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Characterization of fungi (*Fusarium* and *Rhizoctonia*) and oomycetes (*Phytophthora* and *Pythium*) associated with apple orchards in South Africa

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Abstract Several species of fungi and oomycetes including *Fusarium, Rhizoctonia, Phytophthora* and *Pythium* have been reported as root pathogens of apple where they contribute to a phenomenon known as apple replant disease. In South Africa, little is known about specific species in these genera and their pathogenicity toward apple. Therefore, these aspects were investigated along with the development and optimization of qPCR tests for detection and quantification of the most virulent oomycete species. In eight investigated orchards, the oomycete *Phytophthora cactorum* was widely distributed, while nine *Pythium* species were differentially distributed among the orchards. *Pythium irregulare* was the most widely distributed and the most virulent species along with *P. sylvaticum, P. vexans* and *Ph. cactorum*. Seven binucleate *Rhizoctonia* anastomosis groups (AGs) were also differentially distributed among the orchards, with the majority appearing to be non-pathogenic while certain AG-I and AG-F isolates exhibited low virulence on apple. In the genus *Fusarium, F. oxysporum* was widely distributed, but isolates were non-pathogenic. *Fusarium solani* and *F. avenaceum* were less frequently encountered, with only some isolates having low virulence. qPCR data obtained from seedling roots inoculated with the most virulent *Pythium* species (*P. irregulare, P. sylvaticum* and *P. vexans*) and the genus *Phytophthora* were not always reproducible between trials, or isolates of the same species. In general, seedling growth inhibition was associated with the presence of a low amount of pathogen DNA (±40 fg μl^{-1} to 2 pg μl^{-1}) in roots. *Pythium irregulare,* although having the lowest DNA concentrations in roots, was the only species for which a significant negative correlation was found between seedling weight and pathogen DNA concentration.

Keywords Apple replant disease · DNA extraction from plant roots · Growth reduction · Pathogen DNA in roots · TaqMan chemistry

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Abbreviations

AG anastomosis group
qPCR quantitative real-time PCR

Introduction

Several pathogenic fungi and oomycetes are associated with apple tree roots in old orchards, and with roots of young trees grown in soil previously cultivated with apple or closely related crops (Dullahide et al. 1994; Mazzola 1998, 1999). Plant pathogenic microbes can start accumulating in the rhizosphere and roots of apple within 1–2 years after orchard establishment (Mazzola 1999). Some of these pathogens contribute to a phenomenon known as apple replant disease, which refers to the poor growth of apple trees on soil previously cultivated to apple or closely related species. The primary fungal and oomycete genera reported as containing species that are pathogenic toward apple, include the fungal genera *Cylindrocarpon*, *Fusarium* and *Rhizoctonia,* and the oomycete genera *Phytophthora* and *Pythium* (Sewell 1981; Dullahide et al. 1994; Mazzola 1998; Manici et al. 2003).

Fusarium species are widely distributed, and the genus contains over 70 species (Leslie and Summerell 2006). Many species inhabit soil ecosystems where they are rhizosphere and/or endophytic plant colonizers (Sutton et al. 1998). The interaction of *Fusarium* species with plants can range from highly pathogenic to beneficial plant growth stimulation (Fravel et al. 2003). *Fusarium* is frequently isolated from diseased apple tree roots (Dullahide et al. 1994; Mazzola 1998), but most isolates do not seem to be pathogenic.

The genus *Rhizoctonia* is comprised of a diverse group of fungi that can broadly be divided into multinucleate and binucleate groups based on the number of nuclei per hyphal cell (Yamamoto and Uchida 1982; Sneh et al. 1991). Isolates are classified further into anastomosis groups (AGs), based on their hyphal compatibility with known tester isolates (Carling et al. 2002). Members of the genus *Rhizoctonia* include economically significant soilborne plant pathogens, but a considerable number have been reported to function as saprophytes or establish mycorrhizal associations with plants (Sneh et al. 1991). On apple, some multinucleate *Rhizoctonia solani* Kühn AGs have been reported as pathogens, causing severe root rot and death of apple transplants. Some isolates within a few binucleate *Rhizoctonia* AGs are also pathogenic towards apple (Mazzola 1997).

The genus *Cylindrocarpon* includes approximately 125 taxa (species and varieties). Species of *Cylindrocarpon* are generally considered as weak or minor pathogens, but they can sometimes have a significant economic impact (Mantiri et al. 2001). On apples, a few *Cylindrocarpon* species, mostly *C. destructans* (Zinnsm.) Scholten, have been implicated as root pathogens of apple (Dullahide et al. 1994; Mazzola 1998; Manici et al. 2003).

The oomycete genera *Phytophthora* and *Pythium* belong to the Kingdom Straminipila, Phylum Heterokonta (Dick 2001a,b). The genus *Pythium* contains several species that are facultative plant pathogens, causing severe decline in crops acting either individually or in complexes with other organisms (Lévesque and De Cock 2004). There are also some *Pythium* species that can have plant growth-promoting effects and/or have potential for biocontrol (Picard et al. 2000; Mazzola et al. 2002). The interaction of *Pythium* with apple can range from highly virulent to that of promoting plant growth (Mazzola et al. 2002). Most *Phytophthora* species are plant pathogens and can cause severe devastation to a large variety of trees, ornamentals and crop plant species, including apple. There are, however, a few species such as *Ph. gonapodyides* (Petersen) Buisman and *Ph. inundata* Brasier that are in general considered as only weakly virulent and thus of minor importance (Cooke et al. 2007).

Molecular techniques have become very valuable for the identification, detection and quantification of plant pathogenic fungi and oomycete genera. Although species level identifications have traditionally been conducted using morphology, gene sequence data are increasingly being used for the rapid and accurate identification of species (Lévesque and De Cock 2004; Geiser et al. 2004; Schena et al. 2008). Techniques for the detection and quantification of pathogens have been developed using gene sequence data generated for species level identification. Quantitative PCR (qPCR) in particular, has become a powerful tool for detection and quantification of different plant pathogenic microorganisms (Schena et al. 2004), since quantification based on culturing techniques is considered relatively

inaccurate and in some cases even unreliable (Goud and Termorshuizen 2003).

In South Africa, little is known about the species of *Fusarium, Phytophthora, Pythium* and *Rhizoctonia* associated with apple roots and their pathogenicity towards apple. In contrast, the pathogenicity of *Cylindrocarpon* species has been reported recently (Tewoldemedhin et al. 2010). Van Schoor et al. (2009) found that *Cylindrocarpon*, *Fusarium, Phytophthora, Pythium* and *Rhizoctonia* were associated with apple roots in the Western Cape region, but the specific species and their pathogenicity were not investigated. Oomycete species previously reported from apple in South Africa include *P. minus* Ali-Shtayeh, *P. sylvaticum* Campbell & Hendrix, *P. helicandrum* Drechsler (McLeod et al. 2009) and *Ph. cactorum* (Zondo et al. 2007).

The aim of this study was to investigate the specific *Fusarium, Phytophthora, Pythium* and *Rhizoctonia* species associated with apple roots and soil (only oomycetes) in South Africa, and to determine their pathogenicity towards apple seedlings in glasshouse experiments. Furthermore, root material from the pathogenicity trial was used to optimize and develop qPCR tests for the detection and quantification of species (*Ph. cactorum, P. irregulare* Buisman, *P. sylvaticum* and *P. vexans* de Bary) that were found to be the most virulent towards apple. The qPCR techniques were then evaluated to determine whether a correlation exists between pathogen biomass (DNA) in seedling roots and the degree of seedling growth inhibition.

Materials and methods

Sample collection

Root and soil samples were collected from eight orchards that all contained trees that were grafted onto rootstock MM793. Five of the orchards were located in the Grabouw area (GF, GG, GE, GR and GM) and three in the Ceres area (CV, CD and CE). Orchards GF, GE, GR and GM were two-year-old trees that were replanted in orchard soil previously cultivated with apple for more than 15 years, whereas orchards GG, CV, CD and CE consisted of apple trees that were more than 15 years old. Samples were collected from all study sites during autumn (April) or spring (October) from 2006 to 2007. The soil sample for orchards GG, GE, GM, CV, CD and CE consisted of five sub-samples, which were taken at different locations in a "W" shaped pattern at a depth 5–30 cm, at least 10 cm from the trunk of trees. The samples of each orchard were thoroughly mixed together into one compound sample that was split into six replicates for each of the glasshouse experiments. Root samples for orchards GF and GR consisted or roots collected from 12 different trees.

Fungal and oomycete isolations from apple roots

Two different approaches were used for isolating fungi and oomycetes from soil and root samples. For orchards GF and GR, isolations were made directly from apple roots collected from orchard trees. These roots, however, predominantly yielded saprophytic fungi. Therefore, a second approach was used for isolations from the remaining orchards (GG, GE, GM, CV, CD and CE). Apple seedlings were grown in these orchard soils under glasshouse conditions for 3 months, after which isolations were made from seedling roots. For each orchard, six replicates, each consisting of one plastic bag (1 l) containing three 4-week-old seedlings, were planted.

Roots from orchard trees and harvested seedlings were washed under tap water to remove adhering soil, followed by dipping in 99% ethanol for 5 s, and air drying in a laminar flow hood. Small root segments were plated onto: (1) water agar (Agar Bacteriological, Biolab Diagnostics, Midrand, South Africa) containing 100 μg ml^{-1} streptomycin (WA^+), (2) potato dextrose agar (Biolab Diagnostics, Midrand, South Africa) containing 100 μg ml^{-1} streptomycin (PDA^+), (3) PARP (Jeffers and Martin 1986) and (4) PARPH (Kannwischer and Mitchell 1978). The isolation plates were incubated in the dark at 25°C. Individual colonies that developed on the four media were transferred to WA^+ and purified by hyphal-tipping and single-sporing (only *Fusarium*).

The number of roots plated from the field grown trees and glasshouse seedlings varied. For the GF and GR orchard trees, 12 root segments from each of 12 trees per orchard were plated on each of the four media (WA^+, PDA^+,PARP and PARPH), resulting in a total of 576 root segments being plated for each orchard. For the other six orchards, where seedlings

were first grown in orchard soils in a glasshouse, 24 root segments representative of the six soil replicates of each orchard, were plated onto each of the four media, resulting in a total 96 root segments being plated for each orchard.

Soil samples from each of the six replicates used per orchard were also baited for the presence of *Phytophthora* and *Pythium* spp. by floating 24 avocado leaf discs on a soil-water mixture. The floating discs (144 per orchard) were kept in the dark, and rinsed in de-ionized water after 3 days of incubation (Singleton et al. 1992). The discs were plated onto PARP and PARPH media. Individual colonies that developed on these media were transferred to WA^+ and purified by hyphal-tipping.

Isolates that were sub-cultured onto WA^+ were identified to the genus level. Isolates were identified as *Fusarium* according to Leslie and Summerell (2006), as *Phytophthora* using Erwin and Ribeiro (1996), as *Pythium* according to Dick (1990) and as *Rhizoctonia* according to Sneh et al. (1991).

All single-spored (*Fusarium*) and hyphal-tipped (*Phytophthora, Pythium* and *Rhizoctonia*) cultures were stored for subsequent species identification and pathogenicity testing. Pure cultures of *Fusarium* were stored in 15% glycerol at −85°C and on PDA discs in water at 15°C. All *Rhizoctonia* isolates were stored on sterile wheat grains using a slight modification (vermiculite was excluded) of the inoculum preparation method of Sneh et al. (1986). Cultures on wheat grain were stored at −20°C. Oomycetes were stored on grass leaves in water at 15°C, V8 agar discs in water at 15°C and on potato carrot agar slants (20 g potato, 20 g carrot and 20 g agar) at 15°C.

Molecular identification of fungal and oomycete species

Isolates that were morphologically identified as belonging to the genera *Phytophthora, Pythium* and *Rhizoctonia* were identified to the species level using sequencing data from the internal transcribed spacer (ITS) 1, ITS2 and the 5.8 S gene. The species identity of *Fusarium* isolates was conducted using partial EF-1α gene sequence data.

DNA extraction DNA was isolated from 5- to 10-day-old oomycete cultures growing on V8 agar (Miller 1955), and from 5-day-old *Fusarium* and *Rhizoctonia* cultures growing on PDA plates. DNA was extracted using a slight modification of the protocol described by Lee and Taylor (1990).

PCR amplification and sequencing of oomycete and Fusarium isolates The ITS regions and 5.8 S gene of oomycetes were amplified using primers ITS6 (Cooke and Duncan 1997) and ITS4 (White et al. 1990). For a few isolates where amplification could not be obtained using these primers, the primer pair UN-UP18S42 and PyLo28S22 (Lévesque and De Cock 2004) was used. The EF-1α gene was amplified from *Fusarium* isolates using primers EF-1 and EF-2 (O'Donnell et al. 1998). PCR reaction and amplification conditions were carried out as previously described (McLeod et al. 2009), except that an annealing temperature of 55°C was used for oomycetes and 53°C for *Fusarium*. PCR products (5 µl aliquot) were visualized on agarose gels. PCR amplicons were cleaned and sequenced as previously described, using the PCR amplification primers (McLeod et al. 2009).

Polymerase chain reaction (PCR) amplification, cloning and sequencing of Rhizoctonia isolates The ITS regions and 5.8 S gene of *Rhizoctonia* isolates were amplified using primers ITS6 and ITS4. The PCR reaction reagent and PCR amplification conditions were similar to that described for *Fusarium.* Direct PCR product sequencing resulted in poor quality sequence data, since *Rhizoctonia* isolates contain multiple nuclei containing multiple copies that can be polymorphic within the same isolate. Cloning and sequencing of *Rhizoctonia* ITS products was conducted as previously described (McLeod et al. 2009).

BLAST analyses of sequences for species identification Sequences obtained for *Phytophthora, Pythium* and *Rhizoctonia* isolates were submitted to BLAST analyses in Gen-Bank (http://www.ncbi.nlm.nih.gov/Genbank/). For *Pythium* species identification, only sequences submitted by Lévesque and De Cock (2004) were used or published sequences of recently described new species. Sequences from *Fusarium* isolates were submitted to BLAST analyses in the FUSARIUM-ID v. 1.0 database (http://fusarium.cbio.psu.edu) (Geiser et al. 2004).

Morphological identification of *Pythium* species

Two isolates of each of the different *Pythium* species that were identified through sequence data were selected for morphological identification. Isolates were morphologically identified to the species level as previously described (McLeod et al. 2009).

Pathogenicity assays

In the pathogenicity trials, two isolates from each identified species (oomycetes and *Fusarium*) or anastomosis group (*Rhizoctonia*) were used. In cases where only one isolate was obtained, this single isolate was used. All cultures used in these trials were submitted to the Stellenbosch University culture collection (STE-U6741, 6742, and 7185–7226).

The plant growth media (pasteurized bark and sand medium in 2:1 v/v) was inoculated using either millet seed (fungi) or sand-bran (oomycetes). Sterile millet seeds, prepared according to Strauss and Labuschagne (1995), were inoculated with 10 (6 mm diam.) mycelial discs from 2-wk-old *Fusarium* and *Rhizoctonia* cultures grown on PDA. Control bottles were inoculated with discs of PDA only. Inoculated bottles were incubated at 25°C for 2 weeks and shaken every other day to ensure thorough seed colonization. Sand-bran inoculum of *Phytophthora* and *Pythium* was prepared according to Lamprecht (1986). The sand-bran medium consisted of 200 g of sterile, washed river sand, 20 g of wheat digestive bran and 30 mL of distilled water that were added to 500 ml Schott bottles and autoclaved for 20 min at 120°C on 2 consecutive days. Each bottle was inoculated with 10 (6 mm diam.) mycelial discs from a 2-wk-old oomycete culture grown on CMA (corn meal agar, Difco Laboratories, MI, USA). Control bottles were inoculated with discs of CMA agar only. The inoculated bottles were incubated at 25°C for 2 weeks for *Phytophthora* and 1-wk for *Pythium* and shaken every other day to ensure thorough colonization. Millet seed and sand-bran inoculum were added to soil at a rate of 1% (v/v) and soils were incubated for 24 h at 20°C–25°C prior to planting.

Four-week-old apple seedlings were produced as previously described (Tewoldemedhin et al. 2010). The pathogenicity of each isolate was tested using six replicates, each replicate consisting of a 1 l plastic bag containing three apple seedlings. The experiment was set up as a complete randomized block design and was repeated once. The inoculated seedlings were grown in a glasshouse at temperatures ranging from 20°C to 25°C and at relative humidity ranging from 60% to 70%, and were harvested after 3 months.

At harvest, seedling root systems were washed and three parameters were used to evaluate the pathogenicity and virulence of isolates, i.e. (i) seedling height, (ii) seedling weight and (iii) root rot. Root rot was rated on a 0–4 scale, with 0=no obvious symptoms; 1=moderate discolouration of root tissue; 2=extensive discolouration of tissue; 3=extensive discolouration of tissue with girdling lesions; and 4=plant dead. Increase in seedling weight and height was determined by subtracting measurements taken at the start of the trial, from those taken at trial termination. In order to fulfill Koch's postulates, isolations for fungi and oomycetes were made from discoloured fibrous roots of seedlings. Isolation and identification of each organism to the genus level was made as described above. Root material (three replicates from each of the two trials) from seedlings grown in *P. irregulare, P. sylvaticum, P. vexans* and *Ph. cactorum* infested soils as well as the control treatment were stored at −85°C and used for subsequent DNA extractions, and qPCR analyses.

Statistical analysis Levene's variance ratio test (Levene 1960) was performed to test for homogeneity of trial variances between the trial repeats. The error variance ratios for root rot and seedling weight were $P=0.1079$ and $P=0.2756$ respectively, and the data of the two repeat trials could thus be combined. However, the error variance ratio for seedling height between the two trials was significant ($P=0.0016$), and therefore weighted analysis was conducted (only for height) in order to combine the data of the two repeat trials (John and Quenouille 1977). Data of the two independent trials were considered block treatments providing that Levene's variance ratio test showed homogeneity in trial variance. Data were also subjected to analysis of variance (SAS 1999), and the Shapiro-Wilk test was performed to test for normality (Shapiro and Wilk 1965). In cases where deviations from normality were due to kurtosis and not skewness, the data were accepted as reliable and the results were interpreted without transformation (Glass et al. 1972). The Student's t-Least Significant Difference was calculated at the 5% confidence level to compare treatment means.

Quantitative real-time PCR (qPCR) analyses using pure culture DNA

Sensitivity testing Oomycete DNA, extracted using a Qiagen DNeasy plant mini kit (Qiagen GmbH, Hilden, Germany), was used for optimization and sensitivity testing of the qPCR techniques. All qPCR analyses were conducted using either SYBR® Green I or TaqMan chemistry and SensiMix™ dT (Quantace, London, UK) on a Rotor-Gene™ 6000 real-time rotary analyzer (Qiagen GmbH, Hilden, Germany). The generated qPCR data were analyzed using Rotor-Gene 6000 Series Software 1.7. All primer pairs and the probe that were used are listed in Table 1, along with the regions that they amplify.

qPCR analyses for *Phytophthora, P. irregulare* and *P. sylvaticum* were conducted using SYBR® Green I reactions in 100 µl PCR strip tubes (Qiagen GmbH, Hilden, Germany). The total reaction volume of 40 µl contained 4 µl pure culture DNA extract (10 ng μl^{-1}), 20 µl SensiMix, 1 µl of SYBR® Green I and 0.3 µM of each primer Yph1F and Yph2R for the genus *Phytophthora* (Schena et al. 2006; Table 1), or 0.2 µM of each primer Syl1F and Syl2R for *P. sylvaticum* (Schroeder et al. 2006; Table 1), or 0.3 µM of primer PirF1 and 0.9 µM of primer PirR3 for *P. irregulare* (Spies 2010; Table 1). Each set of qPCR reactions included two replications of each sample and a negative (water) control. Cycle conditions for the genus *Phytophthora* consisted of initial denaturing at 95°C for 10 min followed by 40 cycles of denaturing at 95°C for 10 s, annealing at 62°C for 15 s and extension at 72°C for 30 s. Cycle conditions for *P. sylvaticum* consisted of initial denaturing at 95°C for 10 min followed by 40 cycles of denaturing at 95°C for 10 s, annealing at 65°C for 5 s and extension at 72°C for 10 s. Cycle conditions for *P. irregulare* consisted of initial denaturing at 95°C for 10 min followed by 40 cycles of denaturing at 95°C for 10 s and annealing at 65°C for 5 s and extension at 72°C for 20 s.

qPCR analysis for quantification of *P. vexans* was conducted using TaqMan chemistry. Each 40 µl reaction contained 4 µl pure culture DNA (10 ng μl^{-1}), 20 µl SensiMix, 0.2 µl probe VexP1, 0.9 µM primer PvF1 and 0.3 µM primer PvR1 (Spies 2010; Table 1). Cycle conditions consisted of an initial denaturing at 95°C for 10 min followed by 40 cycles of denaturing at 95°C for 10 s and annealing and extension for 30 s at 60°C.

The qPCR detection limit for each species was determined using two isolates per species. A standard curve was constructed from 10-fold serial dilutions of genomic DNA, yielding concentrations from 10 ng μl^{-1} to 0.01 fg μl^{-1}, with two replicates per concentration. The standard curves were generated by plotting cycle threshold (C_q) values obtained for each specific DNA concentration, versus the log of the initial concentration of species DNA. The standard curves were also used to quantify the amount of pathogen DNA from root material, based on C_q values obtained in the root DNA qPCR assays.

Specificity testing of qPCRs The specificity of the qPCR assays for the different *Pythium* species and genus *Phytophthora* was evaluated by testing whether DNA

Table 1 Oligonucleotide primers and probes used for the quantitative real-time PCR detection of *Phytophthora* and *Pythium* species

Target species	Primer or probe name	Oligonucleotide sequence (5′–3′)	Region amplified	Reference
Phytophthora	Yph1F	CGACCATKGGTGTGGACTTT	Ypt1[y]	Schena et al. 2006
Phytophthora	Yph2R	ACGTTCTCMCAGGCGTATCT	Ypt1	Schena et al. 2006
Pythium irregulare	PirF1	AGTGTGTGTGGCACGTTGTC	ITS1, 5.8S, ITS2 [z]	Spies 2010
Pythium irregulare	PirR3	GATCAACCCGGAGTATACAAAAC	ITS1, 5.8S, ITS2	Spies 2010
Pythium sylvaticum	Syl1F	GTGTCTCGCTGTGGTTGGTATATTTG	ITS1, 5.8S, ITS2	Schroeder et al. 2006
Pythium sylvaticum	Syl2R	CTTCTGCCAATTGCACAAGTGC	ITS1, 5.8S, ITS2	Schroeder et al. 2006
Pythium vexans	PvF1	TTTCCGTTTTGTGCTTGATG	ITS1, 5.8S, ITS2	Spies 2010
Pythium vexans	PvR1	AGCGAACACACCCAATAAGC	ITS1, 5.8S, ITS2	Spies 2010
Pythium vexans	VexP	HEX-CCGTGTCTGCTGGCGGGTC	ITS1, 5.8S, ITS2	Spies 2010

[y] The ras-related protein gene *Ypt1*

[z] The internal transcribes spacer (ITS) regions of ribosomal DNA

from closely related species [based on previous phylogenetic analyses (Lévesque and De Cock 2004)] could be amplified using the species specific primers. The specificity of the qPCR tests for *P. irregulare* and *P. sylvaticum* was evaluated by analyzing DNA from six isolates belonging to other *Pythium* clade F *sensu* Lévesque and de Cock (2004) species (one of each of *P. attrantheridium, P. kunmingense P. mamillatum*, *P. paroecandrum* and *P. spinosum*) and eight species from seven other *Pythium* clades (*P. coloratum*/*dissotocum*, *P. erinaceum*, *P. helicandrum*, *P. oligandrum*, *P. perplexum, P. torulosum*/*folliculosum*, *P. ultimum* var. *ultimum*, and *P. vexans*). The specificity of the *P. vexans* assay was evaluated using DNA of the aforementioned eight species, five *Pythium* clade K *sensu* Lévesque and De Cock (2004) species (*P. chamaehyphon*, *P. helicoides, P. litorale*, *P. mercuriale* and *P. oedochilum*), and one isolate from each of five *Phytophthora* species (*Ph. cactorum*, *Ph. cinnamomi*, *Ph. cryptogea*, *Ph. nicotianae* and *Ph. niederhauserii*). The specificity of the *Phytophthora* assay was evaluated by testing DNA of *Pythium* species from the closely related *Pythium* clade K *sensu* Lévesque and de Cock (2004) (three isolates of *P. vexans* and one isolate of each of *P. chamaehyphon, P. helicoides, P. litorale*, *P. mercuriale* and *P. oedochilum*).

qPCR analyses of genomic DNA extracted from apple seedling roots

DNA extraction from seedling roots DNA was extracted from the roots of apple seedlings infected with *Ph. cactorum, P. irregulare, P. sylvaticum,* or *P. vexans,* as well as seedlings grown in the no treatment control. DNA was extracted for each of three replicates for each treatment from the two repeat pathogenicity trials. Seedling roots were ground to a fine powder using liquid nitrogen and a IKA® A-11 Analytical mill (IKA®-Werke Gmbh & Co., Staufen, Germany). DNA was extracted from 60 mg of root tissue using the NucleoSpin® 96 Plant II kit (Macherey Nagel Gmbh & Co. KG, Düren, Germany), according to manufacturers instructions with a few slight modifications as previously described (Tewoldemedhin et al. 2010).

qPCR amplification from root DNA qPCR analyses on root DNA was conducted using the qPCR reaction and amplification conditions that were optimized for oomycete pure culture DNA. Each 40 µl qPCR reaction contained 40 ng root DNA. For each of the isolates, qPCR was conducted on six independent DNA extractions, i.e. one from each of three replicates in the two repeat trials. Each set of qPCR reactions included two replicates of each DNA sample, a non-template (water) control, a negative control (root DNA obtained from roots grown in control un-infested treatment soil) and two standard curve control (calibrator) samples for each pathogenic species. The presence of inhibitors in extracted root DNA was evaluated by making serial dilutions of a randomly selected subset of extracted root DNA samples, and observing whether a consistent linear decrease in C_q values was observed during amplification (Bustin et al. 2009).

Correlation between oomycete DNA concentration in roots with weight, height and root rot of seedlings Statistical analyses were conducted using the oomycete root DNA concentration data, as determined using qPCR, and the weight, height and root rot data obtained for each oomycete isolate used in the pathogenicity trial. Pearson's product moment correlation test was performed to determine the correlation between the measured parameters (Otto 1998).

Results

Fungal and oomycete isolations from apple roots

A total of 948 isolates that were morphologically identified as belonging to *Fusarium, Phytophthora, Pythium* and *Rhizoctonia* were recovered from the eight investigated orchards. A greater proportion of the isolates examined were recovered from the GF (386 isolates) and GR (276 isolates) orchards. For the other six orchards, varying numbers of isolates were obtained including 71 for GG orchard, 69 for GE orchard, 53 for GM orchard, 26 for CV orchard, 28 for CD orchard and 39 for the CE orchard.

Molecular identification of fungal and oomycete species

A subset of 401 isolates was selected for DNA sequencing, which represented isolates from all the

genera and orchards of interest. More isolates were sequenced from the GF (103) and GR (129) orchards. For the GG, GE, GM, CV, CD and CE orchards 38, 35, 41, 15, 22 and 18 isolates were respectively sequenced.

Several oomycete species within the genera *Phytophthora* and *Pythium* were identified. *Phytophthora* isolates were recovered from five of the orchards and all were identified as *Ph. cactorum* (Fig. 1). The population of *Pythium* spp. consisted of nine different species including *P. attrantheridium, P. dissotocum, P. folliculosum, P. heterothallicum, P. irregulare, P. litorale, P. minus, P. sylvaticum* and *P. vexans.* The species exhibited a differential distribution among the orchards, with *P. irregulare* having the widest distribution (seven orchards) followed by *P. dissotocum* (six orchards), *P. heterothallicum* (five orchards) and *P. sylvaticum* (four orchards). The remaining species were only found in one or two orchards. In general, within specific orchards, *P. dissotocum* and *P. heterothallicum* had the highest frequencies (Fig. 1).

Seven binucleate, but no multinucleate *Rhizoctonia* AGs were recovered from roots. *Rhizoctonia* AG-A were isolated from five orchards, followed by AG-I and AG-L that were each isolated from four orchards. The other AGs (F, G, K and R) were found in two or fewer orchards. *Rhizoctonia* was not isolated from orchard CE (Fig. 2).

Seven *Fusarium* species, *F. avenaceum* (Fr.) Sacc., *F. cerealis* (Cke.) Snyd. & Hans., *F. equiseti* (Corda) Sacc., *F. oxysporum, F. reticulatum* Mont., *F. scirpi* Lambotte & Fautrey and *F. solani,* were identified among the eight orchards. Most of these species, except *F. oxysporum* and *F. solani,* were not present in more than two orchards and consisted of no more than six isolates. *Fusarium oxysporum* was identified in all the orchards (Fig. 3).

Morphological identification of *Pythium* species

Preliminary ITS identifications of *Pythium* species were confirmed through morphological analyses of a subset of the isolates. The morphological analyses were required to specifically identify *P. dissotocum, P. folliculosum* and *P. minus* isolates, since the ITS sequences from these species are identical for more than one *Pythium* species (Lévesque and De Cock 2004).

Pathogenicity assays

There were significant differences in root rot ($P<0.0001$), seedling weight ($P<0.0001$) and height ($P<0.0001$) caused by isolates of different species within the genus *Pythium* (Table 2). Among the *Fusarium* isolates, only two affected plant development; *F. avenaceum* STE-U7206 caused significant root rot ($P=0.05$) and *F. solani* STE-U7214 induced a significant ($P=0.05$) reduction in seedling height. Only two of the *Rhizoctonia* isolates (STE-U7217 and STE-U7222) from AG-F

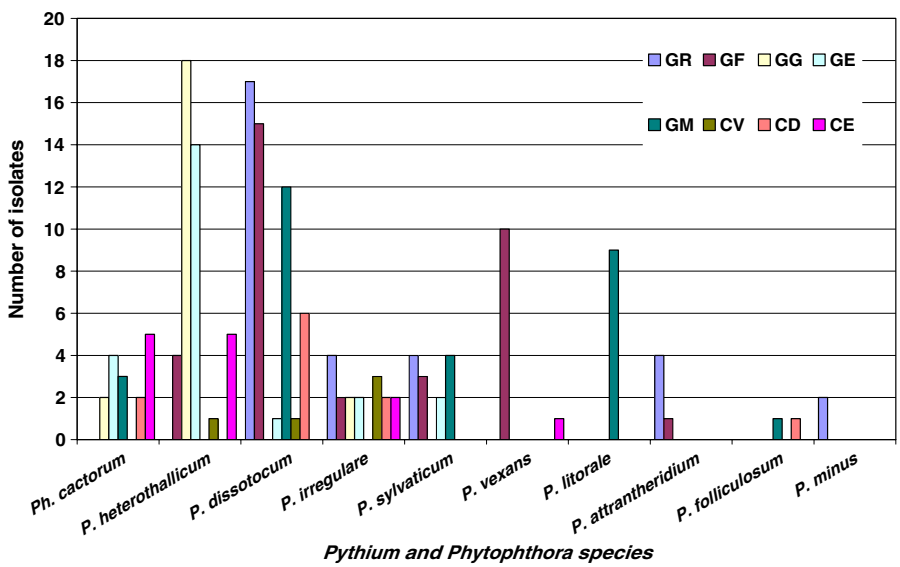

Fig. 1 Distribution of *Phytophthora* (*Ph*) and *Pythium* (*P*) species isolated from apple roots and soils obtained from eight orchards (GR, GF, GG, GE, GM, CV, CD and CE) situated in the major apple growing regions of South Africa

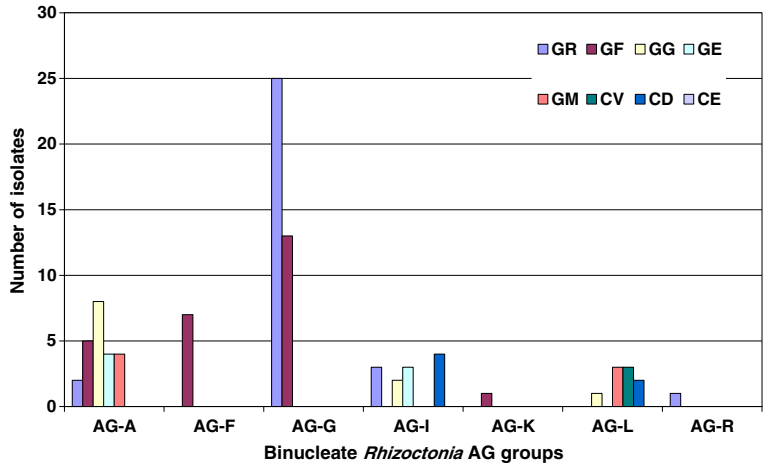

Fig. 2 Distribution of binucleate *Rhizoctonia* anastomosis groups (AGs) isolated from apple roots and soils obtained from eight orchards (GR, GF, GG, GE, GM, CV, CD and CE) situated in the major apple growing regions of South Africa

and AG-I caused a significant (P=0.05) reduction in the weight of seedlings (Table 3).

The oomycete species, as well as isolates within a specific species varied in their ability to affect root rot and increase in weight and height. All isolates of *Ph. cactorum, P. irregulare*, *P. sylvaticum* and *P. vexans* examined were highly virulent, and significantly affected all three measured plant parameters (Table 3). *Pythium dissotocum, P. folliculosum* and *P. heterothallicum* were considered as moderately virulent as isolates within these species only affected one or two of the three measured parameters, typically to a level that was lower than the highly virulent species. The two isolates of *P. attrantheridium* varied in pathogenicity with the one isolate only causing root rot, whereas the other isolate was considered non-pathogenic. *Pythium litorale* were considered weakly virulent, only causing significant root rot. The two *P. minus* isolates were considered to be non-pathogenic since they did not significantly affect any of the three measured parameters (Table 3). In all instances, the introduced oomycete species was recovered from plants grown in infested plant media and was not isolated from plants grown in the non-treated control medium.

Quantitative Real Time PCR (qPCR) analyses using pure culture DNA

Sensitivity testing The sensitivity of qPCR assays for detection of the test oomycetes varied among the different species. Relative sensitivity of detection was

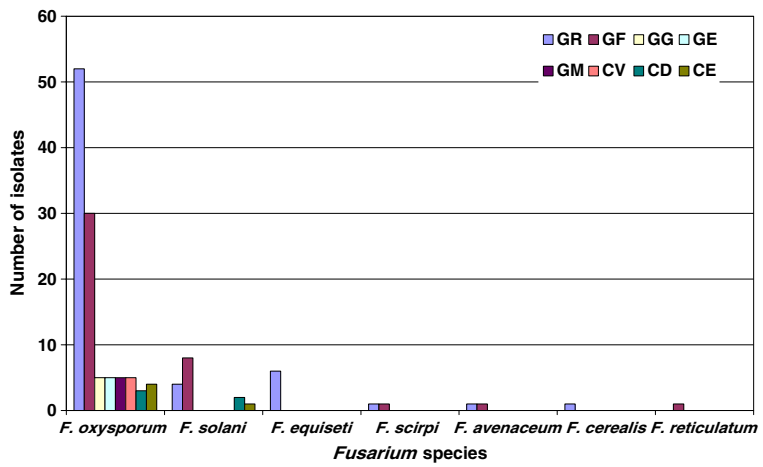

Fig. 3 Distribution of *Fusarium* species isolated from apple roots and soils obtained from eight orchards (GR, GF, GG, GE, GM, CV, CD and CE) situated in the major apple growing regions of South Africa

Table 2 Analysis of variance of the effect of 44 isolates within the genera *Fusarium, Phytophthora, Pythium* and *Rhizoctonia* on mean root rot, weight and height of apple seedlings grown for 3 months under glasshouse conditions

		Root rot		Weight		Height	
Source	DF[x]	MS[y]	SL[z]	MS	SL	MS	SL
Trial	1	0.75	0.0025	8818.37	<0.0001	0.91	0.2661
Block(Trial)	10	0.06	0.6580	49.46	0.7220	0.94	0.2434
Pathogen	4	7.28	<0.0001	3715.24	<0.0001	22.21	<0.0001
Isolate(Pathogen)	39	0.63	<0.0001	376.91	<0.0001	2.03	<0.0001
Isolates within *Control*	1	0	.	197.23	0.0782	1.35	0.0619
Isolates within *Fusarium*	9	0.04	0.6573	31.37	0.8483	0.51	0.4634
Isolates within *Phytophthora*	1	0.26	0.1987	1.98	0.9140	0.16	0.6259
Isolates within *Pythium*	17	1.40	<0.0001	799.37	<0.0001	4.11	<0.0001
Isolates within *Rhizoctonia*	11	0.02	0.9585	57.17	0.2403	0.31	0.9545
Error	473	0.08		70.36		0.74	
Corrected Total	527						

[x] DF degree of freedom, [y] MS mean sqare, [z] SL Significance level

lowest for the genus *Phytophthora* (100 fg μl^{-1}), followed by *P. sylvaticum* (10 fg μl^{-1}), and *P. irregulare* and *P. vexans* (1 fg μl^{-1}). This is most likely due to the single copy gene (*Ypt1*) that was used for quantification of the genus *Phytophthora,* versus the multi-copy ITS region used for the quantification of the *Pythium* species. Standard curves showed a linear correlation between input DNA and cycle threshold (C_q) values with correlation coefficients (r^2) of 0.988 for *P. irregulare*, 0.999 for *P. vexans*, 0.993 for *P. sylvaticum* and 0.994 for the genus *Phytophthora*.

Specificity testing Specificity testing of the qPCR assays showed that the assays were specific and did not detect any of the closely related species that were evaluated for each target species.

qPCR analyses of genomic DNA extracted from apple seedling roots

qPCR amplification of root DNA qPCR amplifications employing root DNA from the *Phytophthora* and the *Pythium* (*P. irregulare, P. sylvaticum* and *P. vexans*) treatments were successful. No amplification product was obtained using DNA from roots of apple grown in control planting media infested with uncolonized sand-bran medium. Analyses of a subset of the root DNA from representative treatments for the presence of PCR inhibitors such as humic acids showed that inhibitors were absent.

The amount of pathogen DNA detected in apple roots differed for some of the isolates within a species, and/or between trials (Table 4). This variation was not seen for the weight and height of seedlings that were inoculated with different isolates from the same species, or for isolates between the two trials (data not shown). *Pythium irregulare* isolate STE-U7194 was detected at a higher DNA concentration in trial 1 than that of *P. irregulare* isolate STE-U7193. A similar difference was observed for the two isolates of *P. sylvaticum*. For *P. vexans*, isolate STE-U6742 was detected at the highest DNA concentration among all the oomycete isolates examined, while isolate STE-U 6741 was not detected at a C_q of 40. The two *Ph. cactorum* isolates were detected at comparable DNA concentrations in apple roots within a trial, but for trial 2 the DNA concentrations were much lower.

Correlation between oomycete DNA concentration in roots with weight, height and root rot of seedlings A significant negative correlation was observed between pathogen DNA concentration in roots and seedling weight, and a significant positive correlation for root rot for some, but not all, species. For *P. irregulare,* a significant negative correlation was observed between pathogen DNA concentration in roots and seedling weight (r=−0.526; P=0.0248). For *Phytophthora* and *P. sylvaticum* a significant positive correlation was observed between pathogen DNA in roots and root rot (r=0.503; P=0.0334 and r=0.572; P=0.0132, respectively). However, no significant correlation was observed between *P. vexans* DNA in roots and any disease assessment parameter (Table 5).

Table 3 Mean root rot, weight and height of apple seedlings grown in steam pasteurized media artificially infested with *Fusarium, Pythium, Phytophthora* and *Rhizoctonia* isolates

Isolate code[y]	Parameter evaluated[x]		
	Root rot[z]	Weight	Height
CON1	0.00 j	55.06 a–e	32.14 a–e
CON2	0.00 j	60.79 a	35.61 a
Pythium attrantheridium STE-U 7185	0.38 ef*	51.49 c–f	31.09 a–f
Pythium attrantheridium STE-U 7186	0.19 f–j	51.87 c–f	28.40 d–i
Pythium dissotocum STE-U 7187	0.33 efg*	45.34 fgh*	28.10 e–i
Pythium dissotocum STE-U 7188	0.33 efg*	51.19 def	31.70 a–f
Pythium folliculosum STE-U 7189	0.13 g–j	43.75 gh*	26.02 g–k*
Pythium folliculosum STE-U 7190	0.22 f–j	40.03 hij*	29.04 c–h
Pythium heterothalicum STE-U 7191	0.49 de*	49.01 efg	26.84 f–j*
Pythium heterothalicum STE-U 7192	0.24 f–i*	46.70 fgh*	29.06 c–h
Pythium irregulare STE-U 7193	0.81 bc*	33.17 k*	20.71 l*
Pythium irregulare STE-U 7194	0.68 cd*	35.35 jk*	21.65 kl*
Pythium litorale STE-U 7195	0.24 f–i*	55.84 a–d	31.43 a–f
Pythium litorale STE-U 7196	0.25 f–i*	59.07 ab	33.05 a–e
Pythium minus STE-U 7197	0.19 f–j	56.73 a–d	31.13 a–f
Pythium minus STE-U 7198	0.11 g–j	59.37 ab	33.70 abc
Pythium sylvaticum STE-U 7199	1.22 a*	36.74 ijk*	20.27 l*
Pythium sylvaticum STE-U 7200	1.10 a*	40.19 hij*	22.85 jkl*
Pythium vexans STE-U 6741	0.79 c*	40.03 hij*	25.12 h–l*
Pythium vexans STE-U 6742	0.79 c*	44.09 gh*	23.46 i–l*
Phytophthora cactorum STE-U 7203	1.11 a*	42.16 hi*	23.41 i–l*
Phytophthora cactorum STE-U 7204	1.03 ab*	42.73 ghi*	22.21 jkl*
Fusarium avenaceum STE-U 7205	0.17 f–j	60.59 ab	32.08 a–e
Fusarium avenaceum STE-U 7206	0.31 e–h*	58.08 abc	34.18 ab
Fusarium cerealis STE-U 7207	0.11 g–j	56.50 a–d	34.19 ab
Fusarium equiseti STE-U 7208	0.22 f–j	56.88 a–d	34.44 ab
Fusarium equiseti STE-U 7209	0.17 f–j	55.78 a–d	32.10 a–e
Fusarium oxysporum STE-U 7210	0.17 f–j	58.86 ab	33.95 abc
Fusarium oxysporum STE-U 7211	0.14 g–j	57.21 a–d	30.66 a–g
Fusarium reticulatum STE-U 7212	0.19 f–j	55.14 a–e	34.14 ab
Fusarium solani STE-U 7213	0.15 f–j	56.16 a–d	32.41 a–e
Fusarium solani STE-U 7214	0.11 g–j	58.06 abc	30.52 b–g*
Rhizoctonia AG-A STE-U 7215	0.06 ij	56.24 a–d	32.09 a–e
Rhizoctonia AG-A STE-U 7216	0.14 g–j	56.63 a–d	33.03 a–e
Rhizoctonia AG-F STE-U 7217	0.17 f–j	51.98 c–f*	34.71 ab
Rhizoctonia AG-F STE-U 7218	0.13 g–j	54.69 a–e	31.28 a–f
Rhizoctonia AG-G STE-U 7219	0.10 hij	55.39 a–e	33.40 a–d
Rhizoctonia AG-G STE-U 7220	0.08 hij	54.20 a–e	31.34 a–f
Rhizoctonia AG-I STE-U 7221	0.08 hij	55.32 a–e	33.12 a–e
Rhizoctonia AG-I STE-U 7222	0.17 f–j	53.88 b–e*	30.92 a–g
Rhizoctonia AG-K STE-U 7223	0.10 hij	57.48 a–d	31.00 a–g
Rhizoctonia AG-L STE-U 7224	0.14 g–j	58.85 ab	32.12 a–e
Rhizoctonia AG-L STE-U 7225	0.14 g–j	57.43 a–d	31.43 a–f
Rhizoctonia AG-R STE-U 7226	0.11 g–j	59.63 ab	33.19 a–d
$LSD_{P=0.05}$	0.228	6.729	5.051

[x] Values are means of six replications of two repeated trials. Data were pooled over two trials. Means within a parameter (column) followed by the same letter do not differ significantly ($P=0.05$). Values within a parameter (column) that differ significantly from the CON1 control for oomycetes (*Phytophthora* and *Pythium*) and the CON2 control for fungi (*Fusarium* and *Rhizoctonia*) are indicated by "*"

[y] Two controls were used. CON1 is the control for sand-bran inoculum used for inoculation of *Pythium* and *Phytophthora* isolates, and CON2 is the control for millet seed used for inoculating *Fusarium* species and *Rhizoctonia* anastomosis groups (AG) isolates. All cultures were submitted to the Stellenbosch University culture collection (STE-U)

[z] Root rot was evaluated on a 0–4 scale, with 0=no obvious symptoms; 1=moderate discolouration of root tissue; 2=extensive discolouration of tissue; 3=extensive discolouration of tissue with girdling lesions; and 4=plant dead. Seedling weight (g) and height (cm) were determined by subtracting values obtained at the termination of the trial, from those at the initiation of the trial

Discussion

This study showed that, the fungal genera *Fusarium* and *Rhizoctonia,* and the oomycete genera *Phytoph-*

Table 4 Mean pathogen (*Pythium irregulare, P. sylvaticum, P. vexans* and *Phytophthora*) DNA concentrations detected through quantitative real-time polymerase chain reaction analyses of DNA isolated from apple seedling roots

Genus	Species and isolate	Mean pathogen DNA (pg μl^{-1})[y] ± STD[z]	
		Trial 1	Trial 2
Pythium	*P. irregulare* STE-U7193	0.04±0.03	0.08±0.06
	P. irregulare STE-U7194	0.33±0.12	0.04±0.06
	P. sylvaticum STE-U7199	13.17±4.07	0.57±0.94
	P. sylvaticum STE-U7200	2.28±1.55	2.62±1.79
	P. vexans STE-U6741	0±0	0±0
	P. vexans STE-U6742	99.03±56.02	104.77±26.29
Phytophthora	*Ph. cactorum* STE-U7203	43.46±23.1	2.61±1.86
	Ph. cactorum STE-U7204	34.24±30.1	7.91±7.42

[y] Pathogen DNA concentration is the amount of pathogen DNA amplified from 40 ng of root genomic DNA that was extracted from 60 mg of root tissue

[z] The mean DNA concentration for each isolate within a trial was determined from three DNA root samples (three differentent replicates) from two independent pathogenicity trials (Trial 1 and Trial 2). The standard deviation (STD) (±) is shown for the three replicates within a trial. Two qPCR reactions were conducted on each root DNA sample

thora and *Pythium* are associated with apple tree roots in South African apple orchards. Investigations in eight orchards identified seven species of *Fusarium*, nine species of *Pythium*, one species of *Phytophthora* and seven binucleate *Rhizoctonia* AGs. The species and AGs were differentially distributed across the orchards, with some having a wider distribution than others. Pathogenicity testing of representative isolates demonstrated that not all of the species from the different genera were pathogenic towards apple seedlings. The most virulent species were found within the genera of *Phytophthora* and *Pythium* and included *Ph. cactorum, P. irregulare, P. sylvaticum* and *P. vexans*. qPCR analyses effectively detected these three *Pythium* species and the genus *Phytophthora* in roots of seedlings grown in artificially infested soils. However, the qPCR data varied quantitatively for different isolates within a species and/or between glasshouse repeat trials. Correlation analyses of pathogen DNA concentration and seedling weight and height showed that a significant negative correlation was only found for seedling weight and *P. irregulare* DNA concentrations.

Table 5 Pearson correlation (r) values between pathogen DNA concentrations obtained from artificially infected apple seedling roots with the weight, height and root rot of these seedlings that were grown in media artificially inoculated with these pathogens

Pathogen	Parameter		
	Weight	Height	Root rot
Phytophthora cactorum	−0.211	−0.098	0.503*
Pythium irregulare	−0.526*	−0.387	0.309
Pythium sylvaticum	−0.436	−0.484	0.572*
Pythium vexans	−0.294	−0.460	0.365

Values indicated with "*" are significantly different from 0 at a significance level of $P=0.05$

Pathogenicity and virulence of the oomycete species varied among species as well as between isolates within the same species. Isolates of *Ph. cactorum*, *P. irregulare*, *P. sylvaticum* and *P. vexans* were highly virulent causing extensive root rot and seedling growth reductions (height and weight). Previous studies also identified *P. irregulare* (Sewell 1981), *P. sylvaticum* (Sewell 1981; Mazzola 1998) and *Ph. cactorum* (Mazzola 1998) as species that are highly virulent towards apple. This is, however, the first report of *P. vexans* being pathogenic and highly virulent towards apple. *Pythium ultimum* and *P. intermedium* are also well known species reported to be highly virulent toward apple (Sewell 1981; Jeffers et al. 1982; Mazzola 1998), but these species were not identified in the current study. *Pythium folliculosum, P. dissotocum* and *P. heterothallicum* were moderately virulent, although it is difficult to deduce the pathogenic and virulence potential of these species since only two isolates of each species were evaluated. This was also true for *P. attrantheridium* and *P. litorale* that seemed to have low virulence, only causing root rot, whereas *P. minus* isolates were not pathogenic. This is the first report of *P. folliculosum* and *P. litorale* functioning as pathogens of apple, and the first report of an association between *P. minus* and apple. For some of the species, such as *P. dissotocum, P. folliculosum* and *P. heterothallicum,* the two tested isolates varied in their virulence. Sewell (1981) and Mazzola et al. (2002) also found

that not all oomycete isolates within the same species (*P. coloratum* (syn. *P. dissotocum*), *P. echinulatum, P. intermedium, P. oligandrum* and *P. sylvaticum*) caused the same response in apple seedlings.

The qPCR methods that were optimized for the highly virulent oomycete species (*P. irregulare, P. sylvaticum, P. vexans* and the genus *Phytophthora*) will be useful for accurate detection of these pathogens in a high throughput manner from many orchard and nursery materials, although these methods may be less useful for quantification of the pathogens from roots. For example, the one *P. vexans* isolate could not be detected from roots even though it was highly virulent, whereas the other isolate was consistently detected at high DNA concentrations in roots. In addition, inconsistencies in the quantity of *Ph. cactorum* DNA detected in infected seedlings were documented between the two pathogenicity trials. Lastly, the qPCR methods may be less useful for meaningful quantification of the pathogens from roots, since a significant negative correlation was not found between DNA concentration and seedling height and weight for most species, except for *P. irregulare*. Hypotheses for the aforementioned three observations could include; (i) that the isolates and species have different mechanisms of affecting plant growth negatively, for example some isolates may produce more cell wall degrading enzymes and effectors that induce cell death, and thus do not proliferate extensively in root tissue, (ii) the invasion by secondary saprophytes differed for the species and trials, which negatively influenced DNA quantification of the pathogens, and (iii) *Pythium* spp. are known as root pruners (Cook et al. 1987) and therefore sampling of roots may not be optimal for determining the level of damage they cause, especially since necrotic root tissue can be lost during seedling harvest. The current study, in addition to the study of Bent et al. (2009), are the only studies that have thus far attempted to correlate the DNA concentration of tree fruit root pathogens in roots with plant growth inhibition, using qPCR.

Among *Rhizoctonia* isolates, multi-nucleate isolates (AG-5 and AG-6) are highly virulent towards apple, whereas in tree fruits binucleate isolates are in general non-pathogenic or have low virulence (Mazzola 1997; Manici et al. 2003). In the current study only binucleate isolates were recovered. This is the first report of the association of *Rhizoctonia* AG-F, AG-K, AG-L and AG-R world-wide with apple, and AG-A, AG-G and AG-I for South Africa. Most of the binucleate isolates were non-pathogenic, except for one (STE-U7217) of the two AG-F isolates and one (STE-U7222) of the two AG-I isolates that had low virulence when compared to the highly virulent oomycete pathogens. AG-I was isolated from four of the orchards, whereas AG-F was only isolated from one orchard. Although Mazzola (1997) also found that only some AG-I isolates were pathogenic towards apple, one of his isolates had a comparable degree of virulence with the highly virulent multinucleate *Rhizoctonia* AG-5 and AG-6 isolates (Mazzola 1997). More isolates of the binucleate AGs recovered from South African orchards will have to be tested for pathogenicity to confirm the current observation that most are non-pathogenic or have low virulence. Even though *Fusarium* isolates were frequently and consistently recovered from all surveyed orchards, most were non-pathogenic towards apple seedlings. The exception was one *F. avenaceum* and one *F. solani* isolate that were both considered as only being weakly virulent. In agreement with this finding, previous studies also showed that *F. solani* was either not pathogenic, or had low virulence towards apple (Mazzola 1998; Manici et al. 2003). *Fusarium oxysporum* isolates, which were obtained from all the orchards, were non-pathogenic, as also previously reported in several other studies (Mazzola 1998; Manici et al. 2003). *Fusarium* species that were found associated with apple in South Africa, which have not been reported world-wide include *F. avenaceum, F. cerealis, F. reticulatum* and *F. scirpi.* For South Africa, this is also the first report of the association of *F. oxysporum, F. solani* and *F. equiseti* with apple orchard soils.

The study showed that although *Fusarium, Phytophthora, Pythium* and *Rhizoctonia* are frequently isolated from apple roots, not all the genera contain highly virulent species. The most virulent species in the seedling bioassays were identified within the genera *Phytophthora* and *Pythium,* specifically *Ph. cactorum, P. irregulare, P. sylvaticum* and *P. vexans.* Oomycetes thus seem to be an important group of organisms that are associated with apple roots, and have potential to cause disease and contribute to ARD in South Africa. Although in the current study and a previous study (Mazzola et al. 2002) only 4–8 week old seedlings were used for evaluating the pathogenicity of oomycetes, some of the species such as *P. cactorum* is a well-known pathogen of mature trees

(Jeffers and Wilcox 1990). Therefore, development of effective strategies to control these pathogens specifically might be instrumental in formulating integrated methods for managing ARD. The optimized qPCR assays used in the current study for detecting highly virulent oomycete species will be useful for evaluating the effectiveness of management strategies in a high throughput manner, and to determine the presence or absence of individual species. Since the qPCR analyses did not detect a negative correlation between pathogen DNA concentrations and plant growth inhibition, except for *P. irregulare*, the significance of the amount of pathogen DNA in roots is currently uncertain. It is, however, important to note that for most species the detection of even small amounts of DNA (0.04–7.91 pg μl^{-1}) in roots was associated with seedling growth inhibition. Although it will be important to target oomycetes in management strategies, suppression of isolates in other genera that have low virulence such as some isolates in the genera *Cylindrocarpon* (Tewoldemedhin et al. 2010), *Fusarium* and *Rhizoctonia* (current study), should not be neglected. These isolates may either increase the severity of damage caused by oomycetes or amongst themselves cause damage especially in association with root lesion nematodes or when plants are under stress.

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