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Cylindrocarpon species associated with apple tree roots in South Africa and their quantification using real-time PCR

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Abstract *Cylindrocarpon* species are known to be a component of the pathogen/pest complex that incites apple replant disease. In South Africa, no information is available on apple associated *Cylindrocarpon* species and their pathogenicity. Therefore, these aspects were investigated. Among the isolates recovered from apple roots in South Africa, four species (*C. destructans, C. liriodendri, C. macrodidymum* and *C. pauciseptatum*) were identified using β-*tubulin* gene sequencing and phylogenetic analysis. This is the first report of *C. liriodendri*, *C. macrodidymum* and *C. pauciseptatum* on apple trees. *Cylindrocarpon macrodidymum* was the most prevalent. Isolates within each of the four species were pathogenic towards apple seedlings, but varied in their virulence. With a single exception, all isolates were able to induce lesion development on seedling roots. Only 57% of the isolates, which represented all four species, were able to cause a significant reduction in seedling weight and/or height. The greatest seedling growth reductions were caused by two isolates of *C. destructans,* and one isolate each of *C. liriodendri* and *C. macrodidymum*. A quantitative real-time polymerase chain reaction (qPCR) method was developed for simultaneous detection of all four *Cylindrocarpon* species. qPCR analyses of *Cylindrocarpon* from the roots of inoculated seedlings showed that the amount of *Cylindrocarpon* DNA in roots was not correlated to seedling growth reductions (weight and height) or root rot. The qPCR method is, however, very useful for the rapid identification of apple associated *Cylindrocarpon* species in roots. The technique may also hold potential for being indicative of *Cylindrocarpon* disease potential if rhizosphere soil rather than roots are used.

Key words Apple replant disease · β-*tubulin* · Soilborne diseases · Ecology

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Introduction

Cylindrocarpon is the hyphomycetous, anamorphic counterpart of *Neonectria* Wollenw. (= *Nectria* (Fr.) Fr. p. p., Ascomycetes, Hypocreales). The genus has a worldwide distribution and occurrs in habitats ranging from tropical rainforest litter to arctic tundra soils (Brayford 1993). Species of *Cylindrocarpon* that attack roots are generally considered as weak or minor pathogens, but they sometimes have a significant economic impact (Mantiri et al. 2001).

Cylindrocarpon has been implicated, along with several other fungi, oomycetes and nematodes, as playing a role in apple replant disease (ARD) (Mazzola 1998). ARD is a soilborne disease that is characterised by the poor growth of trees when established on soils that were previously cultivated to apple. A few *Cylindrocarpon* species have been implicated in ARD in several countries including the USA (Jaffee et al. 1982; Mazzola 1998), Canada (Braun 1991, 1995), Australia (Dullahide et al. 1994), Italy (Manici et al. 2003) and South Africa (Van Schoor et al. 2009). *Cylindrocarpon destructans* has not only been frequently associated with symptomatic apple trees, but has also been shown to be pathogenic towards apple seedlings causing root rot and a reduction in plant biomass. The virulence of isolates varies greatly (Dullahide et al. 1994; Mazzola 1998; Manici et al. 2003). *Cylindrocarpon lucidum*, which causes stunting and black lesions on feeder roots, has also been associated with ARD (Jaffee et al. 1982; Braun 1991, 1995). *Cylindrocarpon heteronema* [anamorph of *Neonectria ditissima* (syn. *Neonectria galligena*] that causes Nectria canker (Swinburne 1975), has been associated with apple roots, but was non-pathogenic towards apple seedlings (Braun 1991). Some *Cylindrocarpon* species can act synergistically with other microbial ARD pathogens such as *Pythium* to cause severe damage to young apple trees (Braun 1995).

Approximately 125 taxa (species and varities) of *Cylindrocarpon* have been described (Mantiri et al. 2001). Booth (1966) was the first to provide a key to segregate 27 species and six varieties of *Cylindrocarpon* into four groups based on the presence or absence of microconida and chlamydospores. Apart from using these morphological characters, correct species identifications have been established using sequencing and phylogenetic analyses of the internal transcribed spacers (ITS) and β-*tubulin* regions (Halleen et al. 2004, 2006; Schroers et al. 2008).

Soilborne plant pathogens such as *Cylindrocarpon*, which form part of a disease complex, are difficult to identify, detect and quantify from soil and plant roots using conventional isolation techniques. Polymerase chain reaction (PCR) based methods that use pathogen specific primers can resolve some of these issues as the methods are usually more sensitive for detecting pathogens, and are also less time consuming and labour intensive (Lievens et al. 2006). Quantitative real-time PCR (qPCR) further has the additional advantage of being more sensitive as well as quantitative in comparison to convential PCR. Therefore, qPCR is very useful for the early and accurate detection and quantification of soilborne plant pathogens in soil and plant material (McCartney et al. 2003; Lievens et al. 2006).

Several PCR primers have been published for the identification and detection of soilborne plant pathogenic *Cylindrocarpon* species. Hamelin et al. (1996) was the first to publish conventional PCR primers specific for the *C. destructans* complex. However, Dubrovsky and Fabritius (2007) stated that these primers would not detect all Californian grapevine *Cylindrocarpon* strains due to a one base-pair mismatch in the primer sequence. They, therefore, designed a new *Cylindrocarpon* genus-specific primer pair for conventional PCR detection of *C. macrodidymum* and *C. liriodendri*, which in theory would also detect *C. destructans* (Dubrovsky and Fabritius 2007). The only qPCR primers that have been published for *Cylindrocarpon,* targeted *Cylindrocarpon destructans* f.sp. *panacis* (Kernaghan et al. 2007).

Cylindrocarpon is known to be associated with ARD in South Africa (Van Schoor et al. 2009). However, the specific species involved and their pathogenicity (ability to damage apple roots) and virulence (amount of damage caused) have not been investigated. Therefore, the first two aims of the study were to determine the *Cylindrocarpon* species associated with apple roots, and to investigate their pathogenicity and virulence towards apple seedlings under glasshouse conditions. Thirdly, a new qPCR technique was developed for simultaneous detection of four *Cylindrocarpon* species associated with apple in South Africa. The qPCR technique was investigated for its ability to (i) quantify *Cylindrocarpon* DNA in roots of apple seedlings and (ii) define the association between *Cylindrocarpon* biomass (DNA) in seedling roots and the degree of seedling growth reduction (weight and height) and root rot.

Materials and methods

Sample collection

Root and soil samples from apple orchards were collected in some of the main apple growing regions

in South Africa. Five orchards were sampled in the Grabouw area (GF, GG, GE, GR and GM), three in the Ceres area (CV, CD and CE) and one each in the Ermelo (ED) and Bethlehem regions (BS). Ceres and Grabouw are located in the south of the country in the Western Cape Province, whereas Bethlehem (Free State Province) and Ermelo (Mpumalanga Province) are located in the north of the country. Root and soil samples were collected from all study sites during autumn (April) or spring (October) from 2006 to 2007.

Isolation of *Cylindrocarpon* from roots

Two different approaches were used for isolating *Cylindrocarpon* from soil and root samples. For orchards BS, GF, GR and ED isolations were made directly from apple roots collected from orchard trees (rootstock MM793) that were nine, three, one and nine years old, respectively. However, these roots yielded many secondary saprophytic fungi due to the fact that old and dying roots can not be differentiated from actively growing roots. A glasshouse experiment can compensate for this in that apple seedlings at this growth stage have a minimal number of old and dying roots and thus contain markedly lower numbers of saprophytes. Therefore, a second glasshouse 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-litre) containing three 4-week-old seedlings were planted.

At seedling harvest roots 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 cabinet. Small root segments were plated onto: (1) water agar (WA) (Agar Bacteriological, Biolab Diagnostics, Midrand, South Africa) containing 100 μg ml^{-1} streptomycin, and (2) potato dextrose agar (PDA) (Biolab Diagnostics, Midrand, South Africa) containing 100 μg ml^{-1} streptomycin. The isolation plates were incubated in the dark at 25°C. Fungal growth that emerged from plated root segments were transferred to WA, and pure cultures were obtained by hyphal-tipping and finally single-sporing.

The number of roots selected for isolation of *Cylindrocarpon* from field grown trees and from seedlings planted in orchard soils and cultivated in the greenhouse varied by orchard site. For orchards GF and GR a total of 288 roots were plated per orchard, whereas for orchards BS and ED a total of only 24 roots were plated per orchard. For isolations from glasshouse grown seedling roots (orchards GG, GM, GE, CV, CD and CE) a total of 48 root segments were plated per orchard.

All single spored *Cylindrocarpon* cultures were stored in 15% glycerol at −85°C, as well as on PDA discs in water at 15°C. A subset of the cultures was submitted to the Stellenbosch University culture collection under accession numbers STE-U6594 to 6633.

Identification of *Cylindrocarpon* isolates to the species level

DNA extractions from Cylindrocarpon cultures DNA was extracted from 5-day-old *Cylindrocarpon* cultures growing on PDA, using a slight modification of the method of Lee and Taylor (1990). The modification consisted of first lysing mycelia in the SDS extraction buffer using glass beads and a mixer/miller (Retsch® MM301, GmbH & Co., Haan, Germany). DNA quantification was conducted using a NanoDrop (NanoDrop Technologies, Wilmington, DE, USA).

PCR amplification and sequencing of the β-tubulin gene A region of the β-*tubulin* gene was PCR amplified from *Cylindrocarpon* isolates (n = 133) using primer pair T1 and T2 (O'Donnell and Cigelnik 1997). Reactions were carried out in a total volume of 40 μl that included 5 μl of diluted DNA (5–10 ng), 0.65 units of BIOTAQ™ DNA polymerase (Bioline, USA Inc., Taunton, MA), 1x PCR buffer (Bioline), 0.2 mg bovine serum albumin (BSA) Fraction V (Roche Diagnostics, Randburg, South Africa), 2 mM $MgCl_2$, 200 μM of each dNTP and 0.2 μM of each primer. Amplification reactions were conducted in a 2700 Applied Biosystems (Foster City, CA) thermal cycler, using amplification conditions consisting of an initial denaturation at 94°C for 5 min, followed by 32 cycles of 94°C for 45 s, 57°C for 45 s and 72°C for 90 s, with a final extension step at 72°C for 7 min. Resulting PCR products (5 μl aliquot) were run on 1% agarose gels, stained with ethidium bromide and visualized under UV illumination.

PCR products were purified using a MSB® Spin PCRapace (Invitek, Berlin, Germany) kit according to

manufacturer's instruction. PCR products were sequenced using primers T1 or T2 and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA), according to manufacturer's instructions. Sequence reaction products were run by the Central Analytical Sequencing Facility at Stellenbosch University using an ABI 3130XL Genetic Analyzer. Geneious Pro 3.6.2 (Biomatters Ltd., Auckland, New Zealand) was used to view ABI trace files, and to obtain consensus double strand sequences for each isolate.

Phylogenetic analyses of the β-tubulin gene sequence data The *Cylindrocarpon* sequences (n = 133) were aligned in Geneious Pro. Many of the sequences had 100% identity to each other, and therefore only a subset of 40 sequences that represented unique sequences were selected for phylogenetic analyses (Fig. 1; Table 1). Nineteen reference sequences were also included from the studies of Halleen et al. (2004, 2006) and Schroers et al. (2008). *Campylocarpon fasciculare* (CBS 113558) and *Fusarium* sp. (U34422) sequences were used as outgroups.

Sequences were aligned online using the software MAFFT alignment program version 6 (Katoh and Toh 2008). Alignments were adjusted manually using the software Geneious Pro 3.6.2 (Biomatters

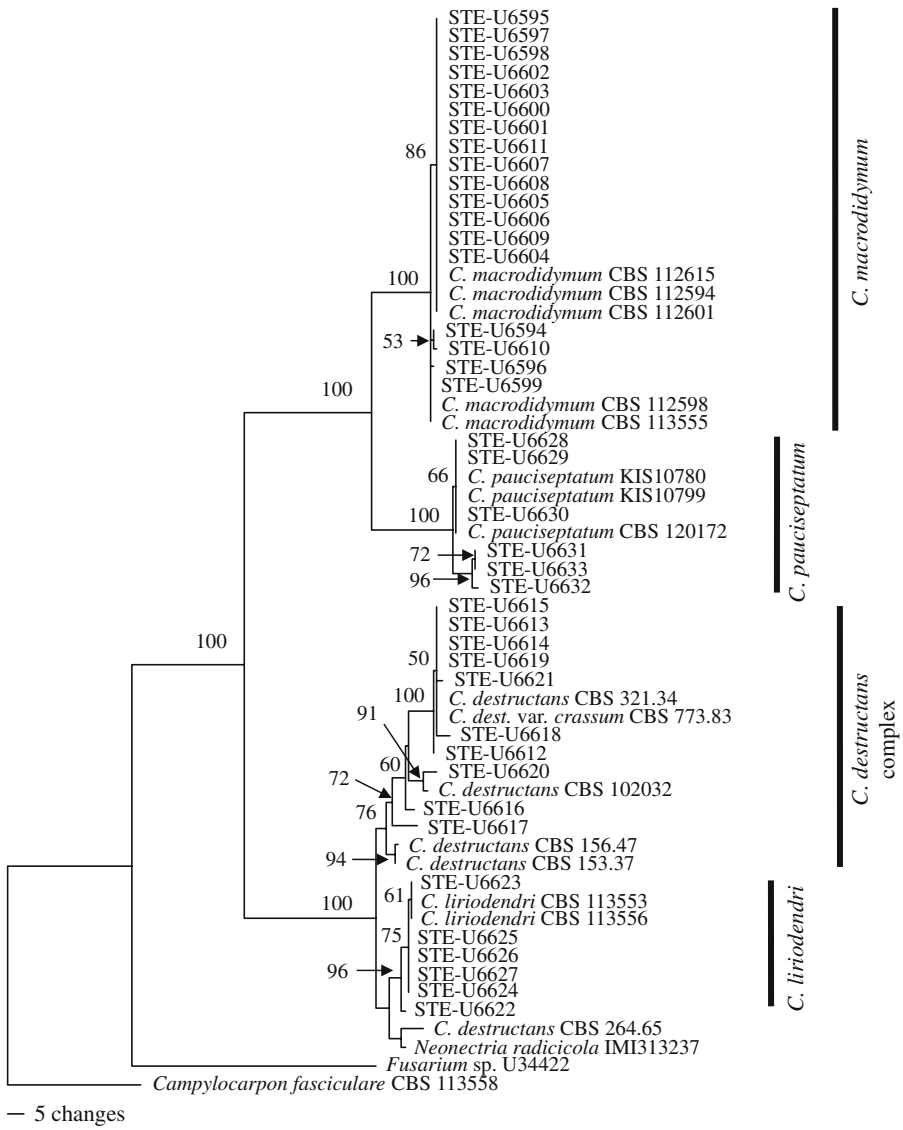

Fig. 1 Phylogeny of *Cylindrocarpon* species based on the partial β-*tubulin* gene region. The tree presents one of 1000 most parsimonious trees obtained from a heuristic search. Numbers within the tree represent the bootstrap values, with values lower than 50% not shown. Length = 400, CI = 0.853, RI = 0.973 and RC = 0.829. *Cylindrocarpon* isolates obtained from South African apple orchards all have "STE-U" codes. The remaining isolates are from the studies of Halleen et al. (2004, 2006) and Schroers et al. (2008). *Campylocarpon* spp. and *Fusarium* sp. were used as outgroups

Table 1 The apple production region of origin and β-*tubulin* GenBank accession numbers of *Cylindrocarpon* isolates from apple roots that were used in pathogenicity studies

Species	Isolate Accession no. [z]	Location	GenBank Accession no.
C. macrodidymum	STE-U6594	Grabouw	GU183630
	STE-U6595	Grabouw	GU183631
	STE-U6596	Grabouw	GU183632
	STE-U6597	Ermelo	GU183633
	STE-U6598	Ermelo	GU183634
	STE-U6599	Ceres	GU183635
	STE-U6600	Grabouw	GU183636
	STE-U6601	Grabouw	GU183637
	STE-U6602	Ceres	GU183638
	STE-U6603	Ceres	GU183639
	STE-U6604	Ceres	GU183640
	STE-U6605	Grabouw	GU183641
	STE-U6606	Ceres	GU183642
	STE-U6607	Grabouw	GU183643
	STE-U6608	Grabouw	GU183644
	STE-U6609	Ceres	GU183645
	STE-U6610	Ceres	GU183646
	STE-U6611	Grabouw	GU183647
C. destructans	STE-U6612	Grabouw	GU183648
	STE-U6613	Grabouw	GU183649
	STE-U6614	Grabouw	GU183650
	STE-U6615	Grabouw	GU183651
	STE-U6616	Bethlehem	GU183652
	STE-U6617	Ermelo	GU183653
	STE-U6618	Ermelo	GU183654
	STE-U6619	Ceres	GU183655
	STE-U6620	Grabouw	GU183656
	STE-U6621	Ceres	GU183657
C. liriodendri	STE-U6622	Grabouw	GU183658
	STE-U6623	Grabouw	GU183659
	STE-U6624	Grabouw	GU183660
	STE-U6625	Grabouw	GU183661
	STE-U6626	Bethlehem	GU183662
	STE-U6627	Ceres	GU183663
C. pauciseptatum	STE-U6628	Grabouw	GU183664
	STE-U6629	Ceres	GU183665
	STE-U6630	Ceres	GU183666
	STE-U6631	Grabouw	GU183667
	STE-U6632	Ceres	GU183668
	STE-U6633	Grabouw	GU183669

[z] Isolates were deposited to the Stellenbosch University culture collection

Ltd., Auckland, New Zealand). Maximum parsimony and distance analysis were conducted in PAUP (Phylogenetic Analysis Using Parsimony) 4.0b10 (Swofford 2002). For distance analysis, neighbour-joining with the uncorrected "p" model was performed. Maximum parsimony analysis was performed using the heuristic search option with a 100 random taxon additions. Tree bisection and

reconstruction (TBR) was used as the branch swapping algorithm with the option of saving no more than 10 trees with a score greater than or equal to 5 (Harrison and Langdale 2006). Gaps were treated as missing data. All characters were unordered and of equal weight. Bootstrap support values were calculated from 1000 heuristic search replicates and 100 random taxon additions. Other measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI) and the rescaled consistency index (RC) values.

Pathogenicity assays

Based on the β-*tubulin* species identifications, a subset of 28 isolates representing the different species were selected for pathogenicity testing. For some species, only six isolates were obtained, and therefore fewer isolates of these species were included. In total ten *C. destructans,* ten *C. macrodydimum,* five *C. liriodendri* and three *C. pauciseptatum* isolates were included in the pathogenicity study.

Apple seeds (Golden delicious) treated with Captan (Dow AgroSciences Southern Africa (Pty) Ltd, Silverton, South Africa) were stratified for 3 months. The stratified seeds were then placed, after another Captan treatment, in perlite for 2 weeks in a growth chamber at ± 25°C. Germinating seeds were transplanted into seedling trays containing a sterile bark and sand (2:1 v/v) growth medium. Seedlings were grown in a glasshouse at 20–25°C for 4 weeks, prior to use in the pathogenicity tests.

Inoculum was prepared using millet seed according to the method described by Strauss and Labuschagne (1995). Control millet seed was inoculated with discs of PDA only. The fungal colonized millet seeds (intact and not crushed) were incubated at 25°C for 3 weeks, after which it was added to pasteurized plant growth media at a rate of 1% (v/v). The plant growth medium consisted of a bark medium and sand (2:1 v/v), of which the pH was corrected to 6.5 by adding dolomitic lime (Gilliam et al. 1982). The medium was pasteurized for 3 h at 82°C and cooled to room temperature overnight. This procedure was repeated once more. After 3 days, millet seed inoculum was mixed into the plant growth medium and was incubated for 24 h at 20 to 25°C prior to planting.

Pathogenicity trials were conducted using 1-litre plastic planting bags. Each treatment was replicated six times, with a replicate consisting of one 1-litre plastic planting bag, containing three 4-week-old seedlings. Seedling weight and height were measured just prior to and after planting, respectively. All trials were conducted in a glasshouse at temperatures ranging from 20 to 25°C and at relative humidity ranging from 60 to 70%. Seedlings were watered twice a week, and on each of these days twice within the same day. The trial was conducted twice.

At 3 months post-planting, seedlings were evaluated for root rot (root discolouration) and increase in seedling weight and height. Root rot was rated on a 0 to 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 prior to planting from those taken at harvest. Re-isolations of introduced *Cylindrocarpon* isolates from seedling roots were conducted as described above, to fulfill Koch's postulate. After the evaluations were conducted, seedling roots from three replicates within each treatment were stored at −85°C for DNA extraction and qPCR analyses (see below).

Statistical analysis Levene's variance ratio test (Levene 1960) was performed to test for homogeneity of variance between the trial repeats. 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 was 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. Analyses of variance were conducted on actual mean weight and height increase of apple seedlings. Pearson's product moment correlation test was performed to determine the correlation between parameters used for evaluating pathogenicity (Otto 1998).

Development of primers for simultaneous detection of four apple associated *Cylindrocarpon* species

Primer selection and design The ITS region was amplified and sequenced from a subset of 19 South African *Cylindrocarpon* isolates using the same PCR reaction and sequencing conditions as described for the β-*tubulin* gene, except that primer pair ITS1 and ITS4 (White et al. 1990) and an annealing temperature of 58°C were used. Alignment of the South African ITS sequences (GenBank accessions GU183670 to GU183676 and GU236522 to GU236533) with published *Cylindrocarpon* sequences showed that the Cyl-R primer of Dubrovsky and Fabritius (2007) perfectly matched these sequences. However, the forward primer Cyl-F had two base pair mismatches, one each at the 5′ and 3′ end, with the South African sequences. Therefore, a new forward primer YT2F (5′-GATGAA GAACGCAGCGAAAT-3′) was designed. The YT2F primer was analyzed for specificity, melting temperature, self-homology, cross-homology, internal stability, PCR product size as well as compatibility with the Cyl-R primer using the NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index).

qPCR sensitivity and specificity testing The ability of primer pair YT2F/Cyl-R to detect all four of the *Cylindrocarpon* species associated with apple was assessed using 32 South African *Cylindrocarpon* isolates that were distributed across the phylogenetic tree (Fig. 1). Sensitivity of the qPCR assay was assessed using standard curves that were constructed for each of the four *Cylindrocarpon* species using two isolates per species. qPCR analysis was conducted using SYBR® Green chemistry on a Rotor-Gene™ 6000 real-time rotary analyzer (Qiagen Inc., Valencia, CA, USA). Reaction mixtures (40 µl) contained 4 µl (10 ng/µl) DNA, 20 µl SensiMix™ dT (Quantace Ltd., London, UK), 1 µl SYBR Green I (Quantace) and 0.2 µM each primer (YT2F and Cyl-R). All reactions were first denatured for 10 min at 95°C, followed by 40 cycles of 95°C for 5 s, 60°C for 5 s and 72°C for 10 s. qPCR data was analyzed using Rotor-Gene™ 6000 Series Software 1.7.

The specificity of the primer pair YT2F/Cyl-R was tested in reactions using pure culture DNA of *Campylocarpon fasciculare*, *Campylocarpon pseudofasciculare, Fusarium oxysporum, F. solani, Pythium irregulare*, *Phytophthora cactorum* and binucleate *Rhizoctonia* AG-G. These species mainly include those that are known to be associated with apple roots, as well as fungi closely related to *Cylindrocarpon.*

qPCR analyses for simultaneous detection of four *Cylindrocarpon* species from apple seedling root DNA

Root DNA extraction Seedling roots from the pathogenicity trial was ground to a fine powder using liquid nitrogen and a IKA® A-11 Analytical mill (IKA®–Werke Gmbh & Co., Staufen, Germany). DNA was

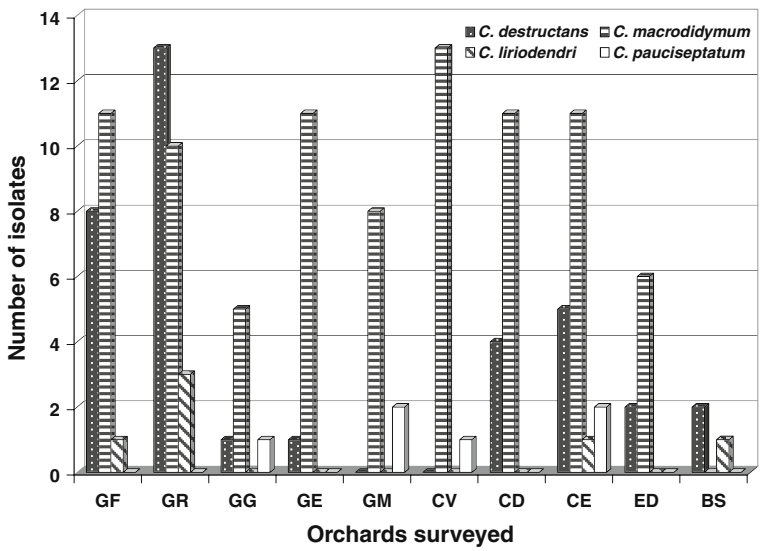

Fig. 2 Distribution of *Cylindrocarpon* species recovered in ten orchards situated in the major apple growing regions of South Africa

extracted using the NucleoSpin® 96 Plant II kit (Macherey Nagel Gmbh & Co. KG, Düren, Germany). Sixty milligram of root tissue was added to 500 µl Buffer PL1 (Macherey Nagel Gmbh & Co.) and 10 µl RNase A, which was mixed vigorously for 30 s in a Retsch® MM301 machine.The samples were incubated at 65°C for 30 min, whereafter the manufacturer's protocol was followed. DNA was eluted with 100 µl of pre-warmed (70°C) Buffer PE (Macherey Nagel Gmbh & Co.).

qPCR analyses on seedling root DNA qPCR reactions and amplifications were conducted as described for pure culture DNA using 40 ng apple root DNA. The amount of *Cylindrocarpon* DNA in seedling roots was quantified using standard curves of each *Cylindrocarpon* species that were constructed using serial dilutions of pure culture DNA spanning nine orders of magnitude (from 40 ng to 0.4 fg), which represented the sensitivity range of the assay.

To investigate the presence of PCR inhibitors in the extracted root DNA a constant amount (10 ng) of *Cylindrocarpon* DNA was added to serial dilutions of root DNA from uninfected apple seedlings, as well as 174 randomly selected root DNA samples. The degree to which the quantification cyles (C_q)-values differed between the serial dilutions was used to determine inhibition.

Statistical analyses Data analyzed included qPCR *Cylindrocarpon* root DNA concentrations, root rot ratings, and seedling weight and height data. Pearson's product moment correlation test was performed to determine the correlation between *Cylindrocarpon* root DNA concentration and: (i) reduction in height, (ii) reduction in weight and (iii) root rot rating (Otto 1998).

Results

Isolation of *Cylindrocarpon* from roots

Among the 540 *Cylindrocarpon* isolates recovered, 133 were identified to the species level, which included isolates representing each orchard in each apple growing region. The number of isolates identified per orchard depended on the frequency of *Cylindrocarpon* isolates recovered in each orchard. In orchards GG, ED and BS, only seven, eight and three isolates were obtained respectively, which were all identified to the species level.

Identification of *Cylindrocarpon* isolates to the species level

The alignment of the β-*tubulin* sequences contained 820 characters of which 155 were parsimony-informative, 105 were variable and parsimony-uninformative, and 560 were constant. The topology of the tree generated with neighbour-joining analysis was similar to the trees obtained using parsimony and is therefore not shown. The β-*tubulin* phylogeny showed that the South African isolates clustered into four well supported clades (76% to 100% bootstrap) representing *C. destructans, C. liriodendri, C. macrodidymum,* and *C. pauciseptatum* (Fig. 1). Therefore, these four species were considered to be the only species associated with apple roots in South African orchards. Within the *C. destructans* clade, which had good bootstrap support (76%), several sub-clades containing South African isolates were evident, indicating the presence of cryptic phylogenetic species in South Africa (Fig. 1).

The South African *Cylindrocarpon* sequences that represented the four species in the phylogeny were used to identify the remaining isolates to the species level. The majority of the isolates were *C. macrodidymum* (85 isolates), followed by *C. destructans* (36 isolates), *C. liriodendri* (six isolates) and *C. pauciseptatum* (six isolates). *Cylindrocarpon destructans* was isolated from all but two orchards (GM and CV), whereas *C. macrodidymum* was isolated from all but one orchard (BS). *Cylindrocarpon liriodendri* and *C. pauciseptatum* were each only isolated from four orchards (Fig. 2).

Pathogenicity assays

Variance for the data from the two independent trials was comparable for root rot based on Levene's variance ratio test (Levene 1960). The error variance ratio for root rot was $P = 0.0568$. Since the error variance ratios for increase in seedling weight and height were $P = 0.0006$ and $P = 0.0016$ respectively, weighted analyses were conducted in order to combine the two trials (John and Quenouille 1977). All data analyses were conducted on the combined data of the two trials.

Almost all of the isolates within each of the species caused a significant ($P = 0.0037$) amount of root rot, which ranged from slight discolouration of roots to severly rotted roots (Table 2; Fig. 3). The exception was the one *C. pauciseptatum* isolate (STE-U6630) that did not cause a significant amount of root rot, nor affected seedling weight and height. Therefore, this isolate was considered non-pathogenic based on the conditions and parameters used in our study. *Cylindrocarpon destructans* isolate STE-U6615 caused the most root rot followed by *C. destructans* isolates STE-U6618 and STE-U6620 and the *C. macrodidymum* isolate STE-U6602 (Table 3). Disease symptoms mainly consisted of black necrotic lesions, which did not have a girdling effect on the root system (Fig. 3). There was no significant correlation between root rot and seedling weight ($r = -0.080$; $P = 0.1365$) or height ($r = 0.103$; $P = 0.0550$) (Table 4).

Isolates within each of the four species differed in their ability to cause a significant reduction in seedling weight ($P < 0.0001$) and height ($P < 0.0001$) (Table 2). Although some isolates within each of the species were able to cause a significant reduction in seedling weight, 39% of the isolates only caused root rot but no significant reduction in seedling weight or height. The latter isolates included individuals within *C. macrodidymum* (STE-U6594, STE-U6599, STE-U6601, STE-U6607 and STE-U6610), *C. destructans* (STE-U6614, STE-U6615 and STE-U6621) and *C. liriodendri* (STE-U6623, STE-U6624 and STE-U6625). Isolates that caused the greatest reduction in seedling weight were *C. destructans* STE-U6616, STE-U6617 and STE-U6619, *C. macrodidymum* STE-U6598 and *C. liriodendri* STE-U6626. These isolates, with the exception of STE-U6619, were also the only isolates that not only caused a significant reduction in weight, but also in seedling height. Analyses showed that there was a positive correlation ($r = 0.662$; $P < 0.0001$) between the ability of isolates to cause a reduction in seedling weight and height (Table 4).

Development of primers for simultaneous detection of four apple associated *Cylindrocarpon* species

qPCR sensitivity and specificity testing Specificity testing with the primer pair, YT2F/Cyl-R showed that the primer pair amplified only DNA derived from the four *Cylindrocarpon* species, but not DNA of the other tested fungal genera. The four *Cylindrocarpon* species differed in amplification sensitivity. The detection level for both *C. destructans* and *C. pauciseptatum* was 10 fg/µl, while the detection level for *C. liriodendri* and *C. macrodidymum* was 1 fg/µl. Standard curves showed a linear correlation between input DNA and cycle threshold (C_q) values with correlation coefficients (r^2) of 0.999 for *C. destructans,* 0.998 for *C. macrodidymum*, 0.997 for *C. liriodendri* and 0.993 for *C. pauciseptatum*.

qPCR analyses for simultaneous detection of four *Cylindrocarpon* species from apple seedling root DNA

Analyses for PCR inhibitors in the extracted root DNA samples indicated the presence of little or no PCR inhibitors in the samples. When low levels of inhibitors were present, very little variation in overall inhibition was observed among different samples.

qPCR analyses showed that roots of seedlings inoculated with the most virulent *Cylindrocarpon* isolates, including STE-U6598, STE-U6616, STE-U6617, STE-U6619 and STE-U6626, did not contain the highest quantity of pathogen DNA (Tables 1 and 5). There was no correlation between the reduction in seedling weight ($r = -0.033$; $P = 0.667$) and height ($r = -0.110$; $P = 0.1517$), and pathogen DNA concentration (Table 4, Fig. 4). Similarly, there was no correlation ($r = 0.196$; $P = 0.1194$) between *Cylindrocarpon* DNA concentration and root rot (Table 4). In general, *Cylindrocarpon* DNA concentrations in seedling roots infected with *C. liriodendri* and *C. pauciseptatum* were lower than that detected in seedling roots infected with *C. destructans* or *C. macrodidymum. C. pauciseptatum* isolate STE-U6630, which was considered non-pathogenic, yielded among the lowest (0.92 pg/µl) *Cylindrocarpon* DNA concentrations in seedling roots (Table 5).

Discussion

Four *Cylindrocarpon* species, *C. destructans*, *C. liriodendri, C. macrodidymum* and C. *pauciseptatum* were associated with apple roots in ten South African

Table 2 Analysis of variance for the effect of 28 isolates from four *Cylindrocarpon* species on mean root rot, and increase in seedling height and weight of apple seedlings grown for 3 months under glasshouse conditions

Source of variation	DF	Root rot		DF	Weight[y]		Height[y]	
		MS	*P*		MS	SL	MS	*P*
Trial	1	1.87	<0.0001	1	13.00	<0.0001	176.06	<0.0001
Block (Trial)	10	0.09	0.5649	10	1.34	0.0407	1.03	0.2946
Species	4	2.37	<0.0001	4	5.32	<0.0001	2.36	0.0287
Isolate within species	24	0.21	0.0037	24	4.06	<0.0001	3.50	<0.0001
CD isolates [z]	9	0.30	0.0120	9	5.89	<0.0001	3.19	0.0093
CM isolates	9	0.13	0.2079	9	5.96	<0.0001	3.91	<0.0001
CL isolates	4	0.15	0.2359	4	8.01	0.0003	2.22	0.0290
CP isolates	2	0.21	0.1213	2	2.27	0.1937	0.50	0.6961
Control	0	.	.	0	.	.	.	.
Error	308	0.10		306	0.69		0.86	
Corrected Total	347			345				

[y] Weighted analyses were done for both increase in weight and height in order to combine data of the two trials

[z] CD = *C. destructans*; CM = *C. macrodidymum*, CL = *C. liriodendri*; CP = *C. pauciseptatum*

orchards. This is the first report of *C. liriodendri*, *C. macrodidymum* and *C. pauciseptatum* on apple trees. *Cylindrocarpon macrodidymum* was the most prevalent species in most orchards, followed by *C. destructans*. Isolates within the *C. destructans* complex were distributed into different sub-clades. This is in agreement with previous suggestions that *C. destructans* may represent a complex of various species (Halleen et al. 2006). Among the species recovered, only *C. pauciseptatum* has not previously been reported from any other host in South Africa. The remaining species have all been reported as pathogens of grapevines in South Africa (Halleen et al. 2004, 2006). The presence of *Cylindrocarpon* species in apple orchards with possible cross pathogenicity towards grapevines may have implications for disease development in young established vines, since old apple orchards are increasingly being replanted with grapevines in South Africa. In South African grapevines, *C. liriodendri* is the most widely distributed *Cylindrocarpon* species followed by *C. macrodidymum,* with both species being equally virulent towards grapevines (Halleen et al. 2004, 2006).

Cylindrocarpon destructans is the only species from the current study that has also been reported in

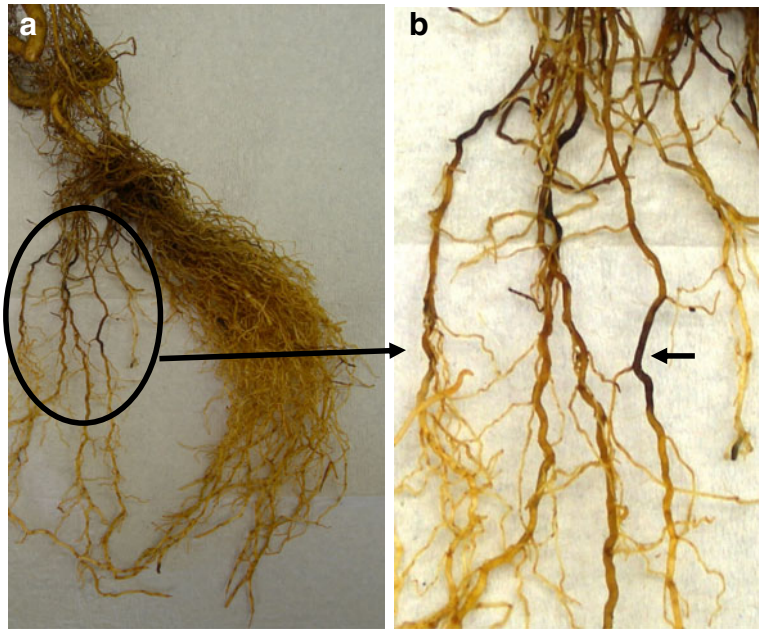

Fig. 3 Three-month-old root system of an apple seedling with root lesions caused by artificial inoculation with a *Cylindrocarpon macrodidymum* isolate: a = the entire root system; b = Closer view of the infected root system, with the black necrotic lesions typically caused by *C. macrodidymum* indicated by an arrow

Table 3 Effect of *Cylindrocarpon* isolates representing four species on root rot severity, and weight and height of apple seedlings

Species	Isolate	Root rot[y]	Weight[y]	Height[y]
Control		0.00 i	23.66 a	9.49 a–i
C. macrodidymum	STE-U6594	0.50 efg	21.84 a–e	8.51 d–m
	STE-U6595	0.65 c–f	19.88 c–g	8.44 e–m
	STE-U6596	0.72 b–e	19.81 c–h	9.83 a–f
	STE-U6597	0.58 c–g	19.02 e–j	8.88 b–k
	STE-U6598	0.75 b–e	14.54 m	7.20 klm
	STE-U6599	0.75 b–e	21.79 a–e	10.79 a
	STE-U6601	0.67 c–f	21.36 a–e	10.55 ab
	STE-U6602	0.83 abc	18.08 f–k	8.69 c–l
	STE-U6607	0.67 c–f	23.39 ab	10.44 abc
	STE-U6610	0.53 d–g	23.00 abc	9.59 a–h
C. destructans	STE-U6612	0.56 d–g	20.34 b–f	9.14 a–j
	STE-U6613	0.78 bcd	19.26 d–i	8.24 f–m
	STE-U6614	0.64 c–g	22.26 a–d	9.91 a–f
	STE-U6615	1.06 a	20.99 a–f	9.74 a–g
	STE-U6616	0.69 b–f	15.99 j–m	7.05 lm
	STE-U6617	0.67 c–f	14.10 m	6.81 m
	STE-U6618	0.94 ab	18.00 f–l	8.93 b–k
	STE-U6619	0.67 c–f	15.22 klm	7.73 h–m
	STE-U6620	0.83 abc	16.48 i–m	8.94 b–k
	STE-U6621	0.61 c–g	21.46 a–e	10.18 a–e
C. liriodendri	STE-U6623	0.50 efg	20.76 a–f	8.51 d–m
	STE-U6624	0.72 b–e	21.67 a–e	10.24 a–d
	STE-U6625	0.53 d–g	21.34 a–e	9.10 a–j
	STE-U6626	0.75 b–e	14.84 lm	7.69 j–m
	STE-U6627	0.67 c–f	16.67 h–m	8.77 c–l
C. pauciseptatum	STE-U6628	0.39 gh	16.95 g–m	8.04 g–m
	STE-U6629	0.44 fgh	16.96 h–m	8.26 f–m
	STE-U6630	0.19 hi	20.84 a–f	7.97 h–m
	LSD[z]	0.256	3.193	1.765

[y] Apple seedlings inoculated with *Cylindrocarpon* were grown for 3 months under glasshouse conditions. Root rot (root discoloration or complete root rot) was evaluated on a 0 to 4 scale, with 0 = no obvious symptoms; 1 = moderate discoloration of root tissue; 2 = extensive discoloration of tissue; 3 = extensive discoloration of tissue with girdling lesions; and 4 = plant dead. Mean 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. Values are the mean of six replications, and data are pooled over two trials. Means within a parameter (in a column) followed by the same letter do not differ significantly ($P = 0.05$)

[z] LSD = least significant difference

Table 4 Correlation matrix (Pearson) of pathogen DNA concentrations as determined by quantitative real-time PCR using DNA extracted from artificially infected apple seedling roots, and the weight and height response of the seedlings after 3 months

Variables	Weight	Height	Root rot	DNA (pg/uL)[z]
Weight	**1**[y]	**0.662**	−0.080	−0.033
Height	**0.662**	**1**	0.103	−0.110
Root rot	−0.080	0.103	**1**	0.196
DNA (pg/uL)	−0.033	−0.110	0.196	**1**

[y] Values in bold are significantly different from 0 at a significance level $P = 0.05$

[z] *Cylindrocarpon* 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

other regions of the world from apple (Braun 1991, 1995; Mazzola 1998). Species that were not found in South African orchards, but that have been reported elsewhere include *C. lucidum* and *C. heteronema* (Braun 1991; 1995). These discrepancies in species distribution could be due to some mis-identifications in previous studies, and new species only being described recently such as *C. liriodendri* (isolates previously identified as *C. destuctans*) (Halleen et al. 2006) and *C. pauciseptatum* (Schroers et al. 2008).

The pathogenicity and virulence of isolates within the four *Cylindrocarpon* species varied. With the exception of *C. pauciseptatum* isolate STE-U6630, all 28 isolates investigated were considered to be

Table 5 Mean *Cylindrocarpon* DNA (pg/μl) concentrations obtained through quantitative real-time PCR analyses of infected apple seedling roots

Species and Isolates	Mean DNA concentration in pg/μL[y] ± STD[z]	
	Trial 1	Trial 2
C. macrodidymum		
STE-U6594	12.56 ± 3.57	47.95 ± 67.97
STE-U6595	15.67 ± 6.69	101.41 ± 80.99
STE-U6596	12.41 ± 2.34	66.98 ± 16.87
STE-U6597	9.96 ± 3.96	33.87 ± 21.90
STE-U6598	7.23 ± 2.39	11.07 ± 6.80
STE-U6599	5.06 ± 0.60	26.71 ± 19.97
STE-U6601	4.80 ± 2.31	32.56 ± 45.45
STE-U6602	29.53 ± 13.88	36.55 ± 50.21
STE-U6607	0.71 ± 0.41	0.20 ± 0.29
STE-U6610	0.09 ± 0.01	0.03 ± 0.03
C. destructans		
STE-U6612	102.78 ± 3.41	1.70 ± 0.59
STE-U6613	31.30 ± 9.08	23.35 ± 35.27
STE-U6614	0.96 ± 0.03	33.54 ± 46.26
STE-U6615	34.45 ± 14.99	122.30 ± 30.28
STE-U6616	11.10 ± 1.07	11.78 ± 4.43
STE-U6617	1.77 ± 0.88	9.56 ± 5.86
STE-U6618	39.19 ± 10.45	0.23 ± 0.06
STE-U6619	4.04 ± 0.87	8.83 ± 3.17
STE-U6620	11.36 ± 5.03	19.64 ± 10.89
STE-U6621	3.53 ± 0.93	3.09 ± 2.79
C. liriodendri		
STE-U6623	2.01 ± 0.67	0.03 ± 0.03
STE-U6624	3.11 ± 0.64	0.33 ± 0.26
STE-U6625	10.02 ± 0.98	0.10 ± 0.15
STE-U6626	9.90 ± 1.82	0.31 ± 0.29
STE-U6627	1.38 ± 1.10	0.10 ± 0.06
C. pauciseptatum		
STE-U6628	1.44 ± 0.38	0.01 ± 0.02
STE-U6629	5.29 ± 2.02	0.05 ± 0
STE-U6630	0.17 ± 0.01	1.67 ± 1.39

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

[z] The mean DNA concentration for each isolate was determined from a total of 12 quantitative real-time PCR reactions, which consisted of two qPCR reactions conducted on six DNA extractions (three per trial) from apple seedling roots inoculated with different *Cylindrocarpon* isolates in two independent trials

pathogenic, since they significantly affected at least one of the evaluated criteria (root rot, seedling weight or height). Significant variation in virulence was observed among isolates within each of the species, with 39% of the isolates being designated as having low virulence and only causing root rot. Although exhibiting low virulence, these isolates may still play an important role within the ARD context, since the necrotic lesions they cause may serve as entry points for more aggressive necrotrophic pathogens such as *P. irregulare* that are known to be associated with ARD (Braun 1995). In contrast to these low virulence *Cylindrocarpon* isolates, four isolates (*C. destructans* STE-U6616 and STE-U6617, *C. macrodidymum* STE-U6598 and *C. liriodendri* STE-U6626) were considered to have high virulence. These isolates caused some of the highest reductions in seedling weight and height. Mazzola (1998) also reported that isolates of *C. destructans* varied in virulence towards apple. On grapevines, variation in the virulence of

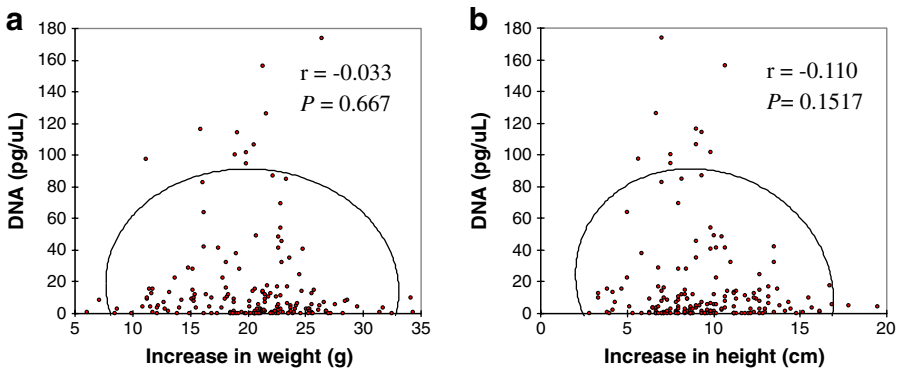

Fig. 4 Scatter plot showing the correlation of *Cylindrocarpon* DNA concentrations obtained through quantitative real-time PCR analyses of artificially inoculated apple seedling roots, with the increase in weight (**a**) and height (**b**) response of the seedlings after 3 months of growth

isolates within *C. macrodidymum* and *C. liriodendri* has also been reported (Alaniz et al. 2009). However, other studies on grapevines did not find differences in virulence between *C. destructans* and *C. macrodidymum* isolates (Halleen et al. 2004; Petit and Gubler 2005).

The molecular detection method developed in this study targeted the four *Cylindrocarpon* species that were recovered from apple, not each of the individual species since: (i) there was no consistent association between species and pathogenicity or virulence, and (ii) the vast majority of isolates within each species were pathogenic. In addition to *Cylindrocarpon*, numerous fungal/oomycete species within a multitude of genera (*Pythium, Phytophthora* and *Rhizoctonia*) must be monitored in studies concerning the cause and management of ARD. As such, the development of a qPCR method for simultaneous detection of the four *Cylindocarpon* species was deemed of greater value, relative to assays for individual species, in the conduct of future ARD investigations. A potential limitation of this multi-species qPCR assay is the possibility for detection of a few other *Cylindrocarpon* species that are phylogenetically most closely related to our four target species. These closely related species include *C. cylindroides, C. faginatum, C. heternema/N. galligena, C. obtusisporum, N. punicae* and *N. ramulariae*. However, among these only *C. heteronema/N. galligena, C. obtusisporum* and some strains of *C. cylindroides* possess 100% homology with our reverse primer and of these only *C. heteronema/N. galligena*, now known as *Neonectria ditissima*, has been reported from apples worldwide. Since *Neonectria ditissima*, which mainly causes cankers, does not occur in South Africa (Carstens et al. 2010) the primer pair will most likely only amplify *Cylindrocarpon* species known to be pathogenic towards apple roots in South Africa.

qPCR analyses showed that the *Cylindrocarpon* DNA concentrations in roots was not correlated with growth reductions observed in apple seedlings. Therefore, qPCR on root DNA might not be a reliable method for use in combination with greenhouse-based seedling bioassays when evaluating the potential of these pathogens to incite host damage on orchard replant sites. Another potential problem with the quantification of *Cylindrocarpon* DNA from roots may be large variance, which was observed between trials for some but not all of the isolates, as well as within a trial for some of the three replicates.

It is difficult to propose a basis for the lack of correlation observed between *Cylindrocarpon* DNA concentrations and seedling growth reductions, since very little is known about the mechanisms involved in *Cylindrocarpon* root colonization, infection and plant growth reductions. One hypothesis could be that the most virulent isolates do not proliferate extensively within the host tissue, but rather cause damage to host cell function due to the excess secretion of cell wall degrading enzymes or toxins. For example, *C. didymum* produces mycotoxins that cause stunting of plant root systems and thus plant growth (Barbetti 2005). Alternatively, *Cylindrocarpon* may also attack fine feeder roots, which are lost during the harvest and washing of seedling roots. Future studies should rather investigate whether the quantification of *Cylindrocarpon* DNA from rhizosphere soil and bulk soil will be a better predictor of host damage by these pathogens. This approach may have potential for the apple pathogen system since qPCR analyses of *C. destructans* f. sp. *panacis* from soil showed that the

pathogen DNA concentrations were significantly correlated with disease severity on ginseng (*Panax quinquefolius*) in artificially and naturally infested soil (Kernaghan et al. 2007).

The identification of four pathogenic *Cylindrocarpon* species in South African apple orchards suggests that they may play a role in the etiology of ARD. Future investigations should be aimed at investigating the interaction of *Cylindrocarpon* with other known replant pathogens and pests in apple orchards. The qPCR method that was developed for apple associated *Cylindrocarpon* species will assist these investigations, since isolation studies are very time consuming and often inaccurate in detecting these pathogens. Another aspect of the role of *Cylindrocarpon* in ARD that needs investigation, is whether these pathogens are only minor pathogens (Mantiri et al. 2001), and whether they act synergistically with other replant pathogens such as plant-pathogenic nematodes (Sutherland 1977) and *Pythium* (Jaffee et al. 1982; Braun 1991, 1995). The aforementioned assessments are required to define the overall contribution of *Cylindrocarpon* to ARD when acting in concert with the complexity of microorganisms resident to orchard replant soils.

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