola *et al*., 2002a; Mazzola *et al*., 2009; Mazzola and Brown, 2010). PARP cornmeal medium is reported to be the most common semi-selective medium reported for the isolation of oomycete species from soil or plant tissue (Erwin and Ribeiro, 1996). Components of PARP include pimaricin, ampicillin (kills gram-positive bacteria), rifampicin (kills gram-negative bacteria), and pentachloronitrobenzene (Erwin and Ribeiro, 1996). PARP can also be amended with hymexazol at 50mg/l (PARPH), which assists in the suppression of fast growing Pythium and Mortierella spp. (Eriwn and Ribeir, 1996; Oudemand, 1999).

In addition to ARD studies, several semi-selective media have been reported for the isolation of oomycetes. Tambong *et al*. (2006) used benomyl (80 µg/ml) in a water agar based semi-selective medium along with the antibiotics vancomycin (200µg/ml) and pimaricin (10 µg/ml) for isolating *Pythium* using soil dilution plating and root baitings. Schmitthenner and Bhat (1994) also included benomyl (25 µg/ml) in their isolation media that they recommend for isolating *Phytophthora,* which they refer to as BARP (25 µg/ml benomyl, 100 µg/ml ampicillin, 30 µg/ml rifampicin and 100 ug/ml pentachloronitrobenzene) that is a 16% unclarified V8 agar based medium (Gevens *et al*., 2007).

A few semi-selective media have also been reported for the isolation of *Rhizocotonia.* Gutierrez *et al*., (1997) used a media containing water agar (Difco) 18 g/L, streptomycin sulfate (100 mg/L), penicillin-G sodium salt (100 mg/L) and sodium hydroxide (800 microliters/L). *Rhizoctonia* grows rapidly on the media and growth can be assessed 24 hours after plating. Water agar containing streptomycin sulfate (100 mg/L) has also been used in some ARD studies (Mazzola, 1997; Mazzola and Brown, 2010; Tewoldemedhin *et al*., 2011b).

Soil dilution

The soil dilution method was first developed for quantification and studying soil bacteria, but was later adapted for soil fungi (Waksman and Tenney, 1927). The advantage of the soil

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dilution method is that it is quantitative. This method can be used for isolation and quantification of fungi and oomycete in the soil.

Soil dilutions have been used in different ARD studies. Mazzola *et al*. (2002a, 2007) used it for isolating *Pythium* spp. and also quantifying soil populations. However, *Pythium* spp. in soil are composed of multi-species, which results in many slow growing *Pythium* spp. not being detected, since fast growing species will prevent their isolation due to overgrowth on the selective media. In another study by this author soil dilutions were used for isolation of fungi (Mazzola, 1998). In Italy, Manici *et al*. (2003) used this method for isolation of fungal and *Pythium* spp. from ARD soils.

Nematode extraction methods

Several methods are available for extracting nematodes from soil and roots (Seinhorst, 1956; Oostenbrink, 1960; Whitehead and Hemming, 1965; Barker *et al*., 1969a; Barker *et al*., 1969b; Viglierchio and Schmitt, 1983b; Robinson and Heald, 1989; Persmark, *et al*., 1992; Bell and Watson, 2001). The extraction efficiency of the method used can be influenced by soil type, nematode genus and the host plant involved (Chapman, 1957; McSorley *et al*., 1984; Prot, *et al*., 1993).

Although there are some inherent advantages and disadvantages to some methods, the procedure used by a laboratory can be influenced by the local supplies of equipment and operating conditions required for the different extraction methods. Equipment that are required for nematode extractions can include sieves, dishes, flasks, funnels, tubing, counting dishes and racks. Metallic ions, especially copper, cause toxic problems during nematode extractions when released into a small volume of static water. Therefore, plastic or stainless steel is better to use than bronze gauze, rings or pans (Pitcher and Flegg, 1968).

For nematode extraction of specific plant materials, nematode genera or species, several modifications have been made to the primary methods initially published (Young, 1954; Chapman, 1957; Sturrock, 1961; Webster, 1962; Tarjan, 1967; Bird, 1971; Gowen and Edmunds, 1973; Viglierchio and Schmitt, 1983a; McSorley *et al*., 1984; Russel, 1987; Robinson and Heald, 1989; Kaplan and Davis, 1990; Griesbach *et al*., 1999). Aerated incubation, modified Seinhorst mist chamber, modified Baermann funnel, and shake incubation are common extraction methods used for extracting endoparasitic nematodes from plant tissue (Lindsey and Cairns, 1971; Georgi *et al*., 1983; Norton and Edwards, 1988; MacGuidwin, 1989; Niblack, 1992; Todd and Oakley, 1996; Forge *et al*., 1998; Lamindia, 2002; Bélair *et al*., 2007). More recent nematode extraction methods such as the modified Baermann funnel technique, which has been reported as one of the most effective extraction methods, relies on the activity of nematodes (e.g. *Pratylenchus*). However, this technique cannot be used for the extraction of sedentary (e.g. *Globodera*, *Heterodera*, *Meloidegyne*, *Rotylenchulus*

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and *Tylenchulus*) and sluggish nematodes (e.g. *Cricnemoides*, *Hemicycliophora* and *Xiphinema*). Therefore, it's better to use maceration filtration or the mistifier technique for these nematodes (Prot *et al*., 1993). Other studies have reported that Seinhorst's mistifier method is more effective than the modified Baermann funnel for extracting *Pratylenchus* spp. (McSorley, et. al., 1984). Different nematode extraction methods have been used in ARD studies, such as pie-pan modification of the Baermann funnel technique (Jaffee *et al*., 1981; Mazzola, 1998; Leinfelder and Merwin, 2006; Yao *et al*., 2006; Laurent *et al*., 2008), Seinhorst's mist chamber (Dullahide *et al*., 1994; Mai and Abawi, 1978) and the centrifugal sugar flotation method (Tewoldemedhin *et al*., 2011a).

MOLECULAR IDENTIFICATION AND QUANTIFICATION OF ARD PATHOGENS

A large number of molecular techniques can be used for the identification, detection and quantification of plant pathogens. These methods are more sensitive, specific and accurate than conventional cultural isolation methods. The polymerase chain reaction (PCR) is the most commonly used method that can amplify DNA gene regions specific for different pathogens or regions common to most pathogens. The Internal transcribed spacer (ITS) region is the most commonly amplified DNA region used in the detection and identification of several plant pathogens. If the DNA region is used for detection of a pathogen within plant tissue or soil, primers must be developed that will only amplify the pathogen of interest (Lévesque and De Cock, 2004). Conventional PCR can only be used for determining the presence or absence of pathogens and cannot be used for quantification of pathogens. Alternatively, conventional PCR using pure culture DNA and general ITS primers can be used for identification of most, but not all plant pathogens. The amplified region can be sequenced to identify the pathogen, or polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) systems have been developed to identify species within genera for some pathogens. PCR-RFLP is based on the use of restriction digest enzymes that cuts the amplified PCR products in a nucleotide specific manner, so that the comparison of the resulting fragment patterns can be used to differentiate species (Mazzola *et al*., 2007; 2009). Quantitative real-time PCR (qPCR) and DNA macro-arrays are two other important molecular techniques used for the detection and quantification of plant pathogens (Tambong *et al*., 2006; Cullen *et al*., 2007; Mazzola and Zhao, 2010). qPCR is a very sensitive, powerful and accurate pathogen quantification method that is less prone to contamination than conventional PCR (Cullen *et al.,* 2007; Vincelli and Tisserat, 2008). DNA macro-arrays have the advantage in that they can be used for accurate and rapid detection of soil-borne pathogens, since a large number of species can be detected simultaneously (Tambong *et al*., 2006).

Several factors can influence the success of PCR based methods during pathogen

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quantification and detection. The use of clean template DNA is very important in qPCR because co-extracted substances in the samples can inhibit the amplification process. Humic acid, acidic polysaccharides, plant starch, fungal polyphosphates and phenolic compounds are examples of inhibitors that are co-extracted from soil and plant tissue and that can cause false negative results (Bickley and Hopkins, 1999). In some cases, the failure of thermal cyclers and reagents can also result in false negatives. The use of an internal control DNA fragment can eliminate these false negative test results (Paterson, 2006; Daniell *et al*., 2012). Another method that can be used to establish the presence of inhibitors is the amplification of serial dilutions of the template DNA. If inhibitors are absent in samples, a consistent linear decrease in C_q (cycle threshold) should be observed (Bustin, 2009). When using qPCR to quantify pathogens, it is important to determine the relationship between qPCR data and the potential of pathogen inoculum to cause yield losses in the host plant. If the relationship between DNA concentration and host damage is achieved for a particular system, qPCR data can be used for example to predict disease potential in soil samples prior to planting (Kernaghan *et al*., 2007).

A few studies have designed PCR primers that are specific for some of the known ARD pathogens. Some studies were on ARD, whereas others were on other crops such as wheat. qPCR assays based on SYBR Green were developed by Schroeder *et al*. (2006) for the identification and quantification of *Pythium* spp. in wheat, and included *P. attrantheridium, P. heterothallicum, P. irregulare, P. sylvaticum,* and *P. ultimum*. Schena *et al*. (2008) developed *Phytophthora* genus specific primers in their study that also developed primers for the detection and quantification of 15 *Phytophthora* species that are damaging to forests and trees. Spies *et al*. (2011) published primers for the quantification of *Pht. vexans* and *P. ultimum* using qPCR Taqman technology. Le Floch *et al.* (2007) published a qPCR assay for *P. dissotocum*, but the usefulness of this assay remains to be seen due to the unresolved taxonomic issues in *Pythium* species complex B2A to which *P. dissotocum* belongs (Robideau *et al*., 2011). *Rhizoctonia solani* AG-5 primers were developed by Mazzola and Zhao (2010) in their ARD study. A SYBR Green assay was developed for *Cylindrocarpon*-like spp. by Tewoldemedhin *et al*. (2011c) known to be associated with ARD in South Africa were identified and quantified using genus specific primers and a SYBR Green assay (Tewoldemedhin *et al*., 2011c).

Oligonucleotides specific for some ARD pathogens that can be used in DNA macro-arrays have also been developed. A macro-array that detected more than 100 *Pythium* species was published by Tambong *et al*. (2006). The array can detect most of the ARD *Pythium* spp. *P. aphanidermatum*, *P. irregulare* and *P. ultimum* were three oomycetes that Zhang *et al*. (2008) developed the DNA array for detection of them. Also for oomycete and large number of fungi this method was developed by Izzo and Mazzola (2009).

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Only a few studies have used molecular methods to detect and characterize in irrigation water oomycete genera associated with ARD, but none of these include oomycete species known to be associated with ARD. Kong *et al*. (2003) used *Ph. nicotianae* species-specific primers to detect the pathogen in naturally infested water samples from Virginia and South Carolina. The molecular method that used DNA extraction from filters or centrifuged water samples, was more sensitive than the conventional filtering and selective media method (Kong *et al*., 2003). Eyre and Garbelotto (2015) used isolation and PCR for detecting *Phytophthora ramorum* in symptomatic leaf baits from streams. Isolation studies only detected the pathogen in spring, whereas the PCR based method allowed detection almost throughout the year. PCR based detections were only made from symptomatic *Rhododendron* bait leaves and not any asymptomatic baits. Thus latent or low levels of infected leaves could have been missed. The failure of isolation success in summer was unclear, but it was noted that all isolations failed when air temperatures exceeded 18˚C (Eyre and Grabelotto, 2015).

Molecular detection of nematodes has only become a practice more recently. For *Pratylenchus* a few species specific primers have been published. Al-Banna *et al*. (2004) developed a PCR based assay for the identification and detection of six *Pratylenchus* species (*Pratylenchus brachyurus, Pratylenchus neglectus, Pr. scribneri, Pr. penetrans, Pr. thornei,* and *Pratylenchus vulnus*) that are common in California. In this assay, five forward species-specific primers were designed from the internal variable portion of the D3 expansion region of the 26S rDNA area, which were each combined with a single, common reverse primer (Al-Banna *et al*., 2004). The β-1,4-endoglucanase gene has also been used for the development of a TaqMan probe qPCR assay for quantification of *Pratylenchus penetrans* by Mokrini *et al.* (2013). A high correlation was reported for the number of *P. penetrans* detected using qPCR and real number using conventional methods (Mokrini *et al*., 2013). A few others studies have also developed qPCR assays for quantification of *P. penetrans* (Sato and Toyota, 2006; Min *et al*., 2012; Yan *et al*., 2013).

One aspect of molecular quantification of plant pathogens that have become more relevant and of interest, is how to express pathogen DNA content in plant tissue and soil. Most often, pathogen DNA in plant tissue is expressed on a biomass basis e.g. 100 fg/mg roots. Alternatively, relative quantification can be used by either using a host plant gene as reference or an internal foreign gene spiked into DNA or extraction buffers. The latter accounts for DNA loss during extraction (Tsai *et al*., 1996; Mumy and Findaly, 2004), as well as PCR machine failure that can cause false negative results (Paterson, 2006) and the presence of inhibitors in DNA extracts (Jackson *et al*., 1997; Okubara *et al*., 2005). Quantification of plant pathogen DNA in plant tissue using relative quantification of pathogen DNA biomass to a plant host gene (Livak and Schmittgen, 2001; Gao *et al*., 2004; van Gent-Pelzer *et al*., 2010; Catal *et al.,* 2013) may cause some problems such as the overestimation of pathogen DNA biomass due to DNA

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degradation which may occur especially during the late stages of infection by necrotrophic pathogenic species (Livak and schmittgen, 2001). To overcome these problems relative quantification using an internal foreign gene spiked into DNA or extraction buffers can be more effective (Eshraghi *et al.*, 2011). This approach has also been used for improving the quantification of microorganisms from soil where DNA extractions were spiked with a muted *E. coli* gene (Daniell *et al*., 2012).

OOMYCETES IN IRRIGATION AND SURFACE WATER

The first study to recognize a relationship between water contaminants and plant disease was done by Bewley and Buddin (1921). Subsequently, the spread of plant pathogens in irrigation water has become well-known, especially for oomycetes since most species produce sporangia in free water that release motile zoospores that disseminate through surface water (river systems, ponds, dams, creeks, lakes and ditches or canal ways) surviving hours to weeks in the water (Porter and Johnson, 2004), depending on the species involved (Erwin and Ribeiro, 1996). Although zoospores are thought to be the main propagule involved in dispersing oomycetes in surface water, oospores and mycelia may also be involved (Porter and Johnson, 2004). Surface water is not only used for irrigation, but also for mixing topical/pesticide sprays (Jones *et al*., 2014). Plant pathogens can enter water sources through various routes including infested soil, water and plant debris, cull piles and field drainage tiles (Hong and Moorman, 2005). In the following section, only information relevant to open irrigation sources will be discussed and not recirculating water used in nursery systems.

Several *Phytophthora* species have been recovered from irrigation water. A few examples of recovered species include *Ph. cambivora, Ph. cactorum* (McIntosh, 1966; Yamak *et al*., 2002), *Ph. citricola* (MacDonald *et al*., 1994; Wilson *et al*., 1998; Yamak *et al*., 2002; Gevens *et al*., 2007; Jones *et al*., 2014), *Ph. capsici* (Gevens *et al*., 2007; Jones *et al*., 2014), *Ph. cinnamomi* (Von Broembsen, 1984; Lauderdale and Jones, 1997; Wilson *et al*., 1998), *Ph. cryptogea* (Bewley and Buddin, 1921; MacDonald *et al*., 1994; Wilson *et al*., 1998), *Ph. nicotianae* (syn. *Ph. parasitica*) (Klotz *et al*., 1959; Lauderdale and Jones, 1997; Wilson *et al*., 1998; Jones *et al*., 2014), *Ph. citrophthora* (Wilson *et al*., 1998), *Phytophthora lacustris* (Jones *et al*., 2014), *Phytophthora hydropathica* (Jones *et al*., 2014), *Phytophthora irrigate* (Jones *et al*., 2014) and *Phytophthora gonapodyides* (Yamak *et al*., 2002; Jones *et al*., 2014). The only report specifically relating to South Africa, was that of von Broembsen (1984) who reported *P. cinnamomi* to frequently occur in rivers in the South Western Cape region.

Reports of specific *Pythium* spp. have been less frequent, but Zappia *et al*. (2014) listed 30 different *Pythium* species isolated from irrigation water and natural waterways. Gevens *et al*. (2007) frequently isolated *Pythium* spp. in surface irrigation sources in Michigan

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when cucumber and pear fruits were used as baits, but did not identify the isolates to species level. Jones *et al*. (2014) in New York State identified *Phytopythium helicoides*, *Pythium catenulatum*, *Pythium marisipium*, *P. myriotylum*, *P. irregulare*, *Pht. vexans*, *P. amasculinum, Pythium mercuriale, Pythium oedochilum* and *Pythium adhaerens.* Sanchez and Gallego (2001) reported the presence of *P. cantenulatum, Pythium diclinum* and *Pythium paroecandrum* in irrigation water in Spain. In Georgia, *P. aphanidermatum, P. irregulare, P. dissoticum, Pythium spinosum, Pht. vexans, Pht. litorale* and 19 other *Pythium* species from ponds in the region. *Pythium irregulare* was found to be the most prevalent among the *Pythium* species isolated (Shokes and McCarter, 1979; Parkunan and Ji, 2013).

In Washington State in the Wenatchee River Valley, *Ph. cactorum,* a highly virulent ARD pathogen has been reported to frequently occur in irrigation water during the summer fruit development period. In this region, the pathogen in irrigation water is problematic since it causes sprinkler rot of immature pear and apple fruit (Grove and Boal, 1991; Yamak *et al*., 2002). Grove and Boal (1991) detected the pathogen in an irrigation canal each week from late June to August 1989 and in 1990 from early June to mid-September and identified it as the major *Phytophthora* species. However, Yamak *et al*. (2002) found in their survey of a total of 19 irrigation canals during 1992 to 1994, 1995 and in 1999, that *Ph. cactorum* occurred infrequently. The proportion of *Ph. cactorum* infected canals during the 7 years varied from 0.1 to 0.7. Several other *Phytophthora* spp. were also isolated during this period, with *Ph. cactorum* comprising only 10% of the isolates and *Phytophthora gonapodyides* being the predominant species. Several *Phytophthora* isolates were also identified that were not closely related to any known *Phytophthora* spp. (Yamak *et al*., 2002).

The oomycete populations in specific surface water sources can vary or stay comparable between sampling periods. Shokes and McCarter (1979) found that the composition of the microflora in the two ponds that they sampled stayed more or less the same during monthly samplings, but that the two ponds differed in their microbial composition. The one pond was dominated by *P. irregulare* and the other by *P. aphanidermatum*.

Water temperature can play an important role in the dissemination of oomycetes in surface water. In Michigan State it was found that the detection of *Ph. capsici* in surface water was positively correlated with temperature, when water was monitored on a weekly basis from May to October over a 4 year period. *Phytophthora capsici* was not detected when temperatures fell below 14˚C or rose above 25˚C (Gevens *et al*., 2007). Oudemans (1999) also found a positive correlation between isolation of *Ph. cinnamomi* and water temperature in New Jersey. However, a negative relationship was found between water temperature and isolation of *Ph. megasperma* (Oudemans, 1999). The impact of water temperature on recovery of *Phytophthora* was hypothesized to be related to the influences of water temperature on the period that zoospores can continue to swim and remain viable (Gevens *et al*., 2007).

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The association of precipitation, turbidity and sunlight with the occurrence or survival of oomycetes in surface irrigation water has been investigated. Gevens *et al*. (2007) did not find a clear indication that precipitation events influenced the recovery of *Ph. capsici* from surface water. Jones *et al*. (2014) also did not find a correlation between precipitation prior to sampling and the presence of hymexazol-insensitive oomycetes. A correlation was found between higher turbidity of the water source and presence of these microbes, which could be due to the turbidity being caused by the presence of larger amounts of debris and components such as clay, silt, organic matter that perhaps play a role in the survival of oomycetes (Jones *et al*., 2014). Porter and Johnson (2004) investigated the effect of solar irradiation on the survival of *Ph. infestans* sporangia, by placing sporangia in water that were then shaded and non-shaded. Spores in water placed in the shade survived on average 4 days longer than those exposed to solar radiation. They also noted that spore survival differed during the year, with lower spore survival during periods of the year when solar intensity was higher and longer, resulting in increased ultra violet radiation of the spores (Porter and Johnson, 2004).

A few studies have investigated and hypothesized on the survival of oomycetes in irrigation water. Whiteside and Oswalt (1973) conducted an interesting study on irrigation water and the presence of *Phytophthora* species pathogenic to citrus in soil banks versus free water. Baits of grapefruit, lemon and oranges were placed in different locations along the river to determine if the pathogen was present in the water and soil sampled from the water's banks. *Phytophthora citrophthora* was isolated from the soil samples and the baits in the river. This was the first evidence of a brown rot epidemic potentially initiated by infested irrigation water (Whiteside and Oswalt, 1973). The data also suggested that the pathogen can survive in the soil at the banks of the river. However, Gevens *et al*. (2007) working with *Ph. capsici* found that based in DNA fingerprinting data of isolates collected over four growing seasons, that the pathogen did not seem to survive in specific surface water locations. Porter and Johnson (2004) found that sporangia of *Ph. infestans* survived for about 4 days longer when sporangia were mixed with soil and water, as opposed to when sporangia were only in water. Shokes and McCarter (1979) found that *P. aphanidermatum* oospores survived for 6 months in a pond, whereas its zoospores did not survive for more than 12 days.

The depth at which water samples are taken in surface water irrigation systems can also influence the pathogens detected and their frequency. Shokes and McCarter (1979) found that *Pythium* spp. propagules ranged from 0 to 0.7 CFU/ml in filtered and unfiltered water, but were much higher in bottom sediment samples (0 to 78 CFU/ml). Ghimire *et al*. (2009) found variable results for the baiting of *Phytophthora* at the surface or at a 0.5m depth. Over their four month sampling, the percentage baits were the same at these depths for two months and for the other months it was significantly higher or lower at the surface versus a 0.5m depth.

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Although oomycete pathogens are frequently isolated from irrigation water using baiting techniques, the ability of the irrigation water to incite actual disease has been less studied. Loyd *et al*. (2014) found that although *Phytophthora* spp. pathogenic to *Pieris* and *Rhododendron* were frequently isolated from irrigation water, these hosts were rarely (~0.5%) infected with *Phytophthora* when irrigated with the contaminated water. They hypothesized that this was due to the fact that the zoospore concentration was below the threshold required for infection of the host, since other studies have shown that for example for *Ph. cryptogea* and *Phytophthora heveae* only between 0 to 20% of their respective host plants became infected at zoospore concentrations of less than 50 zoospores/ml (Benson and Jones, 1980). Kong *et al*. (2010) showed that the disease incidence of *Ph. capsici* on *Capsicum annuum* were less than 5%, approximately 20% and 40% when plants were inoculated with 1, 10 and 100 zoospores respectively. Loyd *et al*. (2014) calculated that they in general only detected two zoospores per plant container and at most 18 zoospores per plant container contamination daily, which is likely too few zoospores required for infection even under favourable environmental conditions. Jones *et al*. (2014) in their survey of New York surface water also encountered low levels of oomycetes that on average ranged between 19 CFU/L (creek samples) to 12 CFU/L (pond samples), although some samples had as high as 200 CFU/L hymexazol-insensitive oomycetes. Hong and Epelman (2001) found that a zoospore concentration of $>10^2$ spores/L of *Ph. nicotianae* were required in irrigation water for inciting disease on *Catharanthus roseus.* Furthermore, disease development was delayed and progresed slowed on plants irrigated every 3 day versus those irrigated daily (Hong and Epelman, 2001). Although Granke and Hausbeck (2010) did not physically irrigate plants with contaminated water, they did investigate the quantity of *Ph. capsici* zoospores required to infect pickling cucumbers. Fruit infection was reduced to <40% at 19˚C and no infection occurred at 12˚C at a zoospore concentration of 1×10^2 zoospores/ml, whereas 100% fruit infection occurred at these temperatures at zoospores concentrations of $>5 \times 10^3$ zoospores/ml. The length of time that zoospores have to swim before finding a host will also influence their infectivity, since less fruit infection occurred with 10 day versus 50 day old zoospore suspensions (Granke and Hausbeck, 2010). The theories regarding density-dependent zoospore plant infection however differ. It has been shown that infection can be regulated by zoosporic extracellular products in zoospore-free fluid, and that this zoospore-free fluid acts interspecifically, i.e. across different species. This could benefit oomycetes, since several different species often infect the same host plant (Kong *et al*., 2010).

From the above it is clear that there are various knowledge gaps on oomycete pathogens in irrigation water that prevents the formulation of risk indicators for specific water sources based on the pathogens detected. Important knowledge gaps identified by Zappia *et al*. (2014) include our lack of understanding the epidemiology of waterborne pathogens, i.e.

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how and what influences pathogen survival, reproduction and spread in irrigation water and what the inoculum thresholds are for infecting host plants when using contaminated irrigation water. Other factors identified were that the pathogenicity of isolates are often unknown and that the identity of species reported prior to the molecular age might be in doubt (Zappia *et al*., 2014). Hong and Moorman (2005) also emphasized the need for determining economic thresholds for pathogens detected in irrigation water, i.e. the quantity and frequency of the pathogens required to pose a real threat to the crop (Hong and Moorman, 2005).

RHIZOCTONIA AND *PRATYLENCHUS* IN IRRIGATION AND SURFACE WATER

Rhizoctonia has rarely been detected in irrigation water. *Rhizoctonia solani* was detected infrequently in irrigation ponds in Georgia, and mostly in the bottom sediment samples (Shokes and McCarter, 1979). Bewley and Buddin (1921) investigated greenhouse water supplies, and detected *R. solani* in 11 of the 41 investigated samples. Another study found *Rhizoctonia* in effluent and stream water (Cooke, 1956).

The first report that showed that plant parasitic nematodes can be dispersed by irrigation water was conducted by Godfrey (1923). A study in Washington State reported that 10-20 % of the total nematode population in irrigation water were comprised of plant pathogenic nematodes. In the Western Cape region in South Africa, irrigation water and rivers have been reported as important sources of nematode distribution (Barbercheck *et al.,* 1985; Van Reenen and Heyns, 1986). *Praylenchus* spp. have specifically been reported in the Western Cape and Mpumalanga from dams and rivers (Smith and Van Mieghem, 1983; Grech *et al*., 1989). *Pratylenchus* spp. have also been reported from canals in Victoria in Australia (Meagher, 1967) and Kashmir Valley in India (Waliullah, 1984, 1989). This species was also found from runoff water in Nebraska (Heald and Johnson, 1969) and Washington (Heald and Johnson, 1969) in the USA.

DISCUSSION

Several studies have used pasteurised soils or fumigated soils to show that ARD is caused by biological agents (Hoestra, 1968; Covey *et al*., 1979; Abawi, 1981; Jaffee *et al*., 1982a; Gur *et al*., 1991). The predominant biological agents involved in ARD that were revealed through pathogenicity studies include selected species of *Pythium*, *Phytophthora*, *Phytopythium*, *Rhizoctonia*, *Cylindrocarpon*-like isolates and *Pratylenchus* (Jaffee *et al*., 1982b; Merwin and Stiles, 1989; Braun, 1991; Mazzola, 1997; 1998; Tewoldemedhin *et al*., 2011b; Mazzola and Manici, 2012). Although the ARD pathogen complex differs at the species level, for the most part, studies have demonstrated that the same genera are implicated in the disease on a world wide basis. The virulence of genera and species may also differ, sometimes even isolates

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within the same species. In South Africa, oomycete species are among the most virulent species since the highly virulent *R. solani* AG-5 and AG-6 have not been identified in South Africa (Tewoldemedhin *et al*., 2011b). The most widespread and virulent oomycete pathogens that have been identified in South Africa include *P. irregulare, Pht. vexans, P. sylvaticum, P. ultimum* and *Ph. cactorum* (Tewoldemedhin *et al*., 2011b).

Apple orchard soils previously planted with apple or closely related species is considered as the main inoculum source of apple replant pathogens (Savory, 1966; Hoestra, 1968; Traquiar, 1984; Mazzola, 1998). Alternative inoculum sources can be planting material and irrigation water. In some orchards, fumigation has been observed to fail in controlling ARD. This can be due to incorrect fumigation practices, or it can indicate that some of the aforementioned external inoculum sources are introduced into orchard soil after fumigation. Furthermore, it is important to determine whether these external inoculum sources could also contribute to the establishment of ARD orchards in new apple orchards that were established on virgin soil, but ultimately end up as ARD soils after several years of tree growth. Irrigation water plays an important role in distribution of oomycete species, and they are known to occur in irrigation water (Whiteside and Oswalt, 1973; Porter and Johnson, 2004; Hong and Moorman, 2005; Gevens *et al*., 2007; Jones *et al*., 2014), and can thus serve as an inoculum source. As for most plant pathogens, planting materials can also be contaminated with plant pathogens and serve as a source of inoculum.

The fields of plant pathogen detection and quantification has evolved rapidly in the last few decades and are continuing to do so (Leivens *et al*., 2006; Schroeder *et al*., 2006; Kernaghan *et al*., 2007; Spies *et al*., 2011). qPCR is widely used to investigate the population dynamics of plant pathogens in plants and soil. Several of the important ARD plant pathogens can be investigated with qPCR since species or genus specific assays have been published (Schroeder *et al*., 2006; Schena *et al*., 2008; Spies *et al*., 2011; Tewoldemedhin *et al*., 2011c. However, little information is available on how qPCR data relate to ARD pathogen isolation data and if a correlation exists between qPCR data and plant growth inhibition. Tewoldemedhin *et al*. (2011a) could only find a significant negative correlation between *P. sylvaticum* DNA concentrations in the roots of apple seedlings grown in ARD soils and reductions in seedling growth, but not for *Ph. cactorum, P. irregulare, P. ultimum,* and the genus *Cylindrocarpon.* This may be due to the fact that in natural soils synergistic interactions between the pathogens are more important in determining the extent of disease, and that quantities are not that important. Alternatively it could be that in this study the whole root system was used for DNA quantification, and not only the first- or second order roots that are the target of ARD pathogens (Emmett *et al.,* 2014). Furthermore, for *Pht. vexans* the qPCR assay that was used differentially quantified different isolates of *Pht. vexans,* most likely since the isolates differed in the copy number of the targeted qPCR gene (Spies *et al*., 2011). Lastly,

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a standard amount of root DNA was used in the qPCR reactions for expressing pathogen quantities (Tewoldemedhin *et al*., 2011a, b), which could have resulted in the incorrect expression of pathogen DNA quantities. Alternatives for expressing pathogen DNA quantities are expression based on root biomass or relative expression using an internal reference gene (Eshraghi *et al.*, 2011; Daniell *et al*., 2012).

One of the aims of the current study was to develop a new *Pht. vexans* qPCR method that quantified different isolates similarly. This assay along with the qPCR assays developed by Tewoldemedhin *et al*. (2011a) were used to investigate the relationship between apple seedling growth reductions caused by the main oomycete apple replant pathogens, their isolation percentages from roots in glasshouse trials and DNA biomass. Data from natural *P. irregulare* infections in nurseries were also used to investigate the significance of DNA quantification. In all of the qPCR studies, the expression of pathogen DNA was conducted on a root biomass basis (absolute quantification), and relative to a foreign internal gene that corrected for DNA inhibition and loss (Daniell *et al*., 2012).

Important possible external inoculum sources of ARD pathogens can be irrigation water and contaminated nursery materials. For these reasons, this thesis was aimed at investigating whether irrigation water is a source of oomycete ARD pathogens, and if nursery tree rootstocks can be an inoculum source of *Pratylenchus* spp., *Cylindrocarpon*-like spp. and oomycete spp. The oomycete inoculum sources were first investigated using traditional culturing techniques and isolation and baiting methods. Additionally, the most important oomycete ARD pathogens in South Africa were also quantified using qPCR. The presence of *Pratylenchus* was investigated using traditional methods, whereas the quantities of the genus *Cylindrocarpon* in nursery material were investigated using qPCR.

The result of this study will be very useful to understand the potential of relative and absolute pathogen DNA biomass quantifications and also percentage root infections for estimating apple seedling growth reductions caused by the main oomycete ARD pathogens in glasshouse trials. This data may also be useful for interpreting the amount of pathogen DNA biomass quantified in ARD orchard management trials. The result will also be helpful to understand the inoculum sources of replant pathogens from apple nursery and orchards irrigation water and determine if any management strategies are required, which can improve the efficacy of currently used orchard fumigations used to manage ARD in replanted orchards.

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CHAPTER 2

Apple seedling growth reduction caused by oomycetes is correlated with feeder root DNA biomass and percentage roots infected only for selected species

ABSTRACT

Investigations into inoculum sources and effective disease management strategies requires knowledge on relationships between pathogen quantification techniques and the extent of plant damage. This was investigated for apple seedling growth reductions caused by five oomycete pathogens (*Pythium sylvaticum*, *Pythium irregulare*, *Pythium ultimum*, *Phytopythium vexans* and *Phytophthora cactorum*), which are major contributors to apple replant diseases (ARD), especially in South Africa. The relationship between seedling growth reduction, percentage seedling root infection and pathogen DNA biomass (absolute and relative) were investigated in glasshouse trials. Pathogen DNA biomass was quantified using quantitative real-time PCR (qPCR) and published assays, except for *Pht. vexans* for which a new qPCR assay was developed. The assay was shown to be specific with a quantification limit of 1000 fg/µL. Data from natural *P. irregulare* infections in nurseries were also used to investigate the correlation of pathogen DNA biomass (absolute and relative) with percentage root infection. Absolute pathogen DNA biomass was calculated using a pure culture pathogen DNA standard curve, whereas relative pathogen DNA biomass was calculated relative to a spiked, foreign internal gene that corrected for DNA inhibition and loss. Percentage root infection and pathogen DNA biomass quantities (absolute and relative) were good predictors (r = -0.56 to 0.77; $P < 0.001$) of apple seedling growth reductions for *P. sylvaticum, Pht. vexans* and *P. ultimum,* when the fine feeder root system of seedlings was targeted. This, however, was not true for *Ph. cactorum* and *P. irregulare* (r = -0.19 to 0.32; $P > 0.19$), except for *P. irregulare* root infections that were indicative of seedling growth reductions (r = 0.50; P = 0.02). Relative pathogen DNA biomass quantifications did not improve absolute DNA biomass quantifications from glasshouse trial roots, since these quantities were highly correlated ($r >$ 0.89; P = 0.001). This was also supported by the quantification of *P. irregulare* from nursery apple roots (r = 92; P = 0.001). The *P. irregulare* DNA biomass (relative and absolute) also correlated with percentage root infections (r = 0.63 to 0.72; $P < 0.001$) for nursery roots. The *P. irregulare* DNA biomass (relative and absolute) also correlated with percentage root infections (r = 0.63 to 0.63; $P < 0.001$) for nursery roots.

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INTRODUCTION

Poor growth of young apple trees that are planted on sites that were previously cultivated with apple trees or closely related species is known as apple replant disease (ARD). ARD has a large economic impact on apple production world-wide due to reductions in fruit yield and quality and for the life-span of the orchard (Mazzola, 1998; Mazzola and Manici, 2012). There is compelling evidence that ARD is caused by biological soilborne agents (Hoestra, 1968; Covey *et al*., 1979; Abawi, 1981; Jaffee *et al*., 1982a; Slykhuis and Li, 1985). The below ground symptoms of ARD, incited by the soilborne agents, is visible as a general reduction in root biomass, with the roots being discoloured and exhibiting necrosis at the root tips (Mazzola and Manici, 2012). Several of the major causative agents of ARD will predominantly colonize the fine feeder roots (first and second order) that ultimately result in the loss of second and third order roots (Emmett *et al.*, 2014). A model, where replant pathogen colonization is concentrated on distal first and second order roots tips, and where the pathogens will not colonize the entire root system, has been proposed by Emmett *et al.* (2014).

Several biological agents have been associated with ARD world-wide, but only a few groups are known to be pathogenic and cause ARD including plant parasitic nematodes, fungi and oomycetes (Mazzola and Manici, 2012). The fungal genera include *Rhizoctonia,* with the most important being *Rhizoctonia solani* AG-5 and AG-6 due to their high virulence, and a few binucleate *Rhizoctonia* groups (Jaffee *et al*., 1982b; Merwin and Stiles, 1989; Braun, 1991; Mazzola, 1997; 1998). Interestingly, in South Africa, *R. solani* AG-5 and AG-6 are not associated with ARD, only binucleate *Rhizoctonia* species have been identified of which most are not pathogenic or only have low virulence (Tewoldemedhin *et al*, 2011b). Another fungal genus involved in ARD is *Cylindrocarpon*-like spp., of which the significance of specific isolates is difficult to determine due to the high variability in pathogenicity and virulence of different isolates (Dullahide *et al*., 1994; Mazzola, 1998; Manici *et al*., 2003; Tewoldemedhin *et al*, 2011c), and large taxonomic changes occurring in the last few years within this fungal group (Chaverri *et al*., 2011; Cabral *et al*., 2012; Lombard *et al*., 2014). Parasitic nematodes involved mainly include *Pratylenchus* spp. in most countries (Mai *et al*., 1957; Jaffee *et al.*, 1982b; Merwin and Stiles, 1989; Utkhede *et al*., 1992; Dullahide *et al*., 1994). Several oomycete species are known to be pathogenic and involved in ARD world-wide, with the most commonly reported species being *Pythium intermedium*, *Pythium irregulare, Pythium sylvaticum, Pythium ultimum, Pythium heterothallicum,* and *Phytopythium vexans*, *Phytophtora drechsleri, Phytophtora cinnamomi, Phytophthora parasitica, Phytophthora cambivora* and *Phytophtora cactorum* (Julis *et al*., 1978; 1979; Sewell, 1981; Jaffee *et al.,* 1982a; Matheron *et al*., 1988; Dullahide *et al*., 1994; Mazzola, 1998; Mazzola *et al.*, 2002; Tewoldemedhin *et al*., 2011b). In South Africa, due to the absence of *R. solani* AG-5 and AG-6, oomycetes form an important component of the ARD disease complex and have been identified in all investigated orchards.

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Pythium irregulare, *Pht. vexans, P. sylvaticum, P. ultimum* and *Ph. cactorum* are known to be widely distributed (Tewoldemedhin *et al*., 2011a, b), and also have high virulence (Mazzola *et al*., 2002a; Tewoldemedhin *et al*., 2011a,b).

The detection and quantification of oomycete ARD pathogens is very important in South Africa, when studying inoculum sources such as irrigation water and nursery trees (Chapter 3), and for evaluating ARD management options in orchard and glasshouse trials. ARD oomycete characterization studies, and studies on the management of these pathogens have used conventional isolation studies or quantitative real-time PCR (qPCR) of pathogen DNA biomass for determining the presence and severity of apple tree or seedling infections. For conventional isolation studies, various semi-selective media such as a *Pythium* semi-selective medium (PSSM) (Mazzola and Brown. 2010), PARP cornmeal medium (Tewoldemedhin *et al*, 2011a) and PARPH (Tewoldemedhin *et al*, 2011a) have been used. PARP and PARPH are well known and widely used media for isolating oomycetes (Erwin and Ribeiro, 1996). Isolation data can be used to express the severity of infection by calculating the percentage infected roots, whereas the quantification of colony forming units per gram of soil through soil dilutions can also indicate pathogen prevalence (Mazzola *et al*., 2002b; 2007). PCR and qPCR based assays have been developed for several of the most important oomycete ARD species, although not always initially on apple, but on other hosts. Assays that have been published include those for *Pht. vexans* (Spies *et al*., 2011), *P. irregulare* (Schroeder *et al*., 2006; Kernaghan *et al*., 2008; Spies *et al*., 2011), *P. ultimum* (Kernaghan *et al*., 2008; Schroeder *et al*., 2006; Spies *et al*., 2011), *Pythium attrantheridium, P. heterothallicum, P. sylvaticum,* (Schroeder *et al*., 2006), *Ph. cactorum* (Schena *et al*., 2008) and the genus *Phytophthora* (Leivens *et al*., 2006; Schena *et al.*, 2008). A genus specific assay is suitable for *Phytophthora* since all species associated with ARD have thus far been identified as pathogens (Matheron *et al*., 1988 Utkhede *et al*., 1992; Mazzola, 1998; Tewoldemedhin *et al*., 2011a, b). For the *Pht. vexans* qPCR assay it was reported that the assay primers, based on the internal transcribed spacer (ITS) region, were not suitable for quantification since different isolates quantified differentially, most likely indicating differences in ITS copy number between isolates of this species complex that consists of three phylogenetic sub-clades (Spies *et al.*, 2011).

qPCR is a more suitable method for investigating inoculum sources and the efficacy of control measures than isolation studies, since it is less labour intensive and more sensitive than conventional isolation studies. However, the technique does have some limitations. These include inaccurate pathogen quantification due to the presence of PCR inhibitors such as humic acids in extracted DNA (Jackson *et al*., 1997; Okubara *et al*., 2005), loss of DNA during the DNA extraction process (Tsai *et al*., 1996; Mumy and Findaly, 2004) and also sometimes PCR machine failure that can cause false negative results (Paterson, 2006). A

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solution to this problem is the use of relative DNA quantification, instead of absolute DNA quantification. In absolute quantification, DNA quantity is determined by relating the PCR signal to standard curves of known DNA concentrations of the target pathogen (Livak and Schmittgen, 2001). Relative quantification is based on the change in the target gene relative to a different reference gene, such as a foreign internal control spiked gene or a plant host gene (Livak and Schmittgen, 2001). Several studies that have quantified plant pathogen DNA in plant tissue, have used relative quantification of pathogen DNA biomass to a plant host gene (Livak and Schmittgen, 2001; Gao *et al*., 2004; Eshraghi *et al*., 2011; van Gent-Pelzer *et al*., 2010; Catal *et al.,* 2013). This approach is not always effective, since when plant cells collapse, DNA degradation often occur especially during the late stages of infection by necrotrophic pathogenic species, which may cause overestimation of pathogen DNA biomass (Livak and Schmittgen, 2001). Most oomycete ARD pathogens including all *Pythium* species are necrotrophic pathogens (Geraats *et al*., 2002), whereas *Phytophthora* depending on the species can be biotrophic or hemi-biotrophic (Haines *et al*., 2003). Not many plant pathogen studies have used pathogen DNA quantification in plant tissue relative to a foreign internal spiked gene, usually located on a plasmid added to the DNA extraction buffer (Livak and Schmittgen, 2001; Eshraghi *et al.*, 2011). This approach, however, have been used by several studies for studying pathogens in animal tissues, and human pathogens in food products (Klerks *et al*., 2006; Damen *et al*., 2008; Halliday *et al*., 2010).

When studying plant disease management, it is important to obtain information on the relationship between qPCR pathogen DNA biomass and growth or yield reductions in the host plant. In South Africa, Tewoldemedhin *et al*. (2011a, b, c) conducted correlation analyses between pathogen DNA biomass concentrations in roots, estimated through absolute DNA quantification, and the increase in height and weight of apple seedlings. Significant negative correlations were only found between DNA concentration and seedling weight for *P. irregulare,* but not for *P. sylvaticum*, *Pht. vexans*, *Ph. cactorum* and *Cylindrocarpon*-like spp. (Tewoldemedhin *et al*., 2011a,b,c). Catal *et al.* (2013) found a significant negative correlation between *Phytophthora* pathogen DNA biomass extracted from infected soybean roots and disease severity index ratings. Similar findings were made by Fraaije *et al*. (2001) for *Stagonospora nodorum* and *Puccinia striiformis* on winter wheat, and by Vandemark and Grunwald (2005) for *Aphanomyces euteiches* on peas.

The aim of this study was to investigate the potential of relative and absolute pathogen DNA biomass quantifications for predicting apple seedling growth reductions caused by the main oomycete ARD pathogens in glasshouse trials. Conventional root isolation studies were also conducted and investigated for their potential to predict seedling growth reductions. The oomycete pathogens that were investigated included *Pht. vexans, Ph. cactorum, P. irregulare, P. sylvaticum* and *P. ultimum.* The qPCR assays used for quantification of pathogen DNA

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biomass were from published studies, except for the *Pht. vexans* assay, for which a new qPCR assay was developed to improve on the quantitative nature of the only previously published *Pht. vexans* qPCR assay by Spies *et al.* (2011). Relative qPCR quantification was conducted by spiking DNA extraction buffer with a foreign gene, specifically a mutated *E. coli* gene from the study of Daniell *et al*. (2012). To further compare pathogen DNA biomass obtained through relative and absolute pathogen DNA quantification under natural field conditions, root samples from nursery rootstocks known to be infected mainly by *P. irregulare,* were also analysed through isolation studies to determine which DNA biomass quantification approach correlated the best with this parameter.

MATERIALS AND METHODS

Phytopythium vexans qPCR assay development

A new Taqman-based qPCR assay was designed for *Pht. vexans* that targets a putative inositol polyphosphate 5-phosphatase region. Primers and probes for amplification of the gene region were identified in sequences of eight *Pht. vexans* complex isolates using Primer3 (Untergrasser *et al*., 2012) in Geneious version 6 (http://www.geneious.com, Kearse *et al*., 2012*). Phytophthora vexans*-specific oligos were selected based on in *silica* specificity testing on an alignment of 182 oomycetes consisting mostly of species of *Pythium*, *Phytopythium* and *Phytophthora* (kindly provided by A. Lévesque, Agriculture and Agri-Food Canada, Ontario, Canada). Regions homologues, but specific to *Pht. vexans* were identified in the alignment. Five primers and one probe were designed to specifically amplify *Pht. vexans*: primers PV390F (CGC CGA CTA CCA GAA CAT CC), PV455R (CTC GGA CAG TCG TCT TCA CC), PV389F (ACG CCG ACT ACC AGA ACA TC), PV456R (GCT CGG ACA GTC GTC TTC AC) and PV387F (GAA CGC CGA CTA CCA GAA CA) and the probe PV412P (FAM-AGC AAG GTC CAG TTC CTC GGC GA). The specificity of the assay was assessed using DNA extracted from seven oomycete species including 12 *Pht. vexans* isolates (STE-U6720, STE-U6745, STE-U6730, STE-U6728, STE-U673, STE-U6703, STE-U6741, STE-U6736, STE-U6718, STE-U6740, STE-U6739, CBS119.80) representing all three phylogenetic *Pht. vexans* groups of this species complex (A, B and C) identified by Spies *et al.* (2011), *Phytopythium oedochilum* (STE-U6748), *Phytopythium mercurial* (STE-U6127), *Phytopythium helicoides* (Chapter 3), *Phytopythium litorale* (Chapter 3), *Ph. cactorum* (STE-U7204) and two isolates that represent a putative new *Phytopythium* species, closely related to *Pht. vexans*. The latter isolates were obtained from a study on novel *Phytopythium* species by S. Langenhoven (Stellenbosch University, Department of Plant Pathology).

Genomic DNA was extracted from all oomycete isolates using the Wizard SV genomic DNA purification system (Promega, MA, USA) according to manufacturer's instructions.

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Conventional PCR was used to screen all six primers in all possible combinations in amplifications that utilized DNA from all oomycete isolates noted above. The final optimized qPCR reaction consisted of 1× PCR buffer (Bioline, London, United Kingdom), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.65 U BIOTAQ (Bioline), 0.05 mg/ml bovine serum albumin (BSA) Fraction V (Roche Diagnostics South Africa, Randburg, South Africa) and 0.2 mM of each primer in a total reaction volume of 40 µl. Amplification was performed in a Biorad PCR machine. PCR amplification conditions consisted of an initial denaturation of 5 min at 94 °C, followed by 32 cycles of 30 s at 94 °C, 30 s at various temperatures, 90s at 72 °C and a final extension of 7 min at 72 °C. The optimal annealing temperature for each primer combination was determined by running a temperature gradient assay from 55-65 °C. The amplified products were run on a 2% agarose gel containing ethidium bromide and were visualized under UV illumination. Since primers PV390F and PV455R were shown to be specific in the conventional PCRs, this primer pair and probe PV412P was used in subsequent qPCR analyses. After evaluation of several primer and probe concentrations, the final optimized qPCR reaction consisted of 1x KAPPA Probe Fast Mastermix universal (Lasec SA, Cape Town, South Africa), 300 nM of each primer and 200 nM of probe (PV412P) in 20µl reaction volumes. The optimized amplification conditions consisted of 3 min. at 95˚C for one cycle and 40 cycles of 95˚C for 10 seconds followed by annealing for 30s at 60˚C. The standard curve consisted of tenfold serial dilutions from 10 ng to 10 fg. The specificity of the assay was evaluated with the optimized assay using isolates of all the previously specified oomycete species. Amplifications were conducted in a RotorGene 6000 real-time rotary analyser (Qiagen Inc., Valencia, CA, USA). All oomycete genomic DNA samples and standard curve controls were included in duplicate.

Glasshouse trial

Inoculum production

The pathogenicity of five oomycete species *Ph. cactorum* (STE-U7204)*, P. ultimum* (STE-OW2187)*, P. sylvaticum* (STE-U7199)*, Pht. vexans* (STE-U6741) and *P. irregulare* (STE-U7193) towards apple seedlings was determined in glasshouse trials. Sand-bran inoculum of the isolates was produced by first growing *Pythium* isolates for 7 days, and *Phytophthora* isolates for 14 days on corn meal agar (Difco Laboratories, MI, USA). Sand-bran was prepared according to Lamprecht (1986) where 200 g washed sand, 20 g digestive bran and 30 ml distilled water were added into a 500 ml Schott bottle, and autoclaved at 120 °C for 20 min. on two consecutive days. Each bottle was inoculated with ten colonized CMA disks (6mm dia.) of each of the respective pathogens, and incubated at 25 °C for one week for *Pythium* and *Phytopyhtium* isolates, and 2 weeks for *Ph. cactorum.* During this incubation period, bottles were shaken only on day 4 to ensure good colonization of the substrate. Control bottles were inoculated with 10 un-colonized CMA plugs (6 mm dia.).

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Seedling production

Golden delicious seedlings were used in all pathogenicity trials. The seedlings were produced from seeds as previously described (Tewoldemedhin et al., 2011c)

Inoculation of seedlings

Seedlings produced in the seedling trays, were transplanted into a bark and sand (2:1 v/v) planting medium. The medium was pasteurized for two hours at 80°C on two consecutive days, and only used after aeration of at least 3 days. The pasteurized medium was inoculated with sand-bran inoculum of the different oomycete species separately at two concentrations, 0.5% v/v and 1% v/v. After thorough mixing of the inoculated plant medium, it was dispensed into 1 L plastic bags. Three week old apple seedlings were planted into the bags, three seedlings per bag. Immediately after planting, the length of each seedling was recorded for use in assessment of increase in length at the end of the trial. For the first trial, seedlings were watered two times per day for 1 min, but after one month the irrigation was increased to three times per day for 2 min. Less irrigation was applied in the second trial and consisted of two times per day for 30 seconds at the time of planting, which was increased after one month to three times per day for 1min. The seedlings were fertilized with Multifeed (Effekto, Bryanston, South Africa) on a weekly basis, 100ml per plant at 5g/L. Trials were analysed 3 months after planting. Each treatment contained six replicates, with each replicate consisting of a 1L planting bag. The trial was a complete randomized designed and the experiment was repeated twice.

Trial evaluation

Growth parameters

At the end of the trial the lengths of the seedlings were determined, which was used to calculate the increase in length over the 3 month growth period.

Isolation studies

Washed root systems from each replicate bag (containing three seedlings) was used for isolation studies. For each replicate, 20 fine feeder roots of the first order (Fig. 1) were plated onto PARP selective media (Erwin and Ribeiro, 1996) containing 0.4g Switch (Syngenta, Johannesburg, South Africa; cyprodinil (375 g/kg) and fludioxonil (250 g/kg)). The plates were incubated in the dark at 25˚C for 2-3 days, and the number of roots yielding oomycete growth was counted, and expressed as percentage infected root fragments. The identity of oomycete growth was confirmed morphologically for representative samples.

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qPCR quantification of pathogens

At the end of the trial, approximately 4ml of washed first order roots (Fig. 1) from each replicate were stored at -20 °C for DNA extraction and qPCR analyses. DNA was extracted from the roots by first lyophilizing the roots. Twenty milligrams of lyophilized roots were placed into a 2 ml centrifuge tube, and fragmented into small pieces using a spatula. DNA was extracted from the roots using the NucleoSpin PLANT II kit (Macherey-Nagel GmbH and Ko, Duren, Germany) according to manufacturer's instructions, except that the roots were first powdered by adding 0.5g glass beads (2mm) to the tube and shaking at maximum speed for 10 min in a Retsch® MM301 mixer mill (GmbH and Co., Haan, Germany). The required amount of extraction buffer PL1 for extraction of all samples was aliquoted and spiked with an internal control plasmid containing a mutated *Escherichia coli* gene (Daniell *et al*., 2012; see below), so that each 400 µl extraction buffer contained 10^9 copies of plasmid. The plasmid was kindly provided by T. Daniell (The James Hutton Institute, Dundee, UK). Subsequently, 400 µl of the spiked extraction buffer was added to each powdered root sample. The tubes were shaken in a Retsch® mill at maximum speed for 5 min. Subsequent steps for the DNA extraction process were conducted according to the NucleoSpin PLANT II kit manufacturer's instructions. In the last step, DNA was eluted from the column using 100 µl of elution buffer.

The extracted DNA was used to quantify the pathogens in seedling roots using qPCR analyses, with either a SYBR® Green or Taqman based assay (Table 1). The species and genus specific primers for the oomycete pathogens and the mutated *E. coli* gene were all previously published (references within Table 1), except for the *Pht. vexans* primers and probe that were developed in the current study. The primer concentrations, annealing time and temperature, which in some instances differed from the published assays, are presented in Table 1. For all qPCR assays, DNA extracted from roots were diluted 1:5, and 2 µl of DNA was used in each qPCR reaction. Each sample was analysed in duplicate. SYBR® Green qPCRs were conducted using the KAPA SYBR FAST qPCR MASTER MIX (Lasec SA, Cape Town, South Africa) in reactions containing a total volume of 20 µl. The general amplification conditions for SYBR Green reactions consisted of 3 min at 95˚C for one cycle, and 40 cycles at 95˚C for 3 s and annealing at 56-60˚C for 20-60 s depending on the pathogen (Table 1). Melt curve analyses were conducted at the end of each run, that consisted of heating samples for 95 °C for 10 s, 65 °C for 15 s, and then heating to 95 °C at a ramp rate of 0.1 °C/s, with continuous acquisition, to confirm the identity of the amplified products. The only Taqman probe based assays were for *Pht. vexans* as described above, and *P. ultimum.* The *P. ultimum* assay was conducted using KAPA probe master mix (Lasec SA, Cape Town, South Africa) in a total reaction volume of 20 µl. Amplification was conducted for 3 min. at 95˚C for one cycle, followed by 40 cycles at 95˚C for 3 seconds and then annealing for 30s at 60˚C. Standard

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curves for *P. ultimum*, *P. irregulare* and *Ph. cactorum* assays were constructed using DNA at concentrations ranging from 1 ng/µL to 1 fg/µL in ten-fold serial dilutions. Standard curves for the *P. sylvaticum* assay were established in assays that used DNA in concentrations that ranged from 2 ng/µL to 120 fg/µL in five-fold serial dilutions. The standard curve was compiled by conducting qPCR analyses for three replicates of each of the concentrations.

The amount of pathogen DNA quantified from root samples in qPCR assays using the pathogen genomic DNA standard curve, will hereafter be referred to as absolute pathogen DNA biomass and quantifications. This is thus the pathogen DNA quantity based on the amount of root weight used in DNA extraction. In addition to absolute pathogen DNA biomass quantifications, relative pathogen DNA biomass was also calculated. The calculation of relative pathogen DNA biomass is based on the principal that the addition of a known quantity of mutated *E. coli* gene copies (Daniell *et al.,* 2012) to the DNA extraction buffer of each sample, can be used to estimate DNA loss during extraction and PCR inhibition in samples. The calculated loss is then used to determine the relative or "real" pathogen DNA quantity in the roots.

The plasmid containing the mutated *E. coli* gene was purified from induced *E. coli* cell pellets with the GeneJet plasmid miniprep kit (Thermo Scientific., Glen Burnie, Maryland, USA). The purified plasmid was linearized using the *NotI* enzyme (Thermo Fisher Scientific) according to manufacturer's instructions. The digested plasmid was cleaned with a MSB Spin PCRapace kit (Invitek, GmbH, Berlin, Germany). The linearized plasmid was quantified using a Nanodrop (Thermo Scientific) and diluted to concentrations of 10 to 10^9 copies per reaction in tenfold dilutions for inclusion in the qPCR standard curves. A 10^9 copies/µl plasmid solution was used to spike the DNA extraction buffer with 1µl in 400µl DNA extraction buffer for all root samples. The relative amount of pathogen DNA biomass was calculated by first determining the ratio of the qPCR quantified pathogen DNA biomass (absolute) to the copy number of mutated *E. coli* gene (i.e. pathogen DNA biomass divide by *E. coli* mutated gene copy number). The ratio was then multiplied by 10^9 copies (amount of *E. coli* gene copies in each DNA extraction) and then divided by 20mg (amount of root tissue used in each DNA extraction).

Analyses of apple nursery trees

Apple trees (1-2 years old) were collected in five different nurseries in the Western Cape region in 2013 and 2014, for which preliminary pilot isolation studies showed that *P. irregulare* was the most prominent oomycete species. In each nursery, five trees were sampled in each of five blocks resulting in a total of 125 trees per sampling year. The roots of the trees were washed, and isolations for *Pythium* were made as described for the glasshouse roots, with 20

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root segments per tree plated onto PARP plus Switch ® media. Hyphal tip cultures were made from growth on the PARP plates after 2-3 days incubation at 25˚C to potato dextrose plates. The species identity of isolates was determined based on ITS-PCR-RFLP analyses as described in Chapter 3. DNA was also isolated from the roots as described for the glasshouse trials. For the 2013 sampling, the roots of the five trees per block were pooled, so that there were a total of 25 root DNA samples. For the 2014 sampling, the roots of each tree were analysed individually, resulting in a total of 125 DNA samples. The percentage roots infected with *P. irregulare* was calculated and *P. irregulare* qPCR analyses were conducted on root DNA as described for the glasshouse samples.

Statistical analyses

All analyses were conducted in Statistica 12 (Dell Software). Analyses of variance were conducted on the apple seedling growth and isolation data of the pathogenicity trials, followed by post hoc testing (Students least significant difference test) at the 95% confidence level. Spearman correlation analyses were conducted in all possible combinations for (i) absolute pathogen DNA biomass, (ii) relative pathogen DNA biomass obtained after corrections based on the internal plasmid control, (iii) increase in plant length and (iv) percentage isolation data. Spearman correlation analyses were also conducted on the nursery relative and absolute *P. irregulare* DNA biomass and percentage *P. irregulare* root infections.

RESULTS

Phytopythium vexans qPCR assay development

Among the combination of primers that were evaluated in conventional PCRs, primers PV390F and PV455R were selected for further qPCR analyses in combination with probe PV412P. The optimized qPCR assay had a relative low sensitivity level of 1000 fg/µL. The assay was specific and did not detect any of the closely related species that were evaluated. The 12 *Pht. vexans* isolates representing *Pht. vexans* phylogenetic clades A to C (Spies *et al.*, 2011) were all quantified equally when each was tested at 10 ng/µl in qPCR reactions.

Glasshouse trial

Trial evaluation

Growth parameters

Analyses of variance for the increase in seedling length showed that there was a significant (P = 0.01) experiment x treatment interaction (Table 2), when the uninoculated control treatment was included, and concentration was not considered as a factor in the analyses. Therefore, the main effect of treatment could not be considered as pooled data of both

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experiments. In experiment 1, almost all pathogen treatments caused significantly more damage (lower increase in length) than in experiment 2, with the exception of *Ph. cactorum* at the 0.5% inoculum concentration (Fig. 2). In experiment 1, almost all of the isolates at both inoculum concentrations significantly reduced seedling length relative to the control, with the exception of *Pht. vexans* and *P. sylvaticum* at the 0.5% concentration. There were no significant difference in disease severity between the two inoculum concentrations for a specific species in experiment 1, with the exception of *Ph. cactorum* and *P. sylvaticum* that caused significantly more disease at the 1% than at the 0.5% inoculum concentrations. In experiment 2, *Ph. cactorum* at both inoculum concentrations was the only species that was virulent based on increase in seedling length (Fig. 2).

In order to access the effect of inoculum concentration, the uninoculated control (0% inoculum) was not included in analyses of variance. This showed that there were no significant interactions ($P \leqslant 0.05$) between any of the including factors experiment, treatment (species) and concentration (Table 3). In terms of the increase in seedling length, there was no significant difference ($P = 0.14$) between the inoculum concentrations. There was a significant difference between pathogen species ($P < 0.001$) for increase in apple seedling length (Table 3), with *Ph. cactorum* being more virulent than the other oomycete species (data not shown).

Isolation studies

For percentage roots infected, there was a significant experiment x treatment interaction ($P < 0.001$) (Table 2), when concentration was not considered as a factor and the uninoculated control was included in the analyses. However, the control treatment did not yield any oomycete infections for any of the treatments and this treatment thus had no variance. Therefore, it was more appropriate to consider the ANOVA where the control was not included (Table 3). This showed that for percentage roots infected there was a significant treatment (pathogen species) x experiment interaction ($P = 0.05$) (Table 3). Post hoc analyses of the weighted means showed that the percentage roots infected for all species were significantly higher in experiment 1 than experiment 2. Within experiment 1 and 2, there were no significant differences between the species in the percentage roots infected, although *Pht. vexans* infections tended to be higher followed by *P. sylvaticum* in both trials. Although in experiment 1, *Ph. cactorum* (1%) and *P. ultimum* (0.5%) were among the most virulent treatments, these species didn't show trends towards increased percentage root infection relative to the other oomycete species (Fig. 3).

qPCR quantification of pathogens

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The limits of quantification were different for the five oomycete species. The pathogen assay that had the highest sensitivity was *P. irregulare* (1 fg/µL) followed by *P. ultimum* (10 fg/µL), *Ph. cactorum* (100 fg/µL), *Pht. vexans* (1000 fg/µL) and *P. sylvaticum* (3200 fg/µL) (Table 4). The efficiency of most of the pathogen assays were comparable (92 to 98%) with that of the mutated *E.coli* gene assay, with the exception of the *P. sylvaticum* efficiency that differed by 12%. This is slightly higher than the 10% difference in efficiency recommended for using relative quantification of two genes (Gao *et al*., 2004). For some oomycete species the negative control was picked-up at C_t values of 36 to 40 (Table 4). For these assays quantification after 35 cycles were not considered as positive.

Not all of the pathogens were consistently quantified from pathogen inoculated replicated treatments. *Pythium sylvaticum*, *P. irregulare*, *P. ultimum* and *Ph. cactorum* were quantified in all treatment replicates in both glasshouse trials. However, although in the first trial *Pht. vexans* was quantified in all treatment replicates, in the second trial the pathogen was only detected in 42% of the replicates. This contrasted with the isolation studies of the second trial that showed a slightly higher infection of *Pht. vexans* roots relative to the other species (Fig. 3).

Correlation analyses

There was a significant negative correlation between increase in plant length and the three measured parameters (percentage root infection, relative- and absolute pathogen DNA biomass quantification) for almost all of the species except for *Ph. cactorum* and *P. irregulare*. For *Pythium irregulare*, a significant negative correlation for increase in plant length was observed only for percent root infection, but the correlation was low (r = -0.5; P = 0.02) (Table 5; Fig. 4). For *Pht. vexans, P. sylvaticum* and *P. ultimum* the significant negative correlations of increase in plant length with percent roots infected (r = -0.60 to -0.77; $P < 0.001$), relative pathogen DNA biomass (r = -0.60 to -0.72; $P < 0.001$) and absolute pathogen DNA biomass (r = -0.56 to -0.72; $P \leq 0.01$) were comparable (Table 5; Fig. 4). All three parameters will thus be suitable for expressing pathogen damage in these species.

For all five pathogens examined, a high and significant correlation ($P < 0.001$) was evident between the absolute pathogen DNA biomass and relative pathogen DNA biomass. The highest correlation was for *P. irregulare* (r = 0.98), followed by *Pht. vexans* (r = 0.95), *P. ultimum* (r = 0.94), *Ph. cactorum* (r = 0.92) and *P. sylvaticum* (r = 0.89). The absolute- and relative pathogen DNA biomass were both highly correlated and significantly with percentage root isolations for all five pathogens (r = 0.84 – 0.95; $P < 0.001$) (Table 5).

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Analyses of apple nursery trees

Pythium irregulare was identified as the primary oomycete pathogen in the nurseries. Similar to the glasshouse results, for the nursery a high and significant correlation (r = 92; P < 0.001) was found between the absolute *P. irregulare* DNA biomass and relative pathogen DNA biomass. For *P. irregulare* the relative and absolute *P. irregulare* DNA biomass also correlated significantly (r = 0.63 - 0.72; P < 0.001) with percentage root infection, as was found in the glasshouse trial, although the correlation values were somewhat lower than that of the glasshouse trials (r = 0.84 – 0.86; P < 0.001) (Table 5).

DISCUSSION

A new Taqman-based qPCR assays was developed for *Pht. vexans,* based on a putative inositol polyphosphate 5-phosphatase region. The assay was specific, and importantly also yielded similar pathogen DNA biomass values for the 12 investigated *Pht. vexans* isolates. This is an improvement on the previously published assay based on the ITS region, which amplified *Pht. vexans* isolates differentially and were thus not suitable for quantitative analyses of this species (Spies *et al*., 2011). The sensitivity of our *Pht. vexans* assay was low (1000 fg/μL), which could be due to the single copy nature of the gene, unlike the multi-copy ITS region used for quantification of most oomycete species. The published ITS based *Pht. vexans* assay had a very high sensitivity (1fg/μL) (Spies *et al*., 2011). Although multi-copy genes in general are more sensitivity than single copy genes (Kandel *et al*., 2015), it is feasible to obtain at least a 100 fg/μL sensitivity level with a single copy gene as has been shown for the *Phytophthora* genus-specific primers that amplifies the single copy *Ypt1* gene (Schena *et al*., 2008; Tewoldemedhin *et al*., 2011b; current study). For other pathogens such as *Fusarium* the amplification of single copy genes have yielded sensitivities ranging from 500fg to 50pg per reaction (Horevaj *et al*., 2011; Kandel *et al*., 2015). Since this is the first report of the putative polyphosphate 5-phosphatase region being used in qPCR analyses, it is unknown whether the low sensitivity of the assay is inherent to the gene itself. Nonetheless, it is concerning that the *Pht. vexans* assay did not detect the pathogen in all replicates of the glasshouse trials, since all the other oomycete pathogens were detected in all replicates. The low sensitivity of the *Pht. vexans* qPCR assay is most likely the cause of the lack of detection. This was true even for the *P. sylvaticum* assay, which was less sensitive (3200 fg/μL) than the *Pht. vexans* assay, and that further also had similar percentage roots infected than *Pht. vexans*.

The newly developed *Pht. vexans* qPCR assay and the conditions of the *P. sylvaticum* assay used in the current study requires optimization for improving their sensitivity for future use in quantification of inoculum sources and analyses of roots from treatments in

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management trials. In the current study, the sensitivity of the *P. sylvaticum* assay was much lower than that reported by Tewoldemedhin *et al*. (2011b) and Schoeder *et al.* (2006) who reported a sensitivity of 10 fg/ μL. The only likely reason for this is the use of different SYBR Green chemistries. For the *Pht. vexans* assay, evaluating the additional primers designed in this study may improve the sensitivity, since it has been shown that different primer pairs of the same gene region can yield a 16-fold increase in amplification, even though primer design software will show that all primers are suitable for qPCR quantification (Lu *et al.*, 2010). An alternative option for increasing the sensitivity of the assays is the use of different probe chemistries. It has for example been found that locked nucleic acid (LNA) probe chemistry can significantly lower Ct-values compared to Taqman probes (Josefsen *et al*., 2009), which was used in the current study. A new touchdown qPCR assay was recently developed that significantly improved the sensitivity of assays (Zhang *et al*., 2015), which may also have potential for increasing the sensitivity of the two oomycete assays, without compromising specificity. The low efficiency of the *P. sylvaticum* assay (0.87) is also somewhat concerning, and may also be improved with touchdown qPCR since this approach has been reported to also improve the efficiency of qPCR assays (Zhang *et al.*, 2015).

Two glasshouse trials were conducted to generate apple seedlings that differ in disease severity, as measured through increase in seedling length, upon inoculation with five different oomycete species (*P. ultimum*, *P. irregulare, P. sylvaticum*, *Pht. vexans* and *Ph. cactorum*). This was attempted by using two different inoculum concentrations (0.5% and 1% v/v). However, this approach was not effective since there were no significant differences in disease severity for most species within a trial, between the two inoculum concentrations. It was however, possible to generate differences in disease severity between the two trials for each oomycete pathogen, most likely due to the higher irrigation regime used in the first trial. Altogether the two trials provided plant material that differed in disease severity for each oomycete pathogen. This material was used to investigate the correlation of disease severity with percentage roots infected and pathogen DNA biomass (absolute and relative DNA qPCR quantifications).

An important aim of the study was to determine if relative qPCR quantification of pathogen DNA biomass can improve quantification compared to absolute quantification. The correlation between relative and absolute pathogen DNA biomass was high and significant for all five oomycete pathogens (r = 0.98 to 0.89; $P < 0.001$) in glasshouse trials, and correlated equally well with plant growth reduction (see below). The correlation was also investigated in roots collected from nursery trees, since it was hypothesized that the DNA quality of nursery roots that are more suberized than glasshouse seedling roots will be poorer or more DNA loss may occur during extraction. These analyses also revealed a highly significant correlation (r = 0.96; $P < 0.001$) between absolute and relative pathogen DNA biomass quantification for *P.*

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irregulare, the main oomycete pathogen in the nursery material. Therefore, either approach can be used for quantifying ARD oomycete pathogens in glasshouse or nursery tree roots, but since relative quantification is more expensive only absolute quantification can be done. This finding contrasts with findings of Eshraghi *et al*. (2011), who showed that for *Phytophthora cinnamomi* the use of relative quantification with an artificial gene improved pathogen quantification as related to disease severity. It is possible that the DNA loss and purity of our DNA extractions was better or more consistent between samples than that of Eshraghi *et al*. (2011), resulting in relative quantification not improving pathogen DNA biomass estimations. This is also suggested by the high correlations found for the nursery roots between relative and absolute DNA quantifications. The nucleospin DNA extraction kit thus proved to be very successful for consistently extracting high quality DNA from lyophilized roots. The use of only fine feeder roots probably also yielded higher quality DNA extracts than extractions from whole root systems, including suberized pioneer roots.

For most oomycete species, qPCR pathogen DNA biomass (absolute and relative) and percentage root infection had a significant negative correlation with increase in seedling length. This, however, was not true for *Ph. cactorum* and *P. irregulare,* where a significant negative correlation (r = -0.50; P = 0.02) was only found for *P. irregulare* between percentage roots infection and increase in seedling length. For *Pht. vexans, P. sylvaticum* and *P. ultimum* the correlations were all significant and comparable between increase in plant length with (i) percentage root infection (r = -0.60 to -0.77; P < 0.001), (ii) absolute pathogen DNA biomass (r = -0.56 to -0.72; P < 0.001) and (iii) relative pathogen DNA biomass (r = -0.60 to -0.72; P < 0.001). Therefore, all three parameters were deemed suitable for predicting disease severity for these pathogens. The good correlations found between seedling growth reduction and pathogen DNA biomass for *Pht. vexans, P. ultimum* and *P. sylvaticum* differs from results obtained by Tewoldemedhin *et al*. (2011a) who could not identify any significant correlations for these pathogens. This could be due to the fact that Tewoldemedhin *et al*. (2011a) used the whole root system, whereas in the current study only fine feeder roots were dissected from the root system and used for DNA extractions. This is supported by findings of Emmett *et al*. (2014), in that *P. sylvaticum* is more likely to be detected in fine feeder roots, than pioneer roots of apple seedlings. Another reason might be that Tewoldemedhin *et al*. (2011a) in their absolute quantification used the same amount of root DNA in each qPCR reaction, resulting in expression of pathogen DNA biomass per nanogram / femtogram root DNA. In the current study, absolute pathogen DNA biomass was expressed per mg dried roots. The finding that pathogen DNA biomass of *P. irregulare* does not correlate with growth reductions, is similar to findings reported by Emmett *et al*. (2014) for late stages of infection by *P. irregulare*. They found that during the early stages of seedling infection, *P. irregulare* was more likely to be detected in first and second order roots, than in pioneer and third order roots. However, during

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the later stages of infection, which was 9 weeks after inoculation, the pathogen was no longer detected from third order feeder roots, whereas DNA biomass increased drastically in pioneer roots at this time point (Emmett *et al*., 2014). Our trials were evaluated 12 weeks after inoculation, which would have also resulted in only low levels of pathogen being detected in fine feeder roots that were most likely not representative of pathogen presence in the whole root system. The tendency of *P. irregulare* to start colonizing pioneer roots in the later stages of infection, could also explain why Tewoldemedhin *et al*. (2011a) found a significant negative correlation between *P. irregulare* DNA biomass and seedling weight, since the whole root system, including pioneer roots were used in DNA extractions and not only feeder roots as in the current study. *Phytophthora cactorum* may also have a similar colonization behaviour during late stages of seedling infection than *P. irregulare*, which could explain the lack of correlations between growth reduction and pathogen DNA biomass and percentage root infected when quantifications are done from feeder roots.

To conclude, the current study showed that percentage root infection and pathogen DNA biomass quantity (absolute and relative) were good predictors of apple seedling growth reduction for *P. sylvaticum, Pht. vexans* and *P. ultimum,* but not for *Ph. cactorum* and *P. irregulare*, when the fine feeder root system of seedlings was targeted. For *Ph. cactorum* and *P. irregulare,* the whole roots system should be evaluated in future studies to determine if percent root infection and pathogen DNA biomass will be better predictors of seedling growth reductions. It is possible that different parts of the roots system will have to be targeted for different ARD pathogens in inoculum source studies and when treatments in management trials are being evaluated. The current study, however, only used single pathogen inoculations, unlike in ARD orchards where different groups of pathogens act synergistically to cause disease (Tewoldemedhin *et al*., 2011a). Thus, whether the current approach will be useful for predicting tree damage in complex ARD orchard soils must still be determined. Emmett *et al.* (2014) quantified *P. sylvaticum* in roots of apple seedlings in natural ARD soils and could not find a significant correlation between seedling growth reductions and the DNA quantity of the pathogen in any of the root system orders analyzed. Another important aspect that was also elucidated in the current study is that oomycete pathogen DNA biomass in roots can be quantified equally well using relative or absolute qPCR amplification. Since the latter will be more cost effective, this can be used, but a few samples should still be tested for relative quantification within groups of experiments to exclude false negatives. In future studies, relative quantification using an apple gene should also be investigated in qPCR assays to determine if correlations between plant growth reduction and oomycete pathogen DNA biomass can be improved. Another option could be to quantify oomycete pathogen DNA biomass in rhizosphere soil samples to determine if this could perhaps yield significant correlations between plant growth reduction and DNA quantity for all five oomycete pathogens,

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including *P. irregulare* and *Ph. cactorum.* The use of relative qPCR quantification using an internal spiked foreign gene in soil DNA extractions and qPCR assays will be important, since soil is a much more difficult and highly variable matrix to work with (Daniels *et al*., 2012) than fine feeder roots of plants. A new qPCR assay for *Pht. vexans* was developed that will be useful in future studies for equally quantifying all *Pht. vexans* isolates. However, this will need further optimization to ensure that the sensitivity level of the assay is improved. Altogether the study not only yielded knowledge on new approaches that can be used for quantifying oomycete ARD pathogens from apple roots to reflect disease severity, but knowledge was also gained on the colonization pattern of the root system by different oomycete pathogens.

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Table 1. Quantitative real-time PCR (qPCR) primers, probes and conditions used for amplification of oomycete root pathogens and a mutated *Escherichia coli* gene.

Target species	qPCR chemistry	Primer concentrations		Probe	Annealing[b]		Extension[b]		$MgCl_2$ [a]	References
					Temperature	Time	Temperature	Time		
P. sylvaticum	SYBR Green	Syl1F 200 nM	Syl1R 200 nM	_	65	15*[b]	72*	30	500 nM*	Schroeder *et a*l., 2006
P. irregulare	SYBR Green	PirF1 300 nM	PirR1 900 nM	_	60	5	72	20	100 nM*	Spies *et al*., 2011
P. cactorum	SYBR Green	Yph1F 250 nM*	Yph2R 250 nM*	_	62	20*	72	27*	_	Schena *et al*., 2008
E.coli mutated	SYBR Green	342Fmut 200 nM	534Rmut 200 nM	_	54	10	72	10	_	Daniell *et a*l., 2012
P. ultimum	Taqman	PulF2 300 nM	PulR2 300 nM	PulP2 150 nM	60	30	_	_	_	Spies *et a*l., 2011
Pht. vexans	Taqman	PV390P 300 nM	PV455P 300 nM	PV412P 200 nM	60	30	_	_	_	This study

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

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

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Table 2. Analyses of variance on the effect of various oomycete apple root pathogens on increase in length of apple seedlings, and percentage root infection of apple seedlings in two glasshouse experiment.

		Increase in seedling length			Percentage roots infected		
Source of variation	DF	MS	F	*P*	MS	F	P
Experiment	1	2440.85	110.93	<0.001	55227.27	332.59	< 0.001
Treatment	10	160.33	7.29	<0.001	1520.57	212.23	< 0.001
Experiment x treatment	10	54.31	2.47	0.01	974.36	5.84	< 0.001
Error	110	22.00			260.23	3.74	

Table 3. Analysis of variance on the effect of two inoculum concentrations of various oomycete apple root pathogens on increase in length of apple seedlings and percentage root isolations in two glasshouse experiments.

		Increase seedling length			Percentage roots infected		
Source of variation	DF	MS	F	*P*	MS	F	P
Experiment	1	2602.05	110.92	< 0.001	60750.0	212.23	< 0.001
Treatment (species)	4	279.79	11.93	< 0.001	1058.02	3.70	0.01
Inoculum concentration	1	50.77	2.16	0.14	907.50	3.17	0.08
Experiment x treatment	4	46.11	1.97	0.16	702.60	2.45	0.05
Experiment x concentration	1	59.80	2.55	0.11	440.83	1.54	0.22
Treatment x concentration	4	27.28	1.16	0.33	352.81	1.23	0.30
Experiment x treatment x concentration	4	32.78	1.40	0.24	242.40	0.85	0.50
Error	100	23.46			286.25		

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Table 4. Linearity, slope, efficiency and limit of quantification test results for quantitative real-time PCR assays used in this study.

Target species	qPCR Protocol	NTC C_t	Linearity R	Slope M	Efficiency E	Limit of quantification (fg/ µL)
Pythium sylvaticum	SYBR green	37	0.996	-3.34	0.87	3200
Pythium irregulare	SYBR green	36	0.998	-3.35	1.08	1
Pythium ultimum	Taqman	N/D	0.997	-3.36	0.95	10
Phytophthora cactorum	SYBR green	N/D	0.988	-3.30	0.93	100
Phytopythium vexans	Taqman	36	0.996	-3.29	1.01	1000
Mutated *E. coli*	SYBR green	N/D	0.999	-3.32	0.99	100[a]

[a] Copy number of *E. coli* gene.

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Table 5. Correlation analyses of various parameters measured on apple seedlings artificially inoculated with five different oomycete pathogens, and apple nursery trees naturally infected with *Pythium irregulare.*

	% Infected roots (r; *P*)	Increase in plant length (r; P)
NURSERY (*Pythium irregulare*)		
Relative pathogen DNA biomass[a]	*r* = 0.72; *P* < 0.001	Not done
Absolute pathogen DNA biomass[b]	*r* = 0.63; *P* < 0.001	Not done
Percentage root infection[c]	-	Not done
GLASSHOUSE[d]		
Phytophthora cactorum		
Relative pathogen DNA biomass	*r* = 0.84; P < 0.001	*r* = -0.19; *P* = 0.43
Absolute pathogen DNA biomass	*r* = 0.87; *P* < 0.001	*r* = -0.28; *P* = 0.24
Percentage root infection	-	*r* = -0.32; *P* = 0.17
Pythium irregulare		
Relative pathogen DNA biomass	*r* = 0.86; *P* < 0.001	*r* = -0.31; *P* = 0.19
Absolute pathogen DNA biomass	*r* = 0.84; *P* < 0.001	*r* = -0.26; *P* = 0.26
Percentage root infection	-	*r* = -0.50; *P* = 0.02
Pythium sylvaticum		
Relative pathogen DNA biomass	*r* = 0.91; *P* < 0.001	*r* = -0.62; *P* < 0.001
Absolute pathogen DNA biomass	*r* = 0.90; *P* < 0.001	*r* = -0.56; *P* = 0.01
Percentage root infection	-	*r* = -0.70; *P* < 0.001
Pythium ultimum		
Relative pathogen DNA biomass	*r* = 0.95; *P* < 0.001	*r* = -0.72; *P* < 0.001
Absolute pathogen DNA biomass	*r* = 0.94; *P* < 0.001	*r* = -0.72; *P* < 0.001
Percentage root infection	-	*r* = -0.77; *P* < 0.001
Phytopythium vexans		
Relative pathogen DNA biomass	*r* = 0.95; *P* < 0.001	*r* = -0.60; *P* < 0.001
Absolute pathogen DNA biomass	*r* = 0.90; *P* < 0.001	*r* = -0.68; *P* < 0.001
Percentage root infection	-	*r* = -0.60; *P* < 0.001

[a] Amount of pathogen DNA biomass determined by qPCR analysis per mg of lyophilized roots per tree, relative to an internal artificial reference plasmid containing a mutated *E. coli* gene. The data of five nurseries, on average 100 trees per nursery, were used in the analyses.

[b] Total amount of pathogen DNA biomass determined by qPCR analysis per mg of lyophilized roots. The data of five nurseries, on average 100 trees per nursery were used in the analyses.

[c] Twenty roots were plated onto a oomycete selective medium per nursery tree and, the percentage of roots yielding oomycete growth was determined.

[d] For the glasshouse analyses the amount of pathogen DNA biomass, total amount of pathogen DNA biomass and percentage root infection were calculated in a similar manner than for the nursery trees. The exception was that the unit of measurement was not a nursery tree, but replicates (one planting bag containing three apple seedlings) from two independent glasshouse trials, six replicates per species were included from each trial.

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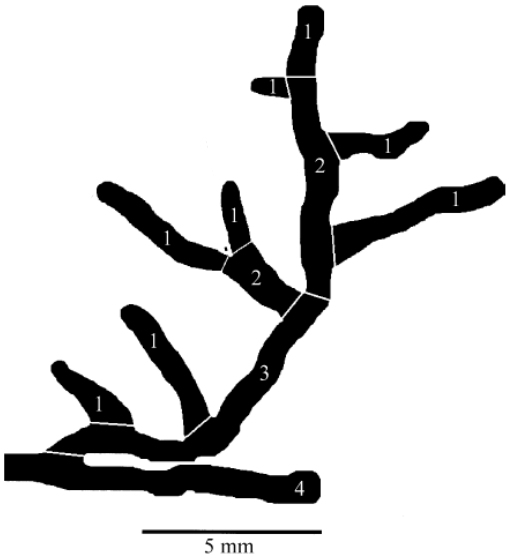

Figure 1. Schematic representation of the branching of a fine root system of a perennial tree. The numbers represent the order of each individual root (Reproduced from Pregitzer *et al.*, 2002 with slight modifications).

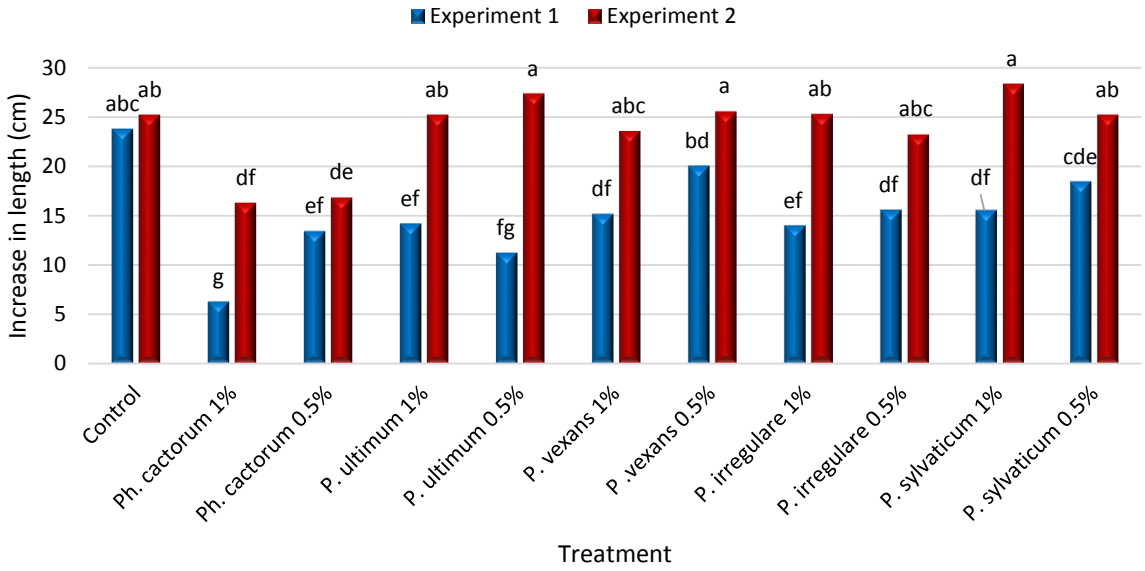

Figure 2. Effect of different oomycete pathogens introduced into growing medium at two inoculum concentrations (0.5% and 1% v/v) on increase in apple seedling length, 3 months after planting. Results are shown from two independent experiments. Bars followed by the same letters do not differ significantly from each other ($P < 0.05$).

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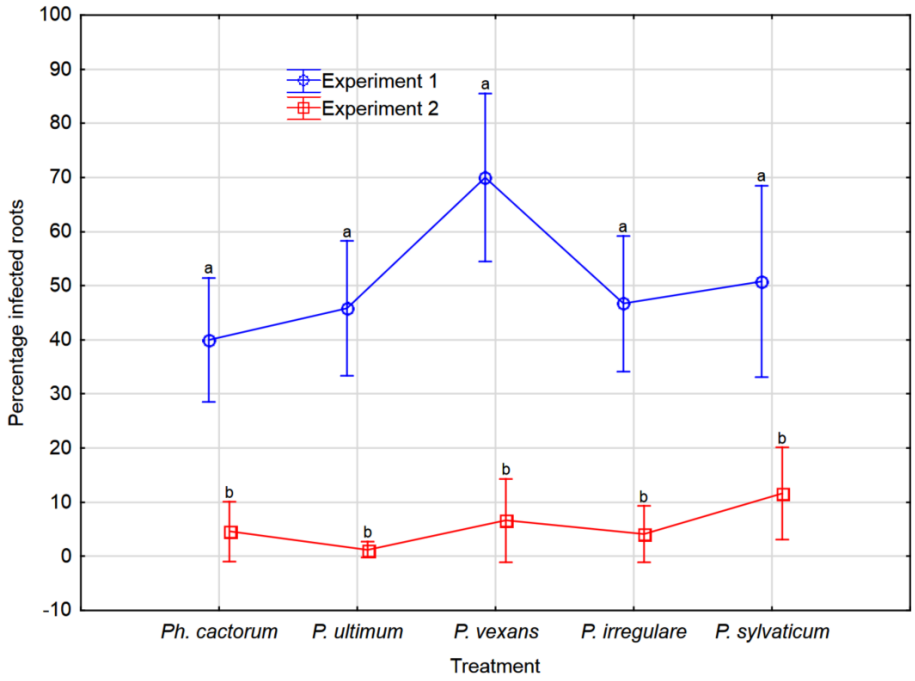

Figure 3. Percentage roots infected by five oomycete species that were introduced into plant growth medium prior to planting apple seedlings in two glasshouse experiments. Line markers followed by the same letters do not differ significantly from each other ($P < 0.05$). Vertical bars denote the 95% confidence intervals.

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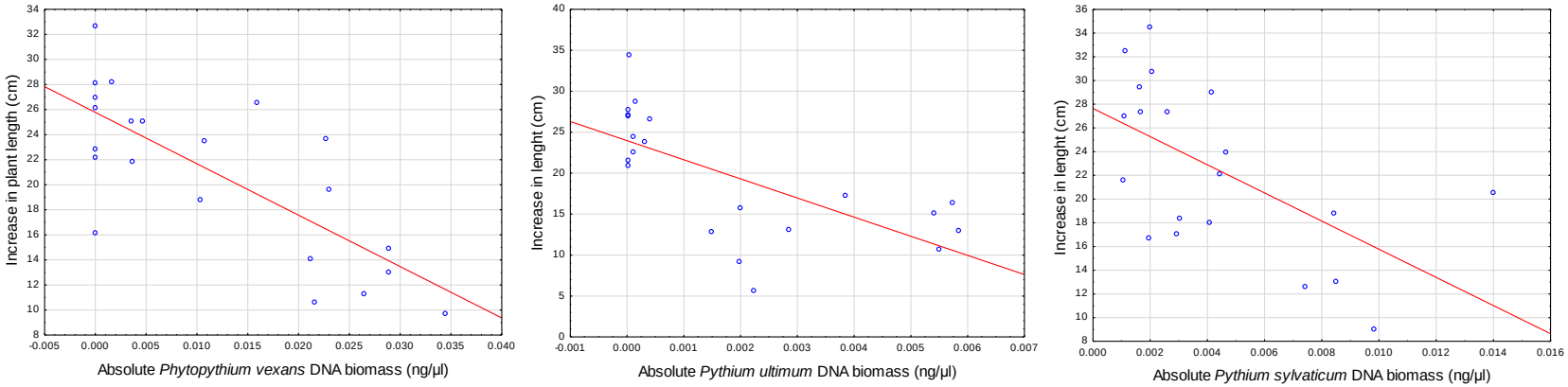
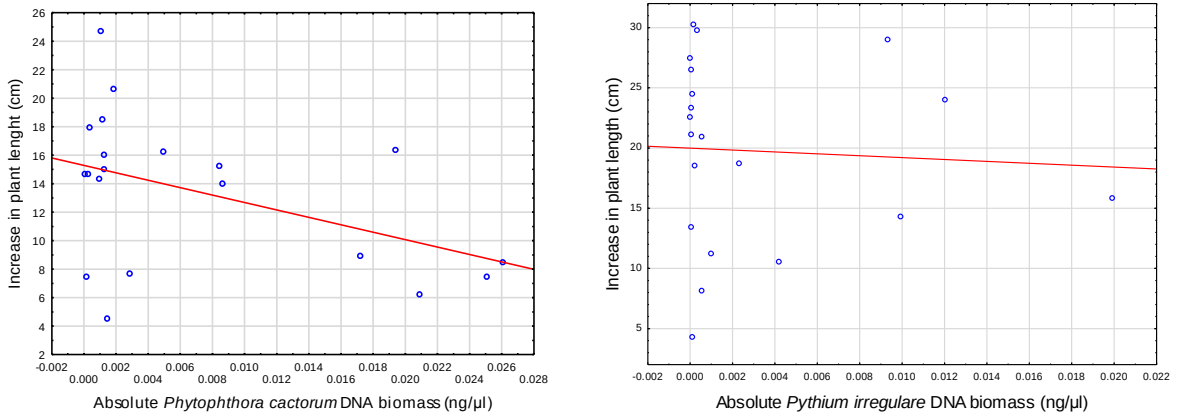


(A) *Phytophythium vexans* (B) *Pythium ultimum* (C) *Pythium sylvaticum*



(D) *Phytophthora cactorum* (E) *Pythium irregulare*

Figure 4. Scatter graphs of Spearman's correlations showing the relation between the amount of pathogen DNA biomass and increase in apple seedling length from a glasshouse trial where the oomycete pathogens (A) *Phytopythium vexans* (r = -0.68; P < 0.001), (B) *Pythium ultimum* (r = -0.72; P < 0.001), (C) *Pythium sylvaticum* (r = -0.56; P = 0.01), (D) *Phytophthora cactorum* (r = -0.28; P = 0.24), and (E) *Pythium irregulare* (r = -0.26; P = 0.26), were inoculated. The absolute pathogen DNA biomass was determined through quantitative real-time PCR and the increase in seedling length by measuring the length of seedlings at the start and end of the 3 month trial. Each point on a graph represents one replicate (1L bag containing three seedlings) in the glasshouse trial.

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CHAPTER 3

Investigating apple nursery trees and irrigation water as inoculum sources of apple replant pathogens

ABSTRACT

Apple replant disease (ARD), mainly caused by *Rhizoctonia solani*, *Cylindrocarpon*-like species, parasitic nematodes (*Pratylenchus*) and oomycetes, is considered as one of the most devastating diseases of apple. Although old apple soils are usually the source of inoculum, additional inoculum sources might be nursery trees and irrigation water. Therefore, apple nursery trees and irrigation water (only oomycetes) was investigated as ARD inoculum sources over two seasons in the apple producing regions of the Western Cape province of South Africa. A combination of isolation and quantitative PCR (qPCR) analyses showed that nursery trees were a large inoculum source of *Pythium irregulare, Cylindrocarpon*-like spp. and *Pratylenchus* spp., and to a lesser extent of *Pythium ultimum. Pythium sylvaticum* was rarely associated with nursery trees*. Phytophthora cactorum* and *Phytophythium vexans* were not detected in any of the nursery trees. The nurseries differed in the occurrence of trees infected with specific pathogens, but none were free of ARD pathogens. All the sampled nurseries and blocks within nurseries were infected with *P. irregulare,* with 95% of trees harbouring this pathogen. *Cylindrocarpon*-like species had an even higher incidence and was identified in all sampled trees. *Pythium ultimum* was present in 60% of nurseries, 55% of the blocks and in 41% of the trees. In the two sampling years, *Pratylenchus* spp. were detected in similar percentages of trees (29 to 39%). However, the percentage of trees having an infestation level higher than 100 per 5g or roots, was higher in 2013 (22%) than in 2014 (6%). Irrigation water was sampled in 16 dams, and at the point of exit of irrigation lines within 13 orchards over a 5 month period in each of two seasons, by exposing leaf and fruit baits for a 5-day period at the water sources. This showed that only *P. irregulare* may potentially be an inoculum source of ARD in irrigation water. Within orchard irrigation water, this pathogen could only be detected using qPCR assays on leaf disk baits, and was present in each of the sampled months in 31 to 82% of the investigated dams. *Pythium irregulare* was also detected at similar levels in dam water. *Pythium ultimum* was only detected in one dam. In dams, 12 non-ARD pathogen species (seven *Pythium* spp., one *Phytopythium* spp. and four *Phytophthora* spp.) were identified. *Phytopythium helicoides* was the most frequently occurring species and was detected in each of the sampling months. The number and

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frequency of occurrences of oomycete species were much lower in water sampled at irrigation pipes within orchards, than in dams. In orchard irrigation water, only five non-ARD pathogen species (four *Pythium* and one *Phytopythium*) were identified.

INTRODUCTION

Apple replant disease (ARD) is caused by a few biological soilborne agents that attack young trees established on soil previously planted to apple or closely related crops. The pathogens cause a reduction in root biomass and damage to roots, which ultimately translates into reduced yields, with the economic viability of orchards being negatively affected world-wide due to ARD. The main groups of ARD pathogens include selected species of fungi (*Rhizoctonia* and *Cylindrocarpon*-like spp.), oomycetes (*Pythium, Phytopythium* and *Phytophthora*) and nematodes (*Pratylenchus* spp.) (Manici *et al*., 2003; Tewoldemedhin *et al*., 2011a; Mazzola and Macini, 2012) During the life span of apple orchards, ARD pathogens build up in soil resulting in large inoculum loads in the soil that can survive long-term (Mazzola 1998; Mazzola and Macini, 2012). This can even start within two years after tree establishment (Mazzola, 1998). It is unknown where the inoculum comes from that initially starts the build-up of pathogens in ARD orchards. Nonetheless at the end of an orchard life-span, when young apple trees, the most susceptible stage of the tree to ARD pathogens, are replanted into the soil, the soil comprises the main source of inoculum.

There are some evidence that additional external inoculum sources could contribute to ARD, including nursery trees. Some support for this hypothesis is the fact that growers in South Africa sometimes don't observe good tree responses after fumigation of orchard soils, the main method used for managing ARD. Nursery trees have been reported as a large inoculum source of some known ARD pathogens including *Phytophthora cactorum* and *Phytophthora cambivora* in New York (Jeffer and Aldwinckle, 1988). In South Africa, nursery trees are suspected as an inoculum source due to the open field production system of rootstocks. The clonally produced rootstocks are produced vegetatively by the "stooling" or mound-layering method, where soil is mounded up around the base of the shoots of mother plants. The shoots will in time form roots, and are subsequently cut and used to establish rootstocks in nursery beds. The mother layering-blocks are used for 5-6 years or sometimes even longer to cut rooted shoots, which can potentially result in the accumulation of ARD pathogens. Some of the known ARD pathogens that have been reported from mother-layer blocks in South Africa is *Pratylenchus* spp. Although nurseries have implemented measures to manage *Pratylenchus,* these are not always successful (personal communication Sheila Story, Nemlab.). The presence of ARD pathogens in a soil that was previously used as a

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nursery has been reported in Washington State, where the oomycetes *Pythium ultimum* and *Ph. cactorum* dominated (Mazzola, 1998).

Another potential external inoculum source of ARD pathogens is surface irrigation water (ponds, rivers, canal ways and dams). Surface irrigation water is known to harbour some ARD pathogens including oomycetes and nematodes. The spread of oomycete pathogens in irrigation water is well-known, since most species produce sporangia in free water that release motile zoospores that disseminate through surface water surviving hours to weeks in the water (Porter and Johnson, 2004), depending on the species involved (Erwin and Ribeiro, 1996). Although zoospores are thought to be the main propagule involved in dispersing oomycetes in surface water, oospores and mycelia are likely also involved (Porter and Johnson, 2004), especially since not all *Pythium* species produce zoospores (Van der Plaats-Niterink, 1981). Several oomycete species within the genera *Pythium, Phytopythium* and *Phytophthora* have been reported from surface irrigation water world-wide (Shokes and McCarter, 1979; Sanchez and Gallego, 2001; Yamak *et al*., 2002; Parkunan and Ji, 2013; Jones *et al*., 2014). In Washington State in the Wenatchee River Valley, *Ph. cactorum,* a highly virulent ARD pathogen, was reported as frequently occurring in irrigation water during the summer fruit development period (Grove and Boal, 1991; Yamak *et al*., 2002). *Pratylenchus* spp. have been reported to occur in the Western Cape and Mpumalanga regions in South Africa from dams and rivers (Smith and Van Mieghem, 1983; Grech *et al*., 1989), but also in other regions of the world (Meagher, 1967; Heald and Johnson, 1969; Waliullah, 1984). Surface water has the potential of becoming contaminated with various microbes including plant pathogens, since they are open and pathogens can easily enter the water from different sources like infested water, infested soil, plant debris and field drainage tiles (Hong and Moorman, 2005).

The identification of ARD pathogens in nursery material and irrigation water can be conducted using conventional isolation or molecular methods, such as quantitative real-time PCR (qPCR) (Kong *et al*., 2003; Tewoldemedhin *et al*., 2011b; Eyre and Grabelotto, 2015). Investigations into irrigation water as an inoculum source can be a bit more challenging than nursery stocks, due to the sheer amount of water having to be surveyed. Therefore, oomycete isolations in surface water can be facilitated through the use of different plant baits, often consisting of leaf disks of specific plant species, or fruits such as pears for detecting *Phytophthora* (Yamak *et al.,* 2002; Gevens *et al*., 2007). To further increase the probability of oomycete isolations from water, baiting traps can be used that consist of floatable containers with the plant baits inside. These have the advantage that they can be exposed to the surface irrigation water sources for several days to increase the probability of oomycete detection (Gevens *et al*., 2007).

In South Africa, the ARD pathogens in replant soils have been characterized thoroughly, but no information, aside from the presence of *Pratylenchus,* is available on

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pathogens associated with nursery rootstocks. The ARD pathogen complex identified in replant orchards in South Africa, mostly agrees with those that have been reported elsewhere in the world (Tewoldemedhin *et al*., 2011b; Mazzola and Manici, 2012). The only exception is the importance of *Rhizoctonia,* for which only bi-nucleate groups have been identified in South Africa. Only a few isolates were found to be pathogenic, with several only having low virulence (Tewoldemedhin *et al*, 2011b). Van Schoor *et al*., (2009) also identified *Rhizoctonia* as the lesser component of ARD agents in South Africa. In contrast, in Washington State in the United States of America and in South Tyrol, the multinucleate *Rhizoctonia solani* AG-5 and AG-6 are important highly virulent members of the complex inciting ARD (Mazzola 1998; Manici *et al*., 2003; Mazzola and Manici, 2012). In South Africa, several oomycete species are very prevalent in ARD orchards and have been identified in all ARD orchards. The most prevalent and virulent species included *Pythium irregulare, Ph. cactorum, Phytopythium vexans* and *Pythium ultimum. Pythium sylvaticum* is also a highly virulent species but occur less frequently in South Africa (Tewoldemedhin *et al*., 2011a,b). *Cylindrocarpon*-like spp. are equally widely distributed and have been identified in all ARD orchards (Van Schoor *et al*., 2009; Tewoldemedhin *et al*., 2011c). The significance of previously identified species belonging to this group of fungi in South Africa is difficult to determine due to isolates varying from non-pathogenic to pathogenic, although most isolates have low virulence (Tewoldemedhin *et al*, 2011a,c). The significance of previously identified species is also confusing and mostly non-informative due to large taxonomic changes that have occurred for isolates with *Cylindrocarpon* anamorphs that were previously linked to the sexual genus *Neonectria* (Chaverri *et al*., 2011; Cabral *et al*., 2012; Lombard *et al*., 2014). *Pratylenchus* spp. have only occasionally been reported from ARD orchards in South Africa by Tewoldemedhin *et al*. (2011a), and were also found to be inconsistently associated with ARD by Van Schoor *et al*. (2009). In Washington state *Pratylenchus* spp. were also considered as having a minor role in ARD development, since although being present in most orchards, they were present below the damage threshold (Mazzola, 1998).

The aims of this study were to investigate nursery trees and surface irrigation water as external inoculum sources of ARD pathogens in South Africa. All the apple nurseries in South Africa, with the exception of one, were surveyed over a two year period. The presence of oomycetes in nurseries were investigated using isolation studies and species level identifications, as well as qPCR quantification of the genus *Phytophthora, P. irregulare, Phytopythium vexans, P. ultimum* and *Pythium sylvaticum.* The prevalence of *Cylindrocarpon*-like spp. in rootstocks were only investigated using genus-specific qPCR analyses that detects *Cylindrocarpon*-like isolates (pathogenic and non-pathogenic) known to occur in ARD orchards in South Africa (Tewoldemedhin *et al*., 2011c). Parasitic nematodes were investigated using only conventional centrifugal sugar flotation method (Tewoldemedhin *et al*.,

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2011a). Surface irrigation water sources were investigated for the presence of oomycetes in the two main apple production regions in South Africa, the Kouebokkeveld and Grabouw/Elgin areas, using leaf and fruit baits exposed to the water for four days. The water was analysed over two seasons on a monthly basis at two points on a farm that included the (i) irrigation pipe within the orchard and (ii) the dam or river from which the irrigation pipe received its water.

MATERIALS AND METHODS

Nursery sampling

Nursery apple trees, 1-2 years old, were sampled from five different nurseries in the Western Cape Province of South Africa. Samples were collected during the spring (late September to mid-December) of 2013 and 2014. In each nursery, five trees from each of four blocks were sampled, resulting in a total of 20 trees being sampled in each nursery at each sampling time. In 2014, only three blocks were sampled in two of the nurseries, resulting in a total of 15 trees per nursery (Table 1). The rootstock types that were sampled, depended on rootstock availability at the individual nurseries. Nursery apple trees were carefully uprooted and placed in plastic bags, and the samples were transported to the laboratory for analyses.

Identifying oomycetes from nursery root samples through isolations

Isolations

Oomycete isolations were made directly from apple roots by first rinsing the roots under running tap water to remove adhering soil. The roots were dried in a laminar flow hood on sterile paper towels. In total, 20 feeder roots were plated per tree, 10 onto PARP media (Kannwischer and Mitchell, 1978) containing 0.4 g/L Switch (375 g cyprodinil/kg, 250g fludopxonil/kgl; Syngenta SA (Pty) Ltd, Halfway House, South Africa), and 10 onto PARP containing 0.8ml/L Benomyl (500g benlate/kg; Villa Crop Protection, Kempton Park, South Africa) as described Tambong *et al*. (2006). The Benomyl solution was prepared by suspending 1.0 g of benomyl in 10 ml of 95% ethanol, and adding 0.8 ml of the suspension to 1 L of autoclaved PARP medium. The plated roots were incubated in the dark at 25 °C for 2 days, and hyphal growth that emerged from the roots was transferred to potato dextrose agar (PDA. Biolab Diagnostics, Midrand, South Africa) containing 0.04g/L streptomycin. Mycelia growing from the hyphal tipped isolates were scraped from the plates and transferred to 2.2 mL centrifuge tubes that were stored at -85°C until subsequent DNA extraction.

DNA extraction

DNA was extracted from the stored mycelia by first lysing the mycelia by adding 0.5g glass beads (2 mm) to each 2.2 mL tube, to which 1 mL of CTAB extraction buffer was added (Lee

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and Taylor, 1990). The tubes were shaken for 5 min. in a mixer miller (Retsch® MM301, GmbH and Co., Haan, Germany) at maximum speed. DNA was isolated from the lysed cells as described by Lee and Taylor (1990).

Polymerase chain reaction (PCR).

The Internal Transcribed Spacer (ITS) region was amplified from oomycete DNA using primers ITS4 (White *et al*., 1990) and ITS 6 (Cooke and Duncan, 1997). The PCR reaction contained 1× PCR buffer (Bioline, London, United Kingdom), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.65 U BIOTAQ (Bioline), 0.05 mg/ml bovine serum albumin (BSA) Fraction V (Roche Diagnostics South Africa, Randburg, South Africa) and 0.2 mM of each primer in a total reaction volume of 40 µl. Amplification was performed in a 2720 Applied Biosystems (Foster City, CA) thermal cycler. PCR amplification conditions consisted of an initial denaturation of 5 min at 94 °C, followed by 32 cycles of 30 s at 94 °C, 30 s at 55 °C, 90s at 72 °C and a final extension of 7 min at 72 °C. The amplified products were run on a 1% agarose gel containing ethidium bromide and were visualized under UV illumination.

Polymerase chain reaction Restriction Fragment Length polymorphisms (PCR-RFLP) *analyses*

The oomycetes isolates were identified to the species level using a PCR-RFLP approach (Mazzola *et al*., 2009). Restriction digest reactions consisted of 1.25 µl of each restriction enzymes *Hinf*I and *Hha*I (Thermo Scientific., Glen Burnie, Maryland, USA), 2.5 µl Tango buffer and 8 µl of PCR product in 20 µl reaction volumes.The reactions were incubated overnight at 37°C. Reactions, 10 µl per lane, were run on a 3% agarose gel along with 100bp DNA ladder. The PCR-RFLP products from isolates obtained from a specific nursery were run on the same agarose gels to ensure selection of isolates that represented similar PCR-RFLP groups within the nursery. One isolate representative of each PCR-RFLP group within a nursery was selected for sequencing. Analyses or 2013 and 2014 were conducted separately.

Sequencing

The ITS PCR products of selected isolates were purified using a MSB® Spin PCRapace (Invitek, Berlin, Germany) kit according to manufacturer's instruction. Direct sequencing was conducted for most isolates using the ITS4 and ITS 6 primers. For a few isolates, good sequencing results could not be obtained, and the PCR products of these isolates were cloned using the InsTAclone PCR cloning kit (Thermo Scientific) according to the manufacturer's instruction. Two clones per cloning reaction were chosen for sequencing. Samples were sequenced by the Central Analytical Sequencing Facility at Stellenbosch University using an

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ABI 3130XL Genetic Analyzer. The sequences were identified using BLAST analyses in GenBank and the *Phytophthora* database (http://phytophthora-id.org/seq-id.html). Identifications were made by selecting sequences from published articles (Levesque and De Cock, 2004; McLeod *et al*., 2009; Robideau *et al*., 2011) that had at least 99% similarity to the submitted sequence in Genbank.

qPCR quantification of ARD pathogens from nursery trees

DNA extraction from roots

Fine feeder roots were dissected from each nursery tree and stored at -20˚C until DNA extraction. For the 2013 samples, the roots of the five trees sampled per block in each nursery were pooled, whereas in 2014, the roots of each tree were analysed separately. DNA extraction from the lyophilized roots, and the spiking of DNA extraction buffer with an internal control plasmid containing a mutated *E. coli* gene were conducted as described in Chapter 2.

qPCR quantification

Oomycete ARD pathogens (*P. irregulare, Pht. vexans, P. sylvaticum, P. ultimum* and the genus *Phytophthora*) were quantified from the extracted DNA as described in Chapter 2. The genus *Cylindrocarpon* was quantified using published SYBR® Green assays (Tewoldemedhin *et al*., 2011c), and the KAPA SYBR FAST qPCR MASTER MIX (Lasec SA, Cape Town, South Africa) in 20 µl reactions. For all qPCR assays, the root DNA was diluted 1:5, and 2 µl of DNA was used in each reaction. Each sample was analysed in duplicate. Melt curve analyses were conducted at the end of each run to confirm the identity of the amplified products. This was done by heating samples at 95 °C for 10 s, 65 °C for 15 s, and then heating to 95 °C at a ramp rate of 0.1 °C/s, with continuous acquisition. A standard curve was conducted from ten-fold serial dilutions of genomic DNA, yielding concentration from 1 ng to 1 fg, with three replicates per concentration.

Pathogen DNA biomass was expressed relative to the internal spiked plasmid containing the mutated *E. coli* gene, i.e. relative pathogen DNA biomass. The relative amount of pathogen DNA biomass was calculated by first determining the ratio of the qPCR quantified pathogen DNA biomass (absolute) to the copy number of mutated *E. coli* gene (i.e. pathogen DNA biomass/*E. coli* mutated gene copy number). The ratio was then multiplied by 10^9 copies (amount of *E. coli* gene copies in each DNA extraction) and then divided by 20mg (amout of root DNA used in each extraction).

Nematode isolation and identification in nurseries

For nematode analyses, roots remaining after oomycete isolation and DNA analyses were sent to Nemlab cc. (Klapmuts, South Africa) for nematode identification and quantification.

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Nematodes were extracted using the centrifugal sugar flotation method (Jenkins, 1964), and parasitic nematodes were identified to the genus level.

Statistical analyses of oomycete isolation and qPCR rootstock data

Analyses of variance and all other analyses were conducted in Statistica 12. Post hoc testing (Students least significant difference test) was conducted at the 95% confidence level. The rootstock data (M7, M109 and M793) that were subjected to ANOVA and post hoc testing when relevant included (i) the amount of relative pathogen DNA biomass (*P. irregulare, P. ultimum* and *Cylindrocarpon*-like spp.) quantified in qPCR assay, (ii) number of *Pratylenchus* and (iii) percentage roots infected with *P. irregulare.* The average of the blocks for each year were taken as replicates.

Survey of irrigation water for the presence of oomycetes

Dams and orchards sampled

Irrigation water was examined for the presence of oomycetes on 14 farms, six farms from the Kouebokkeveld region and eight farms from the Grabouw/Elgin region (Fig. 1). The irrigation water was sampled using baiting techniques at two points on a farm in real time (see "Oomycete baiting from dams and orchards" sections below) that included the (i) sprinkler/drip irrigation pipe within the orchard and (ii) the dam from which the sprinkler irrigation received its water. This represented the water being baited before and after the water had passed through the filter system of the farm. For a few orchards more than one dam was monitored through baiting since during the season irrigation water was sourced from more than one dam for the orchard. The sampling through baiting included eight dams from the Kouebokkeveld region (D1, D2A, D3, D4, D5, D6, D7 and D8) and eight dams from Grabouw/Elgin region (D9, D10, D11, D12, D13, D14, D15, D16). The orchards that were sampled through baiting included six from the Kouebokkeveld region (O1, O2-3, O4, O5-6, O7, O8) and seven from Grabouw/Elgin region (O10, O11, O12, O13, O14, O15, O16). Sampling through baiting of the dams was conducted on a monthly basis from December 2013 to April 2014 and from December 2014 to April 2015. The sampling time period thus ran from approximately one month prior to initiation of orchard irrigation until almost the end of the irrigation season. Sprinkler/drip irrigation sampling was conducted at the same time as the dam sampling, except that it started in January 2014 (O11, O16,) and ended in April 2014.

Oomycete baiting from dams

The dams were sampled by using a home-made plastic basket (28 cm x 20 cm x 15 cm) that were fastened together with tiebacks. The basket was kept afloat by securing two Styrofoam pieces (23 cm x 6 cm x 1.5 cm) to the longest sides of the basket (Fig 2). Inside the basket

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oomycete baits were placed that consisted of six lemon leaf disks (2.5 cm) in 2013/14, and in 2014/15 sampling three lemon and three avocado leaf disks were used. Each basket also contained two 'Packham' pears and one 'Granny Smith' apples. For each dam, one basket was thrown into the dam and secured with a rope to a fixed feature outside the dam. The baskets were left for 4 days in the dams, after which the baskets were collected and transported to the laboratory for further analyses.

Oomycete baiting from sprinkler/drip irrigation water in orchards

Water from the dripper/sprinkler line was sampled within orchards using a plastic baiting container (13 cm dia. x 16 cm height) designed by Citrus Research International in South Africa. Several small holes (4 mm) were drilled just below (about 2.5 cm) the rim of the container to allow overflow of water that entered the container. Each container was closed with a lid that contained a hole (15 mm) into which a dripper line was secured. The dripper line was then connected to the irrigation pipe within orchards (Fig. 3). The oomycete baits that were placed into each container consisted of six lemon leaf disks (25 mm diam) in 2013/14 and in 2014/15 four lemon and four avocado disks (25 mm diam). Each container also contained two 'Packham' pears. In each orchard, one container was placed per sampling period for 4 days, after which the containers were collected for analyses of the baits.

Oomycete isolation and identification from baits (leaf disks and fruits)

For each sampling period, three leaf disks (25 mm diam) from each dam and orchard were rinsed in distilled water for 5 seconds and dried in a laminar flow. The disks were cut in half, and one half of each disk was stored in 2 ml centrifuge tubes for qPCR analyses (see next section), and the other half was plated onto PARP plus Switch selective media as described for the nursery analyses.

Pear and apple fruits that exhibited lesions were dipped in 70% ethanol for 5 seconds and dried in a laminar flow. Fruits that did not exhibit lesions at the time of collection, were incubated in brown paper bags for one week, and isolations were made from lesions if these developed. Small sections from the edge of lesions were plated onto PARP containing Switch selective media.

All isolation plates were incubated in the dark for 2-3 days, after which hyphal growth that emerged from the plated plant material were transferred to PDA containing 0.04 g/L streptomycin. The isolated oomycetes were identified to the species level as described for the nursery samples using PCR-RFLP analyses followed by sequencing of a subset of the isolates.

Molecular identification of known ARD oomycete pathogens from leaf disk baits

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qPCR was conducted for the same oomycete ARD pathogens (*P. ultimum, P. sylvaticum, P. irregulare, Pht. vexans* and the genus *Phytophthora*) that were quantified in the nursery samples. The stored leaf disks were lyophilized and powdered by using scissors and adding glass beads into 2 ml tubes and shaken in a Retsch® mill at maximum speed for 10 minutes. DNA was extracted from 20 mg of powdered leaf disks using the NucleoSpin PLANT II kit (Macherey-Nagel GmbH and Ko, Duren, Germany). The same internal control plasmid used in the nursery root samples was also used to spike the DNA extraction buffer as described in Chapter 2. The internal control was used to check for inhibition in qPCR reactions. For the leaf disk qPCRs, DNA extracted were diluted 1:5, and 2 µl of DNA was added to each 20 µl qPCR reaction. qPCR and amplification conditions were as described for the nursery samples.

Statistical analyses of irrigation water sampling

Correspondence analysis (CA) were conducted with XLStat (Version 2014, Addinsoft, New York, USA). The presence and absence of each oomycete species during each sampling period in each of the dams were used as input data where 0 indicated absence and 1 indicated presence. The analyses were used to visualize the relationships between dams and oomycetes species identified over the five month sampling period. Analyses for the 2013/14 and 2014/15 sampling periods were conducted separately.

RESULTS

Identifying oomycetes from nursery root samples through isolations

Oomycetes within the genus *Pythium* were identified from all the nurseries, but no *Phytophthora* isolates were recovered in any of the years. In order to reduce problems associated with the isolation of Zygomycete fungi, which also lack cross cell walls similar to oomycetes, Benomyl or Switch were included in the PARP medium. A slightly higher percentage of the oomycete isolates were obtained from the Benomyl medium than from Switch medium. In 2013 and 2014, 54.67% and 52.7 % of the isolates were obtained from Benomyl containing PARP, whereas Switch containing media yielded 45.33% and 47.3% of the oomycetes isolates, respectively. The Benomyl containing PARP medium only yielded a few Zygomycete isolates, whereas the Switch containing PARP yielded no Zygomycete isolates.

Two highly virulent ARD *Pythium* species, *P. irregulare* and *P. ultimum,* were identified in the nurseries. *Pythium irregulare* was the most frequently identified pathogenic species and occurred in all of the nurseries in both years (Table 1). A high percentage of the blocks contained *P. irregulare* in 2013 (85%) and in 2014 (89%). Within the blocks a high percentage of trees often contained this pathogen with 49% of the blocks containing 60% or higher

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infected trees across the two sampling years. *Pythium ultimum* was only identified in two nurseries. In nursery 1, the pathogen was detected in both years, but more frequently in 2014 than 2013, and in nursery 2 it was only recovered from one block in 2013 (Table 1).

The only known moderately virulent *Pythium* species in the ARD pathogen complex that was identified belonged to *Pythium* spp. complex B2A, but the occurrence was low (Table 1). *Pythium* spp. complex B2A is a species complex that includes several species (*P. dissotocum, P. coloratum, P. lutarium, P. marinum, P. diclinum, P.* aff. *dictyosporum*, *Pythium* sp. group F and P. sp. '*tumidum*') that cannot be differentiated based on ITS sequence data (Robideau *et al*., 2011). For the purpose of this study, since *P. dissotocum* that is known as a moderately virulent ARD pathogen (Tewoldemedhin *et al*., 2011b) and is included in *Pythium* spp. complex B2A, it is assumed that this group may be of importance when detected in nurseries. However, it is possible that the isolates identified in nursery 4, are not pathogenic and pathogenicity studies would be required to confirm this. *Pythium* spp. complex B2A was only detected in one nursery (nursery 4) in 2013, where it was widely distributed and occurred in all four blocks, although in most blocks a low percentage of trees (≤ 20%) were infected (Table 1).

Considering highly virulent and moderately virulent species, most nurseries contained a high percentage of infected trees. Nursery 5 contained the lowest percentage of infected trees across the two sampling years. Nursery 4 contained more than 55% infected trees in both years (Table 1).

Only two *Pythium* species were identified in the nurseries that are not known pathogens of apple. *Pythium tolurosum* and *Pythium spinosum* were only recovered in 2013 from one block in nursery 1.

A total of seven rootstocks were sampled over the two years. Rootstocks that were sampled most frequently were MM109, MM793 and M7. The remaining rootstocks that included G78, CG228, GC22 and M9, were each only sampled once over the two year period in one nursery. Due to the limited analyses of the latter four rootstocks, conclusions could not be made as to whether specific pathogens were associated with a specific rootstock. *Pythium irregulare* occurred frequently enough to allow statistical analyses of the percentage roots infection per rootstock block sampled. The analyses showed that there were no significant differences (P = 0.42) between the three most frequently surveyed rootstocks (MM109, MM793 and M7) in percentage roots infected, although rootstock MM109 tended to have the lowest percentage root infection (data not shown). Considering all potential pathogenic *Pythium* spp., the percentage of trees infected for the three rootstocks were comparable (47-52%) when considering both sampling years (Table 2).

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qPCR quantification of ARD pathogens from nursery trees

The sensitivity of qPCR assays for detection of the different ARD pathogens differed. The most sensitive assay was for *P. irregulare* (1 fg/ μL) followed by *P. ultimum* (10 fg/ μL), *Ph. cactorum* (100 fg/ μL) and *P. sylvaticum* (3200 fg/ μL) (Chapter 2). The efficiencies, M slope and linearity of the standard curves for the aforementioned pathogens were within acceptable limits (Chapter 2). The limit of quantification was 10 fg/ μL for the *Cylindrocarpon*-like spp. assay. The efficiency was 0.98 for the *Cylindrocarpon*-like spp. assay, and the M slope (M = -3.35) and linearity (R = 0.99) of the standard curve were within acceptable limits.

A few of the oomycete pathogens that were investigated were absent or only present in very few samples based on qPCR analyses. Similar to the isolation studies, *Phytphthora* spp. and *Pht. vexans* were not detected in any of the nursery root samples. *Pythium sylvaticum* was not found in the isolation studies, but was detected by qPCR in one block of nursery 3 in 2014, where 100% of the trees were contaminated (Table 3).

Similar to the isolation studies, *P. irregulare* was also the most frequently detected oomycete species by qPCR analyses (Table 3). The pathogen occurred in all nurseries and all blocks. In 2014, where trees were analysed individually as opposed to the pooled DNA analyses of the five trees per block in 2013, almost 100% of the trees within a block were infected, except in nursery 5 where three blocks had 40-80% of infected trees. The amount of relative *P. irregulare* DNA biomass detected in 2013 was higher than in 2014 in 67% of the total blocks sampled during the two years. Within a given year, nurseries 1, 2 and 5 had comparable *P. irregulare* DNA biomass (0.025 – 0.037 ng/mg in 2013 and 0.001 – 0.060 ng/mg in 2014), whereas nursery 3 had the lowest biomass (0.007 – 0.009 ng/mg) and nursery 4 the highest (0.197 ng/mg in 2013 and 0.030 ng/mg in 2014) (Table 3).

Pythium ultimum was detected more frequently by qPCR analyses than by direct isolation from roots where the pathogen was only determined to be present in two nurseries. qPCR analyses showed that *P. ultimum* was present in the same three nurseries (1,2 and 3) in both years, and in almost all blocks within these nurseries, except for nursery 3 where it was only present in two of the surveyed blocks. Single tree analyses within blocks in 2014 showed that a relatively high percentage of trees were infected within blocks ranging from 42% to 100%. The amount of *P. ultimum* DNA biomass was higher in 70% of the blocks in 2014 compared to 2013. In both years, the quantity of *P. ultimum* DNA biomass detected was lowest in nursery 3(0.003 – 0.051 ng/mg) and highest in nursery 2 (1.010 – 2.351 ng/mg) (Table 3). Based upon results from isolation studies, nursery 1 would have had the highest contamination, followed by nursery 2. No *P. ultimum* was detected in isolation studies in nursery 3 (Table 1).

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Cylindrocarpon-like spp. were present in all nurseries and blocks in both years (Table 3). The average DNA biomass within a nursery was much higher in 2014 (2.355 - 10.720 ng/mg) than in 2013 (0.029 - 1.697 ng/mg). Nursery 1 had among the highest DNA biomass in both years, whereas nursery 2 had the lowest. Nursery 5 had the highest *Cylindrocarpon*-like spp. DNA biomass in 2014, but the lowest in 2013 (Table 3).

The association of ARD pathogens with specific rootstocks were investigated for those species that occurred most frequently in rootstocks M7, M109 and M793. The three rootstocks did not differ significantly ($P = 0.37$) in quantity of *P. irregulare* DNA, similar to results from the isolation studies. The percentage of trees infected for each rootstock was high (88-100%) in 2014 where individual trees were tested for all three rootstocks (data not shown). There were also no significant difference ($P = 0.66$) in the *P. ultimum* pathogen DNA biomass for the three rootstocks. The percentage of trees infected in 2014 was highest for rootstock M109 (60%), followed by M7 (40%) and M793 (32%). For *Cylindrocarpon*-like spp., there was a significant effect for rootstock but only at the 90% confidence level. Rootstock M109 contained significantly higher *Cylindrocarpon*-like spp. DNA than rootstocks M7 and M793 (data not shown). In 2014, all trees of all three rootstocks were infected by *Cylindrocarpon*-like spp.

Nematode isolation and identification in nurseries

The relative lesion nematode infestation detected in 5 grams of apple tree roots differed among the nurseries sampled (Table 4). Nursery 1 and 3 had very low *Pratylenchus* spp. infestation levels in both sampling years, as indicated by the number of *Pratylenchus* (0 to 5 /5 g roots) and percentage infested trees (0 to 27%). These nurseries further contained blocks where *Pratylenchus* spp. were not detected, and there were also no trees with more than 100 *Pratylenchus* spp./ 5g roots. In contrast, nursery 5 had very high *Pratylenchus* infestations in all blocks in both years. The high infestation was evident from the number of *Pratylenchus* spp. (71 to 495 *Pratylenchus*/5 g roots), percentage infested trees (60-95%) and trees with more than 100 *Pratylenchus* spp./5 g roots (30 to 50%). In 2013 this nursery even contained 40% of its trees with more than 300 *Pratylenchus* spp./5g roots. Nursery 2 and 4 also had alarmingly high *Pratylenchus* spp. in 2013, since the nurseries contained 20-40% trees with more than 100 *Pratylenchus* spp./5g roots and 10-20% trees with more than 300 *Pratylenchus* spp./5 g roots (Table 4). The number of *Pratylenchus* spp./ 5 g roots, were significantly ($P = 0.02$) higher in 2013 than in 2014.

The incidence of *Pratylenchus* spp. in rootstocks was investigated for M109, M7 and M793 since these rootstocks were sampled frequently enough. Although there were some trends in infestation levels per year and rootstock, there were no significant differences ($P > 0.31$) between rootstocks in the number of *Pratylenchus* per 5g of roots, percentage trees infested and percentage trees with more than 100 or 300 *Pratylenchus* spp./5 g roots.

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Irrigation water sampling

Dam oomycete composition and characterization

Isolation studies and species identification

During the two sampling periods, a total of 325 isolates were obtained from the baits, with the highest number of isolates (47) obtained in the March 2014/15 sampling. In the 2013/14 surveys, only lemon leaf disks were used as baits, whereas in 2014/15, lemon and avocado leaf disks were used. The lemon and avocado leaf disks yielded a comparable number of oomycete isolates; 52.1% of the isolates were from lemon disks and 47.9% from avocado disks. Some species such as *Ph. lacustris*, were only isolated using pear or apple fruits. All the *Pythium* species and the other three *Phytophthora* species (*Ph. cinnamomi, P. parvispora* and *Ph. macrochlamydospora*) identified in dams were isolated from leaf disks and pear fruit.

Identification of the isolates to the species level through sequence analyses was possible for most of the isolates. However, 7% of the *Pythium* isolates had less than 99% similarity to species in Genbank and more than 12 base pair mismatches, and were therefore designated as *Pythium* spp. Several of the isolates (2.8%) belonged to *Pythium* spp. complex B2A (Robideau *et al*., 2011). As discussed above, this complex contains several species that cannot be differentiated based on ITS sequences, including *P. dissotocum* (Levesque and De Cock, 2004; Robideau *et al*., 2011) that is a known ARD pathogen. A few isolates could not be identified to the species level as their ITS sequences were highly similar to more than one species. These included isolates that were identified as *P. cantenulatum* or *P. rhizo-oryzae*, *P. aqatile* or *P. sukuiense* and *P. myriotylum* or *P. zingiberis*.

A total of eight *Pythium, two Phytopythium* and four *Phytophthora* species were identified over the two sampling periods. The *Pythium* spp. included *Pythium cantenulatum/rhizo-oryzae, Pythium myriotylum* or *Pythium zingiberis*, *Pythium apleroticum*, *Pythium* spp. complex B2A, *Pythium oopapillum*, *Pythium aqatile* or *Pythium sukuiense*, *Pythium undulatum* and *Pythium rostratifingenes*. *Phytopythium* spp. included *Phytopythium helicoides* and *Phytopythium litorale*. The *Phytophthora* spp. included *Phytophthora macrochlamydospora* and *Phytophthora parvispora*, *Phytophthora lacustris* and *Ph. cinnamomi*. Of these species, potential replant ARD pathogens included *Pythium* spp. complex B2A (moderately virulent) and *Pht. litorale* (weakly virulent). No highly virulent pathogens were isolated.

qPCR detection of specific species

qPCR analyses for known ARD oomycete pathogens (*Pht. vexans, P. irregulare, P. sylvaticum, P. ultimum* and the genus *Phytophthora*) in the leaf disk baits obtained from dams showed that *P. sylvaticum* and *Pht. vexans* were not detected in any of the dams in the two

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sampling periods. *Pythium irregulare* was detected in more than 62% of the dams. The qPCR analysis indicated the presence of *Phytophthora* spp. in more than 14.2% of dams in both sampling periods (Fig.4).

Monthly variation in prevalent oomycete species based on isolation and qPCR data

The fluctuation in abundance of specific oomycete species during the five month sampling period was plotted for the 2013/14 and 2014/15 samplings. Only species that occurred eight or more times during both sampling periods were investigated since the remaining species occurred too infrequently over the sampling periods to make any conclusions on monthly fluctuations. The species that were identified as prevalent included *P. irregulare* (only detected with qPCR analyses), *Phytophthora* spp. (only detected with qPCR analyses), *Pht. helicoides, P. cantenulatum/rhizo-oryzae, Pythium* spp. complex B2A, *Pythium oopapilum, Pythium* spp. and *P. undulatum* (Fig. 4). In both sampling years, the groups that occurred most frequently in dams were *P. irregulare* followed by *Pht. helicoides, Phytophthora* spp. and *Pythium* spp. For each of the species separately, most were detected in the same percentage of dams over the five months, although there was a tendency for *Pht. helicoides* to decrease in April. Species that were detected less frequently but at both sampling periods included *P. undulatum, P. cantenulatum/rhizo-oryzae*, *Pythium* spp. complex B2A and *P. oopapilum* (Fig. 4).

Association of oomycete species with specific dams and association of dams with each other

Correspondence analyses were used to investigate the association of species that were identified through qPCR analyses and isolation studies with dams. The total variation explained by the first two dimensions in the correspondence analysis of dams and oomycetes species during the 2013/14 sampling period was 37.94 %, whereas the total variation explained during the 2014/15 sampling period was 51.33 %. The closer the distance between the species and the dam, the stronger the relationship between the two. Furthermore, dams that are situated close to each other in a CA map / plot contained more similar species composition, for example in the 2013/14 sampling dams 1,9,15,14 are similar and were associated with species *P. myriotylum/P. zingiberis, P. undulatum* and *P. cantenulatum/P. rhizo-oryzae.* In the 2013/14 sampling period, *P. irregualre, Pht. helicoides, Phytophthora* spp. and *Pythium* spp. were the species that were associated with the largest number of dams (D5, D11, D6, D16, D12, D13 and D4). *Phytopythium litorale* and *Ph. lacustris* were only associated with single dams (Fig. 5A) and did not occur frequently over the sampling period. Since dams from Grabouw and the Kouebokkeveld co-occurred in the CA map in both sampling periods, the species composition in each region was not distinct and determined by the region (Fig. 5). In the 2014/15 sampling period, most of the dams (D10, D12, D15, D3, D4, D7, D8, D14, D16

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and D9) were similar in composition of the population surveyed since they contained most of the species (*P. apleroticum, P. oopapillum, P. aquatile/P. sukuiense, Pythium* spp. complex B2A, *P. irregulare, P. cantenulatum/P. rhizo-oryzae* and *Pht. helicoides. Phytophthora macrochlamydospora)* and *Phytophothora parvispora* was only associated with dam 11, and *Ph. cinnamomi* with dam 6 (Fig. 5B). *Phytophthora* spp. were associated with fewer dams (D13 and D5) in the 2014/15 sampling than in the 2013/14 sampling (Fig. 5). Altogether it is clear that the grouping of dams depended on the combination of species detected, and that the composition of species were not similar between the two sampling periods (2013/14 and 2014/15), which is evident from the different groupings of dams during the two sampling periods as shown by the CA plots (Fig.5). The dams from the Kouebokkeveld were also not more similar to each other, but were dispersed with dams from Grabouw/Elgin regions.

Orchard irrigation water oomycete species composition and characterization

Isolation studies and species identification

In orchard irrigation water samplings the leaf disk baits that were analysed through isolation studies yielded a total of 120 *Pythium* and *Phytopythium* isolates over the two sampling periods that represented seven different species (*Pht. helicoides, Pht. litorale*, *P. apleroticum*, *P. cantenulatum/P. rhizo-oryzae*, *Pythium* spp. complex B2A, *P. oopapillum* and *P. undulatum*). In the 2014/15 season when avocado and lemon disks were used in baitings, 46.5% and 53.5% of the isolates were obtained from avocado and lemon disks respectively. No *Phytopthora* spp. isolates were obtained.

qPCR detection of specific species

qPCR analyses for known ARD oomycete pathogens in the leaf disks obtained at irrigation lines within orchards showed that *P. sylvaticum* and *Pht. vexans* were not detected in any of the orchards' s irrigation water in the two sampling periods. *Pythium ultimum* was detected only once in an orchard in January 2015. *Phytophthora* spp. were not detected that frequently within dams (Fig. 5). Similar than the dam water, the within orchard water analyses showed that *P. irregulare* was the most prevalent species.

Monthly variation in prevalent oomycete species based on isolation and qPCR data

The species that occurred more frequently than others in isolation and qPCR studies included *P. irregulare, P. cantenulatum/P. rhizo-oryzae, Pythiym spp*., *Pht. helicoides.* Other species include *Pythium* spp. complex B2A, *P. oopapillum, P. undulatum, Phytophthora* spp., *P. apleroticum*, *P. ultimun* and and *Pht. litorale* were less occured. Aside from *P. irregulare*, the aforementioned species did not occur in irrigation water from most of the orchards. Less than

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30% of the orchards in each of the sampling months contained each of these specific species (Fig. 6). It was thus difficult to observe clear trends in the monthly fluctuation of these species.

The percentage of within orchard irrigation water that contained *P. irregulare* were comparable in 2013/14 and 2014/15, but the trends over the five month sampling period differed. In 2013/14 the percentage orchard irrigation remained between 70-80% from December to March, but declined to less than 50% in March. In 2014/15 the percentage orchard irrigation remained high from December to February (>60%), then decreased slightly in March and increased again to about 60% in April (Fig. 6).

Association of oomycete species with irrigation water of specific orchards

Correspondence analyses were used to also investigate the association of species that were identified through qPCR analyses and isolation studies with irrigation water sampled at irrigation pipes within orchards. The total variation explained by the first two dimensions in the correspondence analysis of the orchard irrigation water was similar for 2013/14 (52.86%) and 2014/15 (55.36%) (Fig. 7). This was similar to the results from the dams in the 2014/15 sampling.

In both sampling years, some species were only associated with the irrigation water of one or two orchards. In the 2013/14 sampling, *P. undulatum* was only associated with orchard 12, whereas *P. apleroticum* was only associated with orchard 7 (Fig. 7 A). In the 2014/15 sampling *P. undulatum* was associated with two orchards (O14 and O8), and in 2013/14 the species was only associated with one orchard which was a different orchard than the two 2014/15 orchards. Species *P.cantenulatum/P. rhizo-oryzae* was only associated with orchard water 4 and 1, and species *Pythium* spp. and *Pht. litorale* only with orchard 13 (Fig. 7 B).

The irrigation water within the orchards from the Kouebokkeveld were not more similar to each other, than to orchard water from the Grabouw/Elgin orchards, since the orchard water from the two regions were interspersed in the CA plots in both sampling years (Fig. 7). In the 2013/14 samplings, irrigation water from orchards 11, 15 and 16 were similar and associated with *P. irregulare*, whereas the remaining orchard water samples had similar species composition containing *Pht. helicoides*, *Phytophthora spp.*, *P.cantenulatum/P. rhizo-oryzae*, *Pythium* spp. and *P. oopapillum* (Fig. 7 A). In the 2014/15 sampling the orchard samples formed two large groups with O5-6, 7, 11, 12 and 16 being similar and associated with *Pht. helicoides* and *P. irregulare*. The second group consisted of orchards O1, O2- 3, 10, 13 and 15 that were associated with *Pythium* spp. complex B2A and *Phytophthora* spp. (Fig. 7B).

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DISCUSSION

Two approaches were used for identifying oomycete ARD pathogens in apple nurseries, which included isolation studies and qPCR analyses of selected species known to be important in ARD in South Africa. Considering the results of both approaches, apple nurseries are an inoculum source of ARD oomycete pathogens, since the ARD pathogens *P. irregulare, P. ultimum, P. sylvaticum* and possibly *P. dissotocum* belonging to *Pythium* spp. complex B2A, were identified in nurseries. The qPCR assays identified a higher percentage of infected trees than isolation studies. This was expected since in general isolation techniques are known to be less effective than PCR assays (Thorn *et al*., 1996; Goud and Termorshuizen, 2003). Since the other known oomycete pathogens occurring in South Africa, including *Pythium heterothallicum* and *P. folliculosum* were not identified in the nursery surveys, future investigations into oomycete inoculum sources in nursery rootstocks can only use the qPCR assays employed in the current study (*Pht. vexans, P. irregulare, P. sylvaticum, P. ultimum* and the genus *Phytophthora*), without using time consuming isolation studies. An additional qPCR for *P. dissotocum* may be useful, but this will be difficult to design and interpret due to the taxonomy of the *Pythium* spp. complex 2BA to which *P. dissotocum* belongs still being in flux (Robideau *et al*., 2011). Furthermore, the *Pythium* spp. group B2A complex was not frequently identified in nurseries, only in 20% of nurseries, 10% of nursery blocks and 3% of trees.

Isolation and qPCR analyses of rootstocks frequently identified *P. irregulare* in the rootstocks. Isolation studies showed that *P. irregulare* was the most frequently isolated species with 47.6% of trees, 84.66% of blocks and all nurseries being infected with this species. The qPCR analyses identified an even higher incidence of this species since all blocks in the nurseries were infected and a total of 95% of trees. The prevalence of *P. irregulare* in nursery rootstocks in South Africa, could thus perhaps explain why all of the investigated ARD orchards, with the exception of one, have thus far been found to be infected with *P. irregulare* (Tewoldemedhin *et al*., 2011 a, b).

Pythium ultimum was identified as the second most frequently occurring ARD pathogen in nurseries. Based on qPCR assays *P. ultimum* was detected in 60% of nurseries, 55% of blocks and 41% of trees, while isolation studies only identified the pathogen in 40% of nurseries, 12.5% of nursery blocks and 6.5% of trees. The relative high occurrence of the pathogen in nursery trees (41%) may also be indicative that rootstocks are a source of inoculum, since *P. ultimum* has been identified in all investigated ARD orchards using qPCR analyses (Tewoldemedhin *et al*., 2011a), although investigations into additional orchards using only isolation studies could not identify the pathogen in any of the orchards (Tewoldemedhin *et al*., 2011b). *Pythium ultimum* thus seems to be difficult to isolate using

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PARP or PARP and Switch ® media, and qPCR analyses must be used to identify the pathogen from roots.

In South Africa, apple nurseries are not inoculum sources of *Ph. cactorum* and *Pht. vexans,* since these species were not identified through isolation or qPCR analyses*. Pythium sylvaticum,* is also not an important pathogen associated with nursery material since it was only identified in one nursery and one block, and only using qPCR analyses. Previously in ARD orchards, *P. sylvaticum* was also not frequently identified an occurred in less than 50% of orchards (Tewoldemedhin *et al*., 2011a,b). It was somewhat unexpected to not detect *Pht. vexans* in any of the analysed rootstocks, since this species have frequently been reported from soils of fruit crops including apples and grapevines in South Africa. In vineyards, Spies *et al*. (2011) isolated *Pht. vexans* from 16.7% of grapevine nurseries. In ARD orchards the pathogen was detected in all orchards using a very sensitive (1 fg/ μL) ITS based assay (Tewoldemedhin *et al.*, 2011a). In the current study, a newly developed qPCR assay was used based on a single copy gene that had a much lower sensitivity (1000 fg/ μL) (Chapter 2) than the ITS assay. The lack of detection of *Pht. vexans* in nurseries could thus be due to the low sensitivity of the newly developed qPCR, but this would have to be confirmed in future studies using a more sensitive qPCR assay.

It was surprising that almost no non-pathogenic *Pythium* species were associated with nursery rootstocks. *Pythium spinosum* and *Pythium tolurosum* infected rootstocks were found in only one nursery at a low frequency. In contrast, other studies from apple roots have identified several non-pathogenic species including *Pythium minus* and *Pythium attrantheridium* (Tewoldemedhin *et al*., 2011b), *Pythium rostratifingens* (Mazzola and brown, 2010), *Pythium aphanidermatum*, *Pythium debaryanum*, *Pythium flevoense*, *Pythium iwayamae*, *Pythium macrosporum* and *P. torulosum* (Mazzola *et al*., 2002).

Aside from oomycete pathogens, fungal isolates within the genera *Cylindrocarpon* and *Rhizoctonia* are also important to investigate as nursery inoculum sources of ARD. In the current study, only *Cylindrocarpon* was investigated using qPCR analyses. *Cylindrocarpon*-like spp. was quantified consistently from all nurseries and all analysed trees in both sampling years. This could indicate that nursery trees are also an inoculum source of these pathogens in ARD roots, since members of this group have previously also been identified in all investigated ARD orchards (Tewoldemedhin *et al*., 2011b). The identification to species level of isolates obtained in isolation studies using the latest taxonomic schemes may be helpful in investigating this hypothesis.

Pratylenchus spp. were identified as a very important source of inoculum in apple nursery material, more so in 2013 than in the 2014 samplings. Distribution of *Pratylenchus* spp. varied across nurseries and within blocks. In 2013 all nurseries, 75% of nursery blocks

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and 39% of trees contained *Pratylenchus* spp., with trees from all nurseries having an average of 186.4 *Praytlenchus* spp./5g roots. In 2014, one nursery did not contain infested rootstocks trees, but otherwise the average percentages blocks (55%) and trees (29%) infested were comparable to the 2013 season. However, the average number *Pratylenchus* spp./5g roots was much lower (17.16) in 2014 than in 2014. This resulted in the percentage of infested trees containing more than 100 *Pratylenchus* spp./5g roots being much higher in 2013 (22%) than in 2014 (6%). In general, 3 year old trees should not contain more than 300 *Pratylenchus* spp./5 g of roots, which would require application of nematicides at planting (personal communication S. Story). Since the analysed trees were only 1-2 years old, we considered it reasonable to designate 100 *Pratylenchus* spp./5 g roots as being harmful, requiring treatment, and the infestation level at which a rootstock can serve as a source of inoculum. The significance of trees containing less than this amount of *Pratylenchus* spp./5g roots as potential inoculum sources is unknown.

It is difficult to deduce what the significance is of the detected pathogen DNA biomasses in nursery rootstock roots, especially since ARD pathogens are known to act synergistically (Tewoldemedhin *et al*, 2011a). If considering the pathogen DNA biomass in a simplistic manner without considering synergistic interactions, it may be informative to compare DNA quantities from the current study to those reported in literature. This may especially be relevant for *P. sylvaticum*, *P. ultimum* and *Pht. vexans,* since under glasshouse conditions using single species inoculations, significant negative correlations have been found between increase in seedling length and pathogen DNA biomass in fine feeder roots, which is not true for *P. irregulare* and *Ph. cacotrum* (Chapter 2). Comparison of nursery *P. ultimum* pathogen DNA biomasses to *P. ultimum* DNA biomasses in the roots of apple seedlings reported in Chapter 2 for trial 2 that resulted in significant reductions in increase in length, showed that nursery rootstock roots contained much higher (3413 fg/ μL) DNA biomass than those of seedling roots (1810 fg/ μL). This could suggest that even though the pathogen cannot be isolated it is presented at potentially damaging amounts in nursery roots. The average amount of *P. sylvaticum* DNA biomass in the nursery roots was much lower (200 fg/ μL) than that of roots in the glasshouse trial (4320 fg/ μL) where disease was incited in seedlings by the pathogen. Although *P. irregulare* DNA biomass in fine feeder roots does not correlate with disease (Chapter 2), it was interesting to note that the average for nursery trees was much lower (501 fg/ μL) than that of glasshouse seedling roots (3770 fg/ μL). It will be important in future nursery surveys to quantify *P. irregulare* in pioneer roots in rootstocks, in addition to feeder roots, which could be more indicative of infection levels than analysing only feeder roots as was done in the current study. Unfortunately it is not possible to compare the DNA quantities obtained in the current study to those reported to occur in apple seedlings grown in

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artificially inoculated soils by Tewoldemedhin *et al*. (2011b), or in ARD soils by Tewoldemedhin *et al*. (2011a) and Emmett *et al*. (2014). Both of these studies expressed their DNA quantities as pathogen biomass/ng root DNA, whereas the current study expressed it as pathogen DNA biomass/mg roots. This is probably why for most pathogens a much higher DNA biomass was detected in nursery rootstock roots than for apple seedling roots reported in these studies. Standardization of the units in which DNA quantities is expressed will be useful in all published studies. The inclusion of an internal standard that is also indicative of DNA loss and qPCR inhibition, as was done in the current study, may also be useful for comparative purposes.

During irrigation water sampling from the Kouebokkeveld and Grabouw regions it became apparent that irrigation dams are less important as an inoculum source than nursery material when only isolation data from baits were considered. The species composition between dams in the two regions were not region specific based on correspondence analyses, and therefore results can be discussed as the average of the two regions. Although oomycetes were frequently identified throughout the five month sampling period in both seasons, ARD pathogens were rarely detected and only included *Pythium* spp. complex B2A and *Pht. litorale*, which occurred in a maximum of 25% and 12.5% dams within a specific sampling month and were not detected in several of the months over the two years. The remaining oomycete species detected in dams are not known ARD pathogens and include seven *Pythium* spp. (*P. cantenulatum/rhizo-oryzae, P. myriotylum* or *P. zingiberis*, *P. apleroticum*, *P. oopapillum*, *P. aqatile* or *P. sukuiense*, *P. undulatum* and *P. rostratifingenes*), four *Phytophthora* spp. (*Ph. macrochlamydospora*, *Ph. parvispora*, *Ph. parvispora* and *Ph. cinnamomi*) and one *Phytopythium* spp. (*Pht. helicoides*). Most of these species were reported previously from surface irrigation water in other regions of the world and include *Pht. helicoides* (Shrestha *et al*., 2013; Jones *et al*., 2014; Zappia *et al*., 2014), *P. catenulatum* (Sanchez and Gallego, 2001; Jones *et al*., 2014; Zappia *et al*., 2014), *P. myriotylum* (Jones *et al*., 2014; Zappia *et al*., 2014), *P. apleroticum* (Shrestha *et al*., 2013; Zappia *et al*., 2014). *Pht. litorale* (Shokes and McCarter, 1979; Parkunan and Ji, 2013; Shrestha *et al*., 2013; Zappia *et al*., 2014), *Pythium* spp. complex B2A (Shokes and McCarter, 1979; Parkunan and Ji, 2013; Zappia *et al*., 2014), *P. undulatum* (Shrestha *et al*., 2013; Zappia *et al*., 2014), *Ph. parvispora* (Zappia *et al*., 2014), *Ph. lacustris* (Shrestha *et al*., 2013; Jones *et al*., 2014; Zappia *et al*., 2014) and *Ph. cinnamomi* (Lauderdale and Jones, 1997; Wilson *et al*., 1998; Zappia *et al*., 2014). *Phytophthora cinnamomi* was the only pathogen from the current study that have previously been reported from surface water (rivers) in South Africa, in the Western Cape region (Von Broembsen, 1984). The current study reported for the first time the presence of *P. oopapillum*, *P. aqatile/ P. sukuiense*, *P. rostratifingenes* and *Ph. macrochlamydospora* from surface irrigation water. Among all the species that were isolated *Pht. helicoides* was most prevalent and found in all dams.

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qPCR analyses significantly improved the detection of ARD pathogens in dam water relative to isolation studies using leaf baits. The qPCR approach was beneficial since in isolation studies ARD pathogens can be overgrown by other oomycete spp. or pathogens can be killed by saprophytes (especially bacteria) during the 5 day baiting period. This is likely the case for *P. irregulare,* which could not be isolated from leaf disks, but for which the qPCR assays indicated that the pathogen was present in each of the sampling months in 62.5% to 87.5% of the dams. The presence of *P. irregulare* in surface water has been reported previously (Shokes and McCarter, 1979; Jones *et al*., 2014; Zappia *et al*., 2014). qPCR analyses also revealed the presence in each of the investigated months of *Phytophthora* spp. that were detected in 14.2% to 50% of the dams. However, whether the main *Phytophthora* species associated with ARD in South Africa, *Ph. cactorum,* was present is uncertain since amplifications yielded more than one melt curve. Since the identity of qPCR amplicons of the *Phytophthora* genus specific qPCR assay that was used in the study is long enough to identify amplicons to the species level through sequencing, the species can be determined but this was not pursued in the current study. There are *Phytophthora* species very closely related to *Ph. cactorum* that could have a similar melt curve than this species, and therefore sequencing of amplicons representative of the several different melt curves identified is required. Therefore, the identity of the *Phytophthora* spp. and their relevance to ARD in dams remains unknown.

In orchard irrigation water, a few ARD pathogens were identified using qPCR and isolations, and included *P. irregulare, Pythium* spp. complex B2A, *Pht. litorale* and possibly *Phytophthora*. The problem with detecting *Phytophthora* only through qPCR in orchards is similar to that experienced in dams, and the significance of this detection thus remains unknown. Similar to dams, *P. irregulare* was only detected using qPCR in orchard irrigation water in each of the investigated months in 36.6% to 81.8% of the dams, depending on the month sampled. The other oomycete species identified in orchard irrigation water included four *Pythium* species, one *Phytopythium* sp.

Almost half of the oomycete species that were found in dams were also found in the orchard irrigation water, and these included *P. irregulare, Pythium* spp. complex B2A, *Phytophthora, P. cantenulatum/rhizo-oryzae, P. apleroticum*, *P. oopapillum, P. undulatum*, *Pht. litorale* and *Pht. helicoides*. Thus considering all irrigation water samplings (dams and orchards), only *P. irregulare* is probably of importance as an inoculum source of ARD. The reduced number of species and prevalence of oomycetes detected in orchards versus dams, is likely due to the fact that irrigation water from dams pass through filtering systems on the farms, which can filter out some oomycete propagules. It is known that oomycetes are for example filtered effectively through sand filters (Kubiak, *et al*., 2015). Another reason could

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be that in the current study, dams were surveyed for oomycetes by placing baiting baskets on the surface of the dams, whereas water is frequently pumped from the bottom of dams.

Although oomycete pathogens are frequently isolated from irrigation water using baiting techniques, the ability of the irrigation water to incite actual disease has been less studied. Loyd *et al*. (2014) found that although *Phytophthora* spp. pathogenic to *Pieris* and *Rhododendron* were frequently isolated from irrigation water, these hosts were rarely (~0.5%) infected with *Phytophthora* when irrigated with the contaminated water. They hypothesized that this was due to the fact that the zoospore concentration was below the threshold required for infection of the host. Oomycete zoospore concentrations are known to be very important in determining if host infections will take place, with no or reduced infections occurring at low concentrations (Benson and Jones, 1980; Granke and Hausbeck, 2010; Kong *et al*., 2010). Jones *et al*. (2014) in their survey of New York surface water encountered low levels of oomycetes that on average ranged between 19 CFU/L (creek samples) to 12 CFU/L (pond samples), although some samples had as high as 200 CFU/L hymexazol-insensitive oomycetes. Hong and Epelman (2001) found that a zoospore concentration of $>10^2$ spores/L of *Ph. nicotianae* were required in irrigation water for inciting disease on *Catharanthus roseus.* Furthermore, disease development was delayed and progression slowed on plants irrigated every 3 days versus those irrigated daily (Hong and Epelman, 2001). In the current study, only quantitative analyses of oomycetes were made using leaf disks, and the amount of pathogen inoculum present is thus unknown. We need to improve our understanding of the epidemiology of waterborne pathogens, i.e. how and what influences pathogen survival, reproduction and spread in irrigation water. Most importantly, we need to determine economic thresholds for pathogens detected in irrigation water, i.e. the quantity and frequency of the pathogens required to pose a real threat to the crop (Hong and Moorman, 2005).

In the current study, isolation studies and qPCR analyses of selected ARD species known to be important in ARD in South Africa revealed that nursery trees can be an inoculum source of ARD pathogens, and surface irrigation water to a much lesser extent. The nurseries and blocks within nurseries in general differed in the prevalence of ARD pathogens, but most tested positive. The most prominent ARD pathogens in nursery rootstocks over the two year sampling period were *Pythiym irregulare* (>95% of trees), *Cylindrocarpon-like* spp. (100% of trees) and *Pratylenchus* spp. (>29% of trees with 6 to 22% of trees containing more than 100 *Pratlenchus* spp./5g roots), followed by *P. ultimum* (>41% of trees). Due to their high frequency of occurrence in nursery trees, these species may contaminate fumigated ARD soils when present on rootstocks. Therefore, it will be important to target these pathogens using additional management approaches, in addition to the currently used fumigation. For example, metalaxyl, fenamisphos and phosphonates can be applied at planting. There were some differences in the prevalence of pathogenic ARD species between the two sampling years,

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but this can be expected. The amount of rainfall and temperature will likely influence the level of rootstock infections and infestations in different years. Surface irrigation water analyses showed that the replant pathogens *P. irregulare, Pht. litorale* and *Pythium* spp. complex B2A could potentially re-infect fumigated ARD orchards. Of these pathogens, only *P. irregulare* is most likely important since this species were also detected in orchard irrigation water, whereas the other two species were not detected in orchard water, but only occurred erratically in dams. However, the biological significance of *P. irregulare* in irrigation water is uncertain since host infection by pathogens is inoculum and environmental dependent. In future studies, this can be investigated by placing apple seedlings in planting bags within orchards for varying irrigation periods, followed by qPCR analyses for ARD pathogens in seedling roots. The importance of *Ph. cactorum* is unknown in irrigation water, also due to the aforementioned fact. Furthermore, whether the species was present in water samples is unknown since only a genus specific assay was used. In future, *Ph. cactorum* specific qPCR assays will be more useful. Future studies should also investigate the detection and quantification of *Pratylenchus* spp. in irrigation water. Altogether, the study was able to show that nursery rootstocks are a real threat to being an inoculum source of ARD, which can reduce the efficacy of fumigation in ARD orchards. Management strategies of this inoculum source, whether at the nurseries or at planting, will be important to develop in future. The biological significance of ARD oomycete pathogens in irrigation water requires further investigation.

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Table 1. Percentage apple nursery trees from which pathogenic *Pythium* species were isolated during surveys conducted in 2013 and 2014.

		P. irregulare		*P. ultimum*		*Pythium* spp. complex 2BA		Average nurseries	
Nursery	Block[a]	2013	2014	2013	2014	2013	2014	2013	2014
Nursery 1	1	80	60	0	0	0	0		
	2	20	60	40	60	0	0		
	3	40	80	0	60	0	0		
	4	0	60	0	60	0	0		
Average		**35**	**65**	**10**	**45**	**0**	**0**	**15**	**36**
Nursery 2	1	0	85	0	0	0	0		
	2	60	100	40	0	0	0		
	3	100	33	0	0	0	0		
	4	20	ND[b]	0	ND	0	ND		
Average		**45**	**73**	**10**	**0**	**0**	**0**	**18**	**24**
Nursery 3	1	20	80	0	0	0	0		
	2	100	20	0	0	0	0		
	3	40	0	0	0	0	0		
	4	60	ND	0	ND	0	ND		
Average		**55**	**33**	**0**	**0**	**0**	**0**	**18**	**11**
Nursery 4	1	60	20	0	0	20	0		
	2	60	80	0	0	20	0		
	3	80	80	0	0	20	0		
	4	60	40	0	0	60	0		
Average		**65**	**55**	**0**	**0**	**30**	**0**	**31**	**18**
Nursery 5	1	20	0	0	0	0	0		
	2	0	20	0	0	0	0		
	3	40	20	0	0	0	0		
	4	80	20	0	0	0	0		
Average		**35**	**15**	**0**	**0**	**0**	**0**	**11**	**5**
Average all		**47**	**48**	**4**	**9**	**6**	**0**	**18**	**19**

[a] In each nursery, five trees were sampled from three or four blocks in 2013 and 2014.

[b]Not done

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Table 2. Percentage of rootstocks sampled in apple nurseries from which pathogenic *Pythium* spp. were isolated.

	***Pythium* spp.**	**Nursery 1**		**Nursery 2**		**Nursery 3**		**Nursery 4**		**Nursery 5**		**Average nurseries**	
		2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
MM109	*P. irregulare*	40	60	60	33.3	60	20	60	40	40	20	52	38.6
	P. ultimum	0	0	40	0	0	0	0	0	0	0	4	0
	P. spp. complex 2BA	20	60	0	0	0	0	60	0	0	0	4	0
	Total. Path	40	60	60	33.3	60	20	80	40	40	40	56	38.6
M793	*P. irregulare*	0	60	20	100	20-40[b]	80	60	80	80	20	36.6	68
	P. ultimum	40	60	0	0	0	0	0	0	0	0	4	0
	P. spp. complex 2BA	0	0	0	0	0	0	20	0	0	0	0	0
	Total. Path	0	60	20	100	20-40	80	80	80	80	20	36.6	68
M7	*P. irregulare*	40	60	100	85.7	100	0	60-80[c]	20-80	0	0-20	63	37.9
	P. ultimum	0	60	0	0	0	0	0	0	0	0	4	0
	P. spp. complex 2BA	0	0	0	0	0	0	20	0	0	0	0	0
	Total. Path	40	60	100	85.7	100	0	60-80	20-80	0	0-20	63	37.9

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Table 3. Quantitative PCR analyses for percentage apple nursery trees infected with apple replant pathogens and the amount of pathogen DNA biomass in 2013 and 2014.

Nursery	Block[a]	Average *Pythium irregulare* DNA biomass in ng/mg (% trees)		Average *Pythium ultimum* DNA biomass in ng/mg (% trees)		Average *Cylindrocarpon*-like spp. DNA biomass in ng/mg (% trees)		Average *Pythium sylvaticum* DNA biomass in ng/mg (%trees)	
		2013	2014	2013	2014	2013	2014	2013	2014
Nursery 1	1	0.006	0.006 (100)	0.004	0.003 (80)	6.097	12.096 (100)	0	0
	2	0.002	0.009 (100)	0.004	0.253 (100)	0.033	19.320 (100)	0	0
	3	0.096	0.003 (100)	0.011	0.078 (100)	0.609	10.480 (100)	0	0
	4	0.008	0.005 (100)	0.014	0.089 (100)	0.048	5.989 (100)	0	0
Average		0.028	0.006 (100)	0.008	0.105 (95)	1.697	9.470 (100)	0	0
Nursery 2	1	0.004	0.011 (100)	0.960	2.583 (42)	0.057	2.583 (100)	0	0
	2	0.034	0.190 (100)	1.245	1.085 (42)	0.048	1.085 (100)	0	0
	3	0.006	0.002 (100)	1.065	3.397 (66)	0.045	3.397 (100)	0	0
	4	0.057	ND	0.789	ND	0.038	ND	0	ND
Average		0.025	0.060 (100)	1.010	2.351 (50)	0.047	2.355 (100)	0	0
Nursery 3	1	0.0003	0.012 (100)	0	0.0895 (40)	0.027	1.852 (100)	0	0
	2	0.007	0.0001 (100)	0	0.0662 (80)	0.079	15.120 (100)	0	0
	3	0.0007	0.009 (100)	0.006	0.0041 (60)	0.021	7.055 (100)	0	0.026 (100)
	4	0.030	ND	0.008	ND	0.051	ND	0	ND
Average		0.009	0.007 (100)	0.003	0.051 (60)	0.045	8.010 (100)	0	0.008 (25)
Nursery 4	1	0.540	0.002 (100)	0	0	0.041	4.463 (100)	0	0
	2	0.052	0.010 (100)	0	0	0.098	1.527 (100)	0	0
	3	0.137	0.095 (100)	0	0	0.039	6.863 (100)	0	0
	4	0.060	0.014 (100)	0	0	0.059	11.510 (100)	0	0
Average		0.197	0.030 (100)	0	0	0.059	6.092 (100)	0	0
Nursery 5	1	0.001	0.001 (80)	0	0	0.009	1.288 (100)	0	0
	2	0.020	0.001 (40)	0	0	0.014	14.510 (100)	0	0
	3	0.030	0.004 (100)	0	0	0.012	6.379 (100)	0	0
	4	0.099	< 0.002 (80)	0	0	0.083	20.710 (100)	0	0
Average		0.037	0.000 (75)	0	0	0.029	10.720 (100)	0	0
Average all		0.059	0.020 (95)	0.204	0.501 (41)	0.370	7.320 (100)	0	0.0016 (5)

[a] Five nurseries were sampled in each sampling year. Within a nursery four blocks were sampled and five trees within a block. The averages for the five trees within each block is shown. In the 2013 sampling the data of the five trees were pooled, and the percentage infected trees are thus not shown (ND).

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Table 4. Prevalence of *Pratylenchus* spp. in apple nurseries surveyed in 2013 and 2014.

Nursery	Block[a]	Average number *Pratylenchus*/ 5g roots		% trees with *Pratylenchus*		% trees > 100 *Pratylenchus*/5g roots		% trees > 300 *Pratylenchus*/5 g roots	
		2013	2014	2013	2014	2013	2014	2013	2014
Nursery 1	1	12	0	60	0	0	0	0	0
	2	0	0	0	0	0	0	0	0
	3	4	0	60	0	0	0	0	0
	4	0	0	0	0	0	0	0	0
Average		4	0	30	0	0	0	0	0
Nursery 2	1	102	11.66	80	16	60	0	0	0
	2	80	5.71	60	14	20	0	20	0
	3	170	0	60	0	40	0	40	0
	4	456	ND	60	ND	40	ND	20	ND
Average		202	5.5	65	10	40	0	20	0
Nursery 3	1	0	2	0	20	0	0	0	0
	2	0	14	0	60	0	0	0	0
	3	4	0	20	0	0	0	0	0
	4	0	ND	0	ND	0	ND	0	ND
Average		1	5.33	5	26.6	0	0	0	0
Nursery 4	1	288	0	20	0	20	0	20	0
	2	8	0	20	0	0	0	0	0
	3	560	6	60	40	40	0	20	0
	4	64	12	40	20	20	0	0	0
Average		230	4.5	35	15	20	0	10	0
Nursery 5	1	378	52	40	80	20	20	20	0
	2	622	86	80	100	80	40	60	0
	3	338	50	80	100	60	20	40	0
	4	642	94	40	100	40	40	40	0
Average		495	70.5	60	95	50	30	40	0
Average all nurseries		186.4	17.16	39	29.32	22	6	14	0

[a] In each nursery three to four blocks were sampled, five trees per block in 2013 and 2014. Column values are the averages of the five trees per block.

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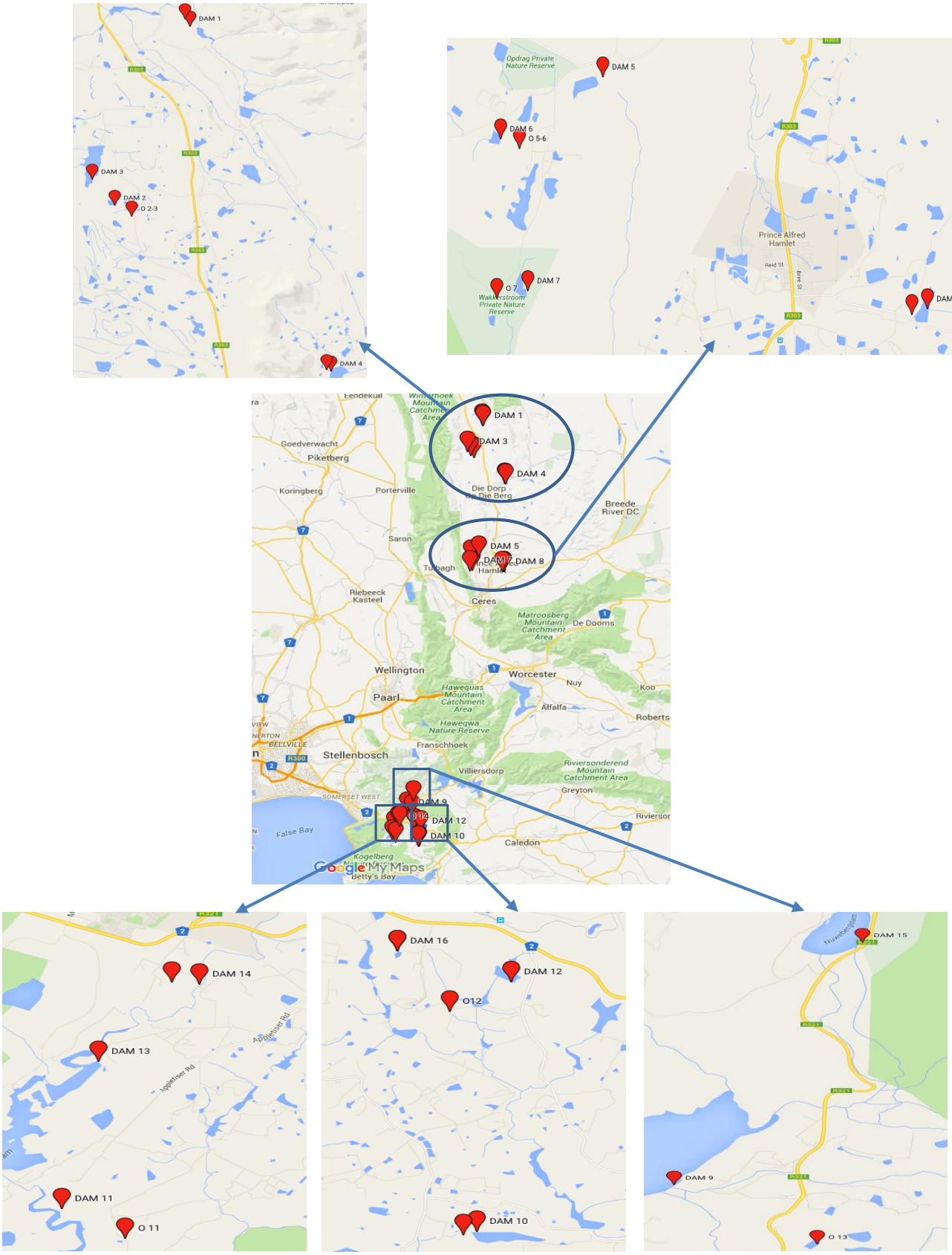

Figure 1. Location of dams (D1 to D16) and orchards (O1 to O16) that were surveyed for the presence of oomycetes in the Kouebokkeveld (top figures) and Grabouw/Elgin (bottom figures) regions.

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Figure 2. Basket baiting container that was used for baiting of oomycetes in dams. Citrus and avocado leaf disks and apple and pear fruit were used as baits inside the basket, which was then closed with tiebacks and floated on dams.

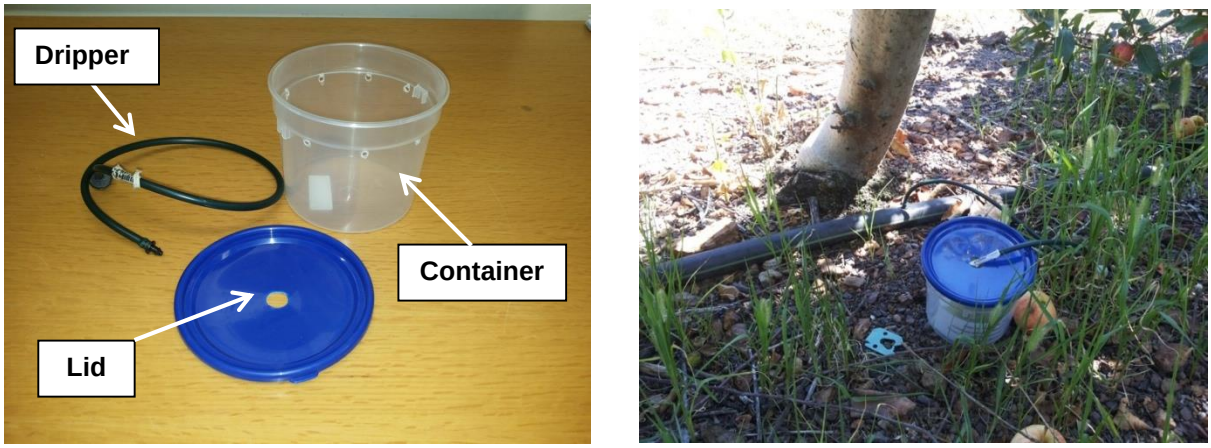

Figure 3. Plastic baiting container used for baiting oomycete species from irrigation water within orchards. The container consisted of a dripper inserted into the lid and drainage holes at the top of the container. The dripper line from the container was connected to the dripper pipe within the orchard.

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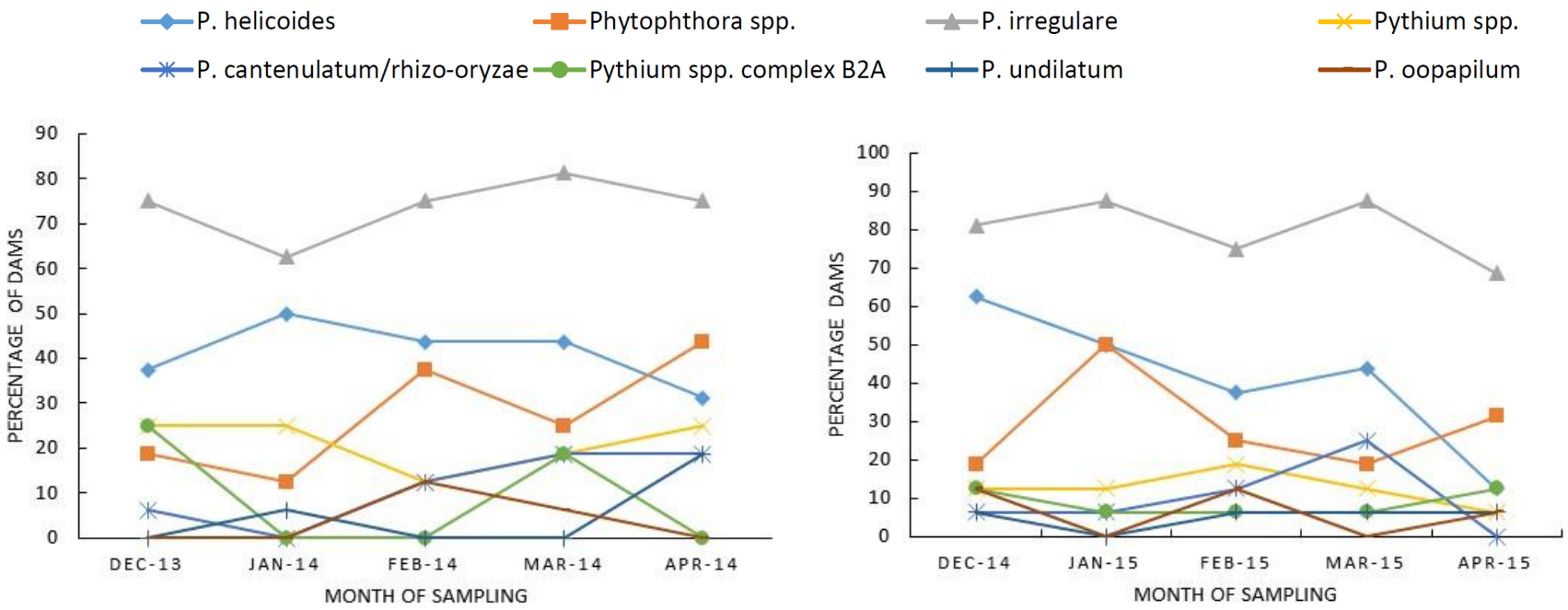

(A) (B)

Figure 4. Percentage of dams containing different *Pythium* and *Phytophthora* species over a five month sampling period in (A) 2013/14 and (B) 2014/15. A total of 16 dams were sampled in each month of sampling, and the presence of species was investigated using isolation and qPCR data. The combined results of the two approaches are shown.

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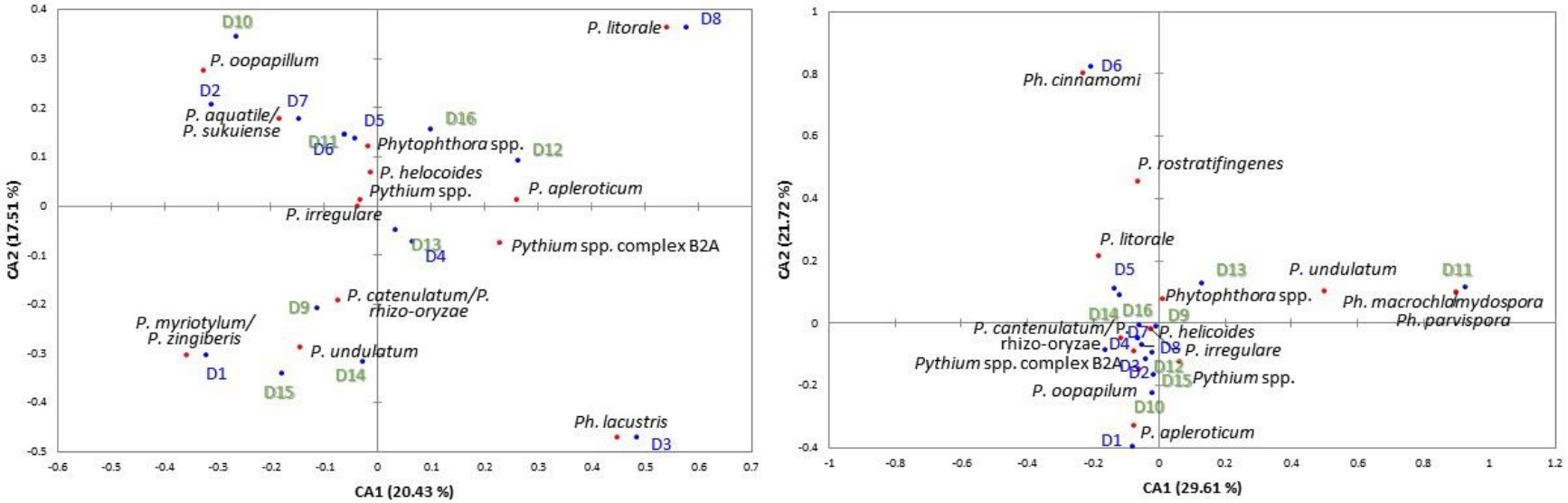
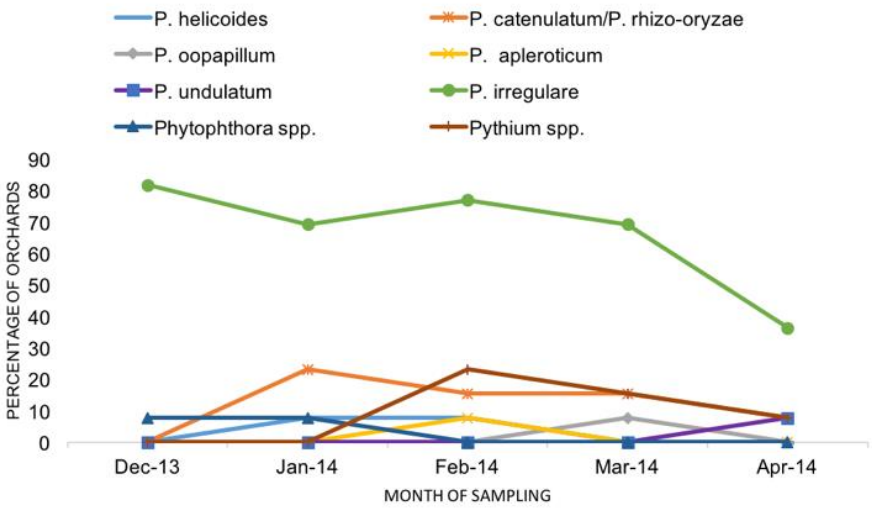
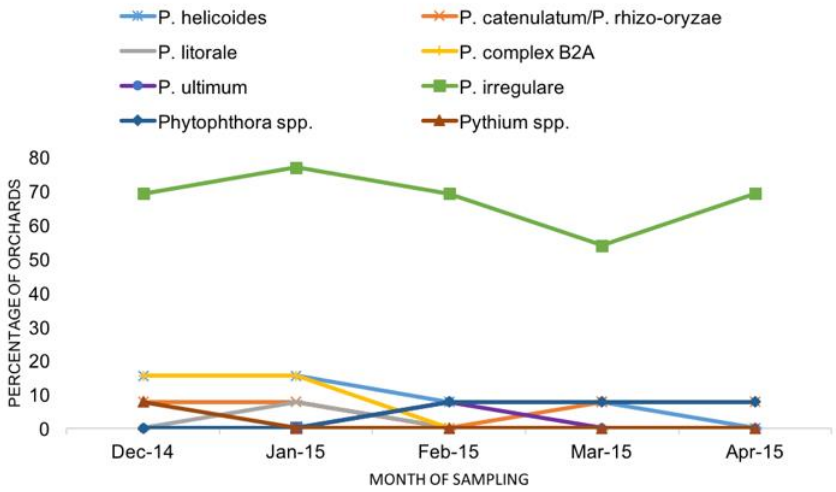


(A) (B)

Figure 5. Correspondence analyses plots of the association of dams with different oomycete species in the Kouebokkeveld and Grabouw regions in (A) 2013/14 and (B) 2014/15 sampling periods. Dams that were located in the Kouebokkeveld are indicated in blue, whereas those from Grabouw/Elgin are indicated in green. Variation accounted for by each correspondence analysis axis is indicated in brackets.

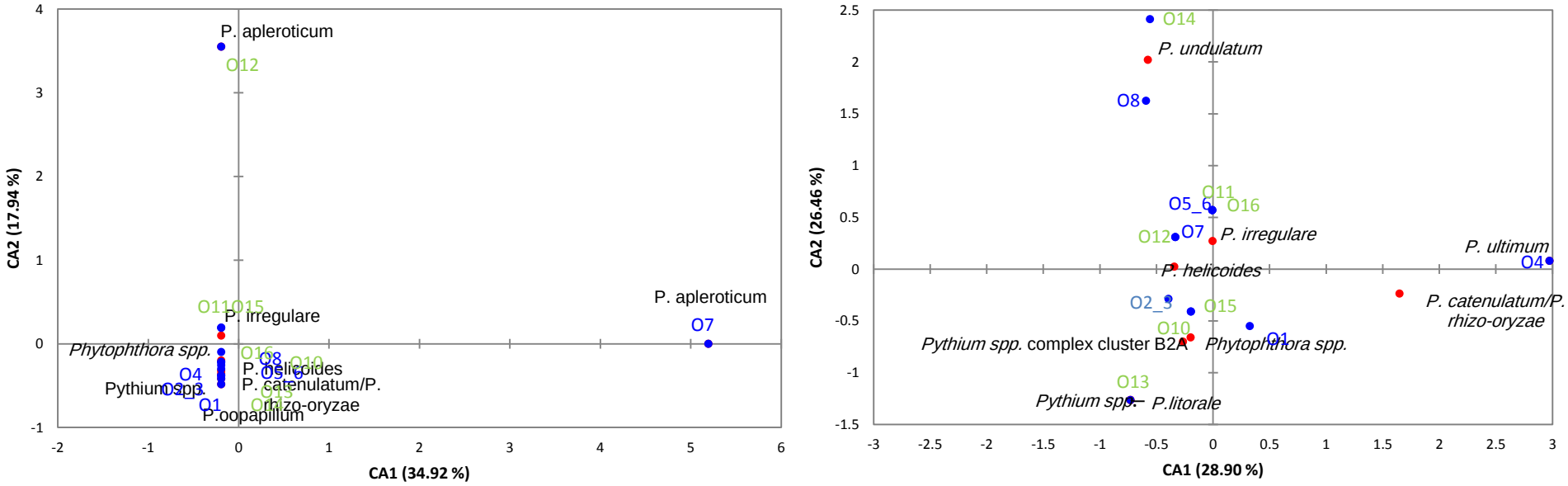


(A)



(B)

Figure 6. Percentage of orchards containing different *Pythium* and *Phytophthora* species over a five month sampling period in (A) 2013/14 and (B) 2014/15. A total of 13 orchards were analysed in each month, and the presence of species was investigated using isolation and qPCR data. The combined results of the two approaches are shown.



(A) (B)

Figure 7. Correspondence analyses plots of the association of different oomycete species identified through baiting in within orchard irrigation water in the Kouebokkeveld and Grabouw/Elgin regions in (A) 2013/14 and (B) 2014/15 sampling periods. Orchards that were located in the Kouebokkeveld are indicated in blue, whereas those from Grabouw/Elgin are indicated in green. Variation accounted for by each correspondence analysis axis is indicated in brackets.

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