Canker and Wood Rot Pathogens Present in Young Apple Trees and Propagation Material in the Western Cape of South Africa

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Abstract

Canker and wood rot pathogens cause dieback and, in severe cases, the death of young apple trees. Recently, a higher occurrence of cankers was observed on 1-year-old apple trees in the Western Cape Province of South Africa. This study aimed to assess the phytosanitary status of nursery trees and propagation material as possible inoculum sources for canker pathogens. Thirteen 1-year-old apple orchards showing canker or dieback symptoms were sampled. Certified nursery apple trees were collected from four nurseries as well as scion and rootstock mother plant material. Isolations were made from the discoloration observed in the vascular tissue of the plant parts and from asymptomatic material. Possible canker and wood rot species were identified with PCR and sequence comparisons of the relevant gene regions and phylogenetic analyses. Similar canker and wood rot species were isolated from 1-year-old diseased apple trees, nursery apple trees, and the propagation material. Forty-five fungal species associated with canker or wood rot symptoms were identified. The top five most abundant fungal species found causing

disease on commercial 1-year-old trees were also found in high numbers causing latent infection in certified apple nursery trees. These species were *Didymosphaeria rubi-ulmifolii* sensu lato, *Diplodia seriata*, *Schizophyllum commune*, *Didymella pomorum*, and *Coniochaeta fasciculata*, with *D. rubi-ulmifolii* sensu lato being the dominant species in both sampling materials. In all, 65% of certified nursery apple trees, 5% of scion shoots used for budding, and 21% of rooted rootstock cuttings from layer blocks had latent infections of canker and wood rot pathogens. Pathogenicity trials were conducted with isolates of 39 species, inoculated onto 2-year-old branches of 14-year-old Golden Delicious trees. All species caused lesions that were significantly longer than the control. This study confirmed the presence of canker and wood rot pathogens in apple propagation material as well as certified nursery apple trees, which will aid the improvement of management practices in nurseries.

Keywords: canker, dieback, *Malus* spp., nursery, wood rot

Dieback of apple trees is caused by both true and opportunistic canker and wood rot pathogens giving rise to general symptoms such as cankers, twig blight, and wood rotting (Borovinova et al. 2012; Brown-Rytlewski and McManus 2000; Menapace et al. 2015). The pathogens responsible for canker and wood rot symptoms infect their hosts through wounds and colonize the vascular tissue, resulting in blockage of the vascular system (Cloete et al. 2011). Infected trunks, branches, and shoots start dying back, eventually leading to the death of the entire tree or even failure of the orchard (Zhang et al. 2014). The farmer can face major financial damages when the infections are severe enough for the orchards to become uneconomical because dieback reduces the yield and longevity of the host (Elfar et al. 2013).

Fifteen canker pathogens have been reported to cause dieback or canker symptoms on apple trees in South Africa. These include *Botryosphaeria dothidea* (Moug.) Ces. & De Not., *Neofusicoccum ribis* (Slippers, Crous & M. J. Wingf.) Crous, Slippers & A. J. L. Phillips, *Diplodia mutila* (Fr.) Mont., *D. seriata* De Not., *N. australe* (Slippers, Crous & M. J. Wingf.) Crous, Slippers & A. J. L. Phillips, *Cryptosporiopsis corticola* (Edgerton) Nannf., *Cytospora schulzeri*

Sacc. & P. Syd., *Diaporthe ambigua* Nitschke, *D. foeniculina* (Sacc.) Udayanga & Castl., *C. leucostoma* (Pers.) Sacc., *Cytospora nivea* (Hoffm.) Sacc., species of the family Didymellaceae, *Phaeoacremonium fraxinopennsylvanicum* (T. E. Hinds) Gramaje, L. Mostert & Crous, and *P. minimum* (Tul. & C. Tul.) Gramaje, L. Mostert & Crous (Adams et al. 2006; Cloete et al. 2011; Crous et al. 2000; Smit et al. 1996; Smit et al. 1997; Sutton et al. 2015). Four Basidiomycetes species are associated with wood rot symptoms on apple trees in South Africa. These include *Bjerkandera adusta* (Willd.) P. Karst., *Chondrostereum purpureum* (Pers.) Pouzar, *Schizophyllum commune* Fr., and *Trametes versicolor* (L.) Lloyd (Crous et al. 2000; Matthee and Thomas 1977; Sutton et al. 2015).

Canker and wood rot symptoms are commonly observed in older apple orchards in South Africa. The decline and eventual death of these trees can take an extended period of time (Smit et al. 1996). In contrast, younger trees infected with canker or wood rot pathogens can die more rapidly, especially when exposed to abiotic stress factors (Marek et al. 2013; Smit et al. 1996). Studies conducted in Europe on the death of young, newly established apple trees found that young trees developed large cankers on the main stems shortly after establishment due to infections by *Cylindrocarpus heteronema* (Berk. & Broome) Wollenw. (Brown et al. 1994) and *Neonectria ditissima* Tul. & C. Tul. Samuels & Rossman (McCracken et al. 2003). Both of these studies concluded that the cankers observed in the young apple orchards originated from latent infections that occurred during the propagation process in the nursery.

Marek et al. (2013) investigated the causal agents of cankers forming on young apple trees that were held in cold storage. The study found that latent infections in nursery trees caused disease symptoms during cold storage or shortly after planting (Marek et al. 2013). A study done by Fujita et al. (1988) found that mature apple trees artificially inoculated with the canker pathogen *Diaporthe tanakae* Tak. Kobay. & Sakuma only exhibited typical canker lesions after 2 years. The time required for symptom development of canker and wood rot together with stress due to cold storage used at producer levels explains the absence of symptoms in the nursery.

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Recently, a higher occurrence of canker development was observed on 1-year-old trees in the same season of establishment in the Western Cape Province of South Africa. Two incidences of serious losses (30 and 70%) of newly planted apple trees due to stem cankers have been reported (Disease Clinic, Department of Plant Pathology, Stellenbosch University). Over the past 6 years, 60 trees between the ages of 1 and 3 years old were sent to the Disease Clinic of Stellenbosch University for analysis. These 60 trees represented 60 commercial orchards in the Western Cape with canker or wood rot symptoms. The deciduous fruit industry of South Africa launched an investigation into the reasons behind observed losses of 1-year-old apple trees of specific rootstocks.

Knowledge regarding latent infections of canker or wood rot pathogens in certified nursery trees in South Africa is lacking. The aim of this study was to assess the phytosanitary status of nursery apple trees and propagation material. The objectives of this study were to (i) determine the occurrence of canker and wood rot pathogens in 1-year-old apple trees showing cankers or dieback, certified nursery apple trees, and plant material used during the propagation of apple trees and (ii) assess the pathogenicity of the isolated fungi.

Materials and Methods

Sampling of planting material. *One-year-old orchards.* Thirteen 1-year-old commercial orchards in the Kouebokkeveld, Hermanus, and Grabouw areas were surveyed in March 2015 and March 2016. These orchards exhibited dieback symptoms within 1 year of establishment. Ten trees exhibiting canker symptoms were sampled per orchard. The trees were removed with their roots to ensure that the dieback symptoms were not due to apple replant. In total, 130 trees were sampled over the 2-year period.

Certified nursery apple trees. Nursery plants with scion Golden Delicious and rootstocks MM109, M793, and Geneva (G222) were collected from four fruit tree nurseries in South Africa. In total, 480 trees (160 trees per rootstock) were collected in August to September 2015. Due to the unavailability of some scion–rootstock combinations, other scion cultivar options were also accepted when the Golden Delicious cultivar was not available. Forty trees with the rootstock CG4202 were also collected instead of G222 rootstock, forming part of the Geneva rootstock sampling. Trees belonging to different size classes were also requested from these nurseries (Supplementary Table S1).

Scion mother block orchards. Scion mother block trees were sampled in February and March 2015 and 2016. Three scion cultivars were used, including Rosy Glow, Golden Delicious, and Early Red One. In all, 6 scion mother blocks per scion cultivar were sampled, which resulted in 18 mother blocks in total, and 15 (2016) or 20 (2015) trees were sampled per block. The first year's results indicated that 15 trees would be adequate, and the number was therefore reduced for the following year's sampling. From each tree, a typical canker symptom and pruning wound, as well as a 1-year-old shoot, were sampled. If there were no cankers present on the tree, two pruning wounds were sampled, and if there were more than one canker present on the tree, two cankers were sampled (Supplementary Table S2).

Rootstock mother blocks. Rootstock plant material was collected in June 2016 in collaboration with the three Plant Improvement Organizations (PIOs) of South Africa. Three rootstock cultivars were selected; namely, G222, M793, and MM109. Three layer blocks per rootstock were investigated, and 45 asymptomatic shoots were randomly removed from each block. In total, 405 asymptomatic rooted rootstocks were collected. In one of the nurseries, dieback symptoms were found in the MM109 and M793 1-year-old nursery blocks. These are the rootstock cuttings, which were removed from the layer blocks and established in the nursery soil in August 2015. Dieback occurred on the rootstock shoots shortly after establishment. In all, 45 M793 and 42 MM109 symptomatic shoots were sampled from the corresponding 1-year-old nursery blocks.

Isolation from planting material. *One-year-old commercial apple orchards.* Canker lesions were excised from the tree and triple surface sterilized by soaking in 70% ethanol solution for 30 s, then

1% NaOCl solution for 60 s, and, finally, in 70% ethanol solution for 30 s. The cankered wood pieces were left to air dry on sterile tissue paper in the laminar flow cabinet. A pruning shear (with blades flame sterilized) was used to cut the wood pieces in half lengthwise, exposing the vascular tissue. Twelve wood pieces measuring approximately 2 by 2 mm were removed from the section between the diseased dark-brown vascular discoloration and the healthy tissue, and placed onto 2% potato dextrose agar (Biolab, Midrand) amended with streptomycin sulfate (PDA+s; 40 mg/liter; Calbiochem, Merck). Dishes were incubated at 23°C under natural light for 2 to 3 weeks, or until substantial fungal growth was observed. Representative subcultures were made, using the hyphal tip method, from each primary isolation dish and incubated under the same conditions. Preparation of plant material and isolations were done throughout the study as described in the sampling of 1-year-old tissues.

Certified nursery apple trees. Isolations were made from four parts found on the nursery trees; namely, wounds made on the scion shoot, the bud union, the pruning wound made when the rootstock was cut back, and additional wounds found on the rootstock. If no wounds were found on the scion or rootstock shoot, asymptomatic pieces were used.

Scion mother blocks orchards. Isolations were made from the vascular discoloration found in the cankers and pruning wounds. Furthermore, eight buds were removed from each scion shoot and placed onto PDA+s. Four disks were also cut through the internodes of the scion shoot and placed onto PDA+s.

Rootstock mother blocks. Isolations were made from asymptomatic tissue. Disks were cut through the buds on the rootstock shoot of the rooted cutting with a sterile pruning shear and placed on PDA+s. Disks were cut into quarters and placed onto PDA+s. Three disks were removed from each rooted cutting and placed onto three PDA+s dishes. Isolations were made from the internal vascular discoloration of the 1-year-old nursery material and fruiting bodies on the canker were scraped off and placed onto PDA+s.

Identification of fungal species. Fungal isolates were arranged into different morphological groups based on cultural and morphological characteristics. Cultural characteristics included colony size, color, texture, and shape. Microscopic slides were made from unknown cultures to observe conidia shape and color. Microscopic slides were examined with a Nikon Eclipse E600 compound microscope. Due to the high diversity of fungal genera, representatives of cultural and morphological groups were selected for further molecular identification. All known saprophytes (e.g., *Fusarium*, *Alternaria*, *Epicoccum*, and *Aureobasidium* spp., among others) were discarded. One isolate per morphological group for each plant part was stored and used for further identification.

Molecular identification.

DNA extraction, PCR, and sequencing. Ten isolates from each morphological group were selected for molecular identification. DNA was extracted from 3 week-old fungal cultures on PDA using the protocol of Damm et al. (2008). Primer pairs were selected according to the taxonomic groups.

For species of Diatrypeaceae, Diaporthales, Basidiomycetes, and unidentified fungal cultures, the internal transcribe spacers (ITS)1 and 2 and the 5.8S ribosomal DNA gene area were amplified with ITS5 and ITS4 (White et al. 1990). In a total reaction volume of 20 µl, the PCR contained 1 µl of DNA, 1× KAPA Taq ready mix (KAPABiosystems), 0.4 µM each primer, and double-distilled (dd) H₂O. Reaction conditions consisted of an initial denaturation step at 94°C for 5 min; followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and a final extension step at 72°C for 7 min.

For species in the Botryosphaeriaceae family and Diaporthales order, the elongation factor 1-α gene (EF1-α) was amplified with primers EF1-728F and EF1-968R (Carbone and Kohn 1999) following the previously described PCR volumes. PCR conditions consisted of an initial denaturation step at 94°C for 5 min; followed by 30 cycles of 45 s at 94°C, 45 s at 53°C, and 90 s at 72°C; with a final extension step at 72°C for 7 min. Fungal cultures with *D. seriata*-like morphological characteristics were identified using the species-specific primers DS3.8 S3 and DS3.8 R6 (Martín et al. 2014). In a total reaction volume of 20 µl, the PCR contained 1 µl of genomic

DNA, 8 µl of 2× KAPA Taq ready mix, 0.8 µl of ITS-5 (0.4 pmol/µl), 0.8 µl of ITS-4 (0.4 pmol/µl), and 8.4 µl of double-distilled H₂O. Reaction conditions consisted of an initial denaturation step at 95°C for 5 min; followed by 35 cycles of 30 s at 94°C, 45 s at 57°C, and 45 s at 72°C; with a final extension step at 72°C for 7 min.

Species of *Phaeoacremonium* and *Diatrypaceae* were identified with the partial β -tubulin gene amplified using the primers T1 (O'Donnell and Cigelnik 1997) and Bt2B (Glass and Donaldson 1995). The same PCR volumes and concentrations were used as described earlier. Reaction conditions consisted of an initial denaturation step at 94°C for 5 min; followed by 36 cycles of 45 s at 94°C, 45 s at 55°C, and 90 s at 72°C; with a final extension step at 72°C for 6 min. All PCRs were performed in an Applied Biosystems 2700 PCR machine. A nontemplate control was also included in each PCR run. PCR products were separated by electrophoresis on a 1% (wt/vol) agarose gel in Tris-acetate-EDTA running buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.5) after ethidium bromide staining. The GeneGenius Gel Documentation and Analysis System (Syngene) were used to visualize the gel under UV light alongside a 100-bp DNA ladder (GeneRuler; Thermo Fisher Scientific).

PCR products were purified using the MSB Spin PCRapase kit (Invitex) and prepared for forward and reverse sequencing. Thermocycler conditions were 1 min at 95°C; 30 cycles of 10 s at 95°C, 5 s at 50°C, and 4 min at 60°C; with a final extension of 30 s at 60°C. The gene areas were sequenced using ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (PE Biosystems) with the primers used in the initial PCRs. The nucleotide order of samples was read in an ABI 3130xl DNA sequencer (Perkin-Elmer) at the DNA Sequencing Unit at the Central Analytical Facility of Stellenbosch University.

Phylogenetic analyses. Forward and reverse sequences for each isolate were aligned in Geneious R 9.1.7 (Biomatters Ltd.) and a consensus sequence was extracted. A Basic Local Alignment Search Tool (BLAST) search was conducted against the NCBI (<https://www.ncbi.nlm.nih.gov/>) nucleotide database. Isolates obtained from this study were aligned with representative sequences from each taxonomic group obtained from GenBank (<https://www.ncbi.nlm.nih.gov/genbank>) in Geneious R 9.1.7. Individual taxonomic group sequences' data sets were aligned with L-INS-I method using the program MAFFT v7.222 (Katoh and Standley 2013; Katoh et al. 2002) in Geneious R 9.1.7. Maximum-likelihood (ML) analyses were performed using PhyML (Guindon and Gascual 2003; Guindon et al. 2010) in Geneious R 9.1.7 under the general time-reversible model. Both the γ distribution parameter and proportion of invariable sites were estimated. Bootstrap support values were calculated from 1,000 replicates. Clades with bootstrap support $\geq 70\%$ were considered significant and highly supported (Hillis and Bull 1993).

Pathogenicity trial. Seventy-two fungal isolates representing 39 species collected and identified from young apple trees were used in the pathogenicity trial. The isolates were placed on PDA+s and incubated at 25°C under natural light. Pathogenicity studies were conducted on 14-year-old Golden Delicious apple trees in Grabouw. Two-year-old healthy shoots were surface sterilized with 70% ethanol. The bark was removed with a sterilized 4-mm cork borer, whereafter a hole of 2 to 2.5 mm deep was drilled using a sterilized 4-mm drill bit. A mycelium plug was placed into the wound and sealed with Parafilm. A PDA+s plug was used as a control. Inoculations were made at least 10 cm from the main branch. Ten shoots were used per tree. The trial layout was a balanced incomplete block design with 73 treatments (including the PDA+s as the control). Each tree was seen as an incomplete block and each treatment was replicated 10 times. The trial was repeated on another 14-year-old Golden Delicious orchard on the same farm. Shoots were harvested after 5 months. Isolations were conducted in the same way as described earlier. The identity of the isolates used in the pathogenicity tests were confirmed by sequencing the relevant barcoding gene region for one isolate per species.

Statistical analyses. To test for differences in the frequencies of the different factors, χ^2 tests were performed using Frequency Procedure (PROC FREQ) of the SAS software, version 9.2 (SAS

Institute Inc.). The percentage of infected trees was calculated. Factor (treatment) means (i.e., PIO, cultivars, rootstocks etc.) were tested using PROC TTEST with SAS software. The number of infections caused by the different pathogens was also expressed as a percentage of the total number of infected trees for the different factors (treatments). Normality of standardized residuals was confirmed by the Shapiro-Wilk test (Shapiro and Wilk 1965). Levene's test was used to verify the homogeneity of factor (treatment) variances (Levene 1960). The data were subjected to analysis of variance (ANOVA) using the General Linear Models Procedure (PROC GLM) of SAS software. Fisher's least significant difference (LSD) was calculated at the 5% level to compare factor (treatment) means (Ott and Longnecker 2001).

For the pathogenicity trials, the data (lesion length and fungal re-isolation percentages) from the two trials were subjected to ANOVA separately according to the experimental design, using PROC GLM of SAS statistical software (version 9.4; SAS Institute Inc.). After testing whether trials were of comparable precision by means of Levene's test for homogeneity of variance (Levene 1960), trial results were combined and investigated in one overall ANOVA, as described by John and Quenouille (1977). Where the requirement of homogeneity of trial variance was not met, a weighted ANOVA (weighing with trial variances) was performed. The Shapiro-Wilk test (W test) was performed to test for normality (Shapiro and Wilk 1965). Lesion length was $\ln(x + 1)$ transformed to improve normality (Snedecor and Cochran 1980). Student's *t* LSD was calculated at the 5% level to compare treatment means. A probability level of 5% was considered significant for all tests.

Results

Diversity of fungal taxa. In total, 45 fungal species belonging to the genera *Bjerkandera*, *Botryosphaeria*, *Cadophora*, *Chondrostereum*, *Coniochaeta*, *Cytospora*, *Diaporthe*, *Didymella*, *Didymosphaeria*, *Diplodia*, *Eutypa*, *Neofusicoccum*, *Peniophora*, *Phaeoacremonium*, *Schizophyllum*, *Stereum*, *Trametes*, and *Truncatella* were identified in this study (Table 1; Supplementary Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15). The majority of the species ($n = 38$) are species of Ascomycetes and only seven species belong to the division Basidiomycetes. *Didymosphaeria rubi-ulmifolii* sensu lato was the predominant fungal species isolated from 276 plant parts throughout the study, and was the predominant pathogen found in the 1-year-old commercial trees (present in 11 of the orchards investigated) as well as the nursery material (241 plant parts) (Table 2). Five pathogens were found in all four sampled plant material types; namely, *S. commune*, *D. seriata*, *D. foeniculina*, *Didymosphaeria variabile*, and *D. rubi-ulmifolii* sensu lato. Apart from the possible canker or wood rot species, a variety of saprophytic species from genera such as *Trichoderma*, *Epicoccum*, *Alternaria*, *Aureobasidium*, and *Penicillium* were also isolated.

One-year-old commercial apple orchards. From the 130 diseased 1-year-old trees, possible canker and wood rot pathogens were found in 55 trees (42.3%). Twenty-one different fungal taxa were isolated from dieback symptoms. The most predominant pathogen was *D. rubi-ulmifolii* sensu lato, isolated from 15 trees, followed by *D. seriata* (isolated from 11 trees) and *Eutypella citricola* (isolated from 5 trees) (Table 2). Typical symptoms observed on the 1-year-old trees were constricting cankers (Fig. 1A). A cross section through the cankers exposed dark-brown vascular discoloration, streaking down into the live tissue of the tree (Fig. 1B, C). The majority of trees sampled had well-developed root systems, indicating that the aboveground symptoms were not due to soilborne diseases. Cankers were found on different parts of the trees. From 55 trees, the majority of the pathogens were isolated from the scion shoot (36 trees), followed by the pruning wound on the rootstock (5 trees), the rootstock (10 trees), and, finally, from the bud union (6 trees). Internal and external symptoms associated with canker on the scion shoot of the tree are illustrated in Figure 1. One pathogen (Fig. 1B, C, D) and in some cases more than one pathogen (Fig. 1E, F) was isolated and, more importantly on some trees, canker and wood rot symptoms occurred on more than one plant part (Fig. 1E, F). Multiple infections were often on the scion shoot and from the pruning wound on the rootstock. Fruiting bodies (pycnidia) of Ascomycete fungi, specifically *Coniochaeta* spp., *D. rubi-ulmifolii*

Table 1. List of isolates identified in this study

Group, strain ^u	Species	Area ^v	Cultivar	Plant part	GenBank accession ^t			Support (%) ^w
					ITS	EF	β-Tubulin	
Amphisphaeriaceae								
STEU 8283	<i>Truncatella angustata</i>	G	African Red	Canker on scion shoot of 1-year-old tree	KY312617	...	...	98
STEU 8284	<i>T. angustata</i>	K	Golden Delicious	Bud union of a nursery tree	KY312618	...	...	...
Basidiomycetes								
STEU 8285	<i>Bjerkandera</i> sp.	R	Royal Beaut/MM109	Pruning wound on rootstock of a nursery tree	KY312619	...	...	87 ^x
STEU 8286	<i>Bjerkandera</i> sp.	R	Royal Beaut/M793	Soft rot in pruning wound of nursery tree	KY312620	...	...	...
STEU 8287	<i>Chondrostereum purpureum</i>	K	Rosy Glow	Pruning wound on a mature tree	KY312621	...	...	100
STEU 8288	<i>Peniophora</i> sp. 2	K	Golden Delicious	Canker on scion shoot of 1-year-old tree	KY312622	...	...	96
STEU 8289	<i>Peniophora</i> sp. 2	L	Golden Delicious/M793	Bud union of a nursery tree	KY312623	...	...	...
STEU 8290	<i>Peniophora</i> sp. 1	K	Rosy Glow	Pruning wound on a mature tree	KY312624	...	...	100
STEU 8291	<i>Peniophora</i> sp. 1	L	Golden Delicious/M793	Bud union of a nursery tree	KY312625	...	...	...
STEU 8292	<i>Schizophyllum commune</i>	L	Golden Delicious/M793	Pruning wound on rootstock of nursery tree	KY312626	...	...	99
STEU 8293	<i>S. commune</i>	K	Royal Beaut	Canker on scion shoot of 1-year-old tree	KY312627	...	...	...
STEU 8294	<i>Stereum hirsutum</i>	K	Royal Beaut	Canker on scion shoot of 1-year-old tree	KY312628	...	...	74
STEU 8295	<i>Trametes versicolor</i>	L	Golden Delicious/MM109	Wound on rootstock of nursery tree	KY312629	...	...	79
STEU 8296	<i>T. versicolor</i>	L	Golden Delicious/MM110	Bud union of a nursery tree	KY312630	...	...	...
Botryosphaeriaceae								
STEU 8297	<i>Botryosphaeria dothidea</i>	R	Gale Gala/G222	Bud union of a nursery tree	...	KY312606	...	73
STEU 8298	<i>B. dothidea</i>	R	Royal Beaut/M793	Bud union of a nursery tree	...	KY312607	...	...
STEU 8299	<i>Diplodia seriata</i>	K	Early Red One	Bud on new shoot	...	KY312608	...	79
STEU 8300	<i>D. seriata</i>	R	Royal Beaut/M793	Bud union of a nursery tree	...	KY312609	...	...
STEU 8301	<i>Neofusicoccum austral</i>	R	Royal Beaut/MM109	Bud union of a nursery tree	...	KY312610	...	80
STEU 8302	<i>N. austral</i>	R	Gale Gala/G222	Wound on scion shoot of nursery tree	...	KY312611	...	...
STEU 8303	<i>Neofusicoccum</i> sp. 4	R	Gale Gala/G222	Pruning wound on rootstock of a nursery tree	...	KY312612	...	59
STEU 8304	<i>N. viticlavatum</i>	K	Fuji Supreme	Canker on rootstock of 1-year-old tree	...	KY312613	...	91
STEU 8305	<i>N. viticlavatum</i>	K	Fuji Supreme	Canker on rootstock of 1-year-old tree	...	KY312614	...	...
STEU 8306	<i>N. stellenboschiana</i>	R	Gale Gala/G222	Pruning wound on rootstock of a nursery tree	...	KY312615	...	62
STEU 8307	<i>N. stellenboschiana</i>	R	Gale Gala/G223	Pruning wound on rootstock of a nursery tree	...	KY312616	...	...

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^t ITS = internal transcribed spacer and EF = elongation factor.^u Taxonomic group and strain number.^v Area sampled: G = Grabouw, H = Hermanus, K = Kouebokkeveld, L = Langkloof, and R = Riviersonderend.^w Bootstrap support value.^x Isolate clades with *Bjerkandera adusta*, *B. fumosa*, and *B. atroalaba*. Isolates cannot be identified up to species level.^y The isolates formed a clade with *Cytospora malicola* (DQ243802) found on an apple tree in Michigan and *C. germanica* found from *Populus* sp. in South Africa (DQ243793).^z *D. eres* is a species complex that has been designated into two groups (A and B) (Guarnaccia et al. 2018).

Table 1. (Continued from previous page)

Group, strain ^u	Species	Area ^v	Cultivar	Plant part	GenBank accession ^t			Support (%) ^w
					ITS	EF	β-Tubulin	
<i>Cadophora</i>								
STEU 8308	<i>Cadophora luteo-olivacea</i>	K	Royal Beaut/MM109	Pruning wound on rootstock of a nursery tree	KY312631	...	...	88
STEU 8309	<i>C. luteo-olivacea</i>	K	Golden Delicious/M793	Pruning wound on rootstock of a nursery tree	KY312632	...	...	...
STEU 8310	<i>Cadophora</i> sp.	K	Golden Delicious/M793	Bud union of a nursery tree	KY312633	...	...	96
STEU 8311	<i>Cadophora</i> sp.	K	Golden Delicious/M793	Bud union of a nursery tree	KY312634	...	...	...
<i>Coniochaeta</i>								
STEU 8312	<i>Coniochaeta fasciculata</i>	K	Gale Gala/CG4202	Pruning wound on rootstock of a nursery tree	KY312635	...	...	100
STEU 8313	<i>C. fasciculata</i>	K	Golden Delicious	Canker on scion shoot of 1-year-old tree	KY312636	...	...	...
STEU 8314	<i>C. velutina</i>	R	Gale Gala/G222	Pruning wound on rootstock of a nursery tree	KY312637	...	...	70
STEU 8315	<i>C. velutina</i>	K	Early Red One	Pruning wound on a mature tree	KY312638	...	...	...
STEU 8316	<i>Coniochaeta</i> sp.	G	Golden Delicious	Fruiting body on canker found on a 1-year-old tree	KY312639	...	...	...
<i>Cytospora</i>								
STEU 8317	<i>Cytospora malicola/germanica</i> complex	K	M793	Canker on rooted rootstock cutting	KY312640	...	...	... 58 ^y
STEU 8318	<i>C. malicola/germanica</i> complex	K	MM109	Canker on rooted rootstock cutting	KY312641	...	...	...
<i>Diaporthe</i>								
STEU 8319	<i>Diaporthe ambigua</i>	K	Early Red One	Pruning wound on a mature tree	KY312642	MH925324	...	61
STEU 8320	<i>D. ambigua</i>	K	Rosy Glow	Pruning wound on a mature tree	KY312643	MH925323	...	...
STEU 8321	<i>D. cynaroidis</i>	K	Gale Gala/G222	Wound on rootstock of a nursery tree	KY312644	MH925322	...	99
STEU 8322	<i>D. eres</i> (B)	R	Gale Gala/G222	Pruning wound on rootstock of a nursery tree	KY312645	MH925321	...	62 ^z
STEU 8323	<i>D. eres</i> (B)	G	Sundowner	Canker on scion shoot of a 1-year-old tree	KY312646	MH925320	...	...
STEU 8324	<i>D. foeniculina</i>	G	Golden Delicious	Pruning wound on a mature tree	KY312647	MH925319	...	88
STEU 8325	<i>D. foeniculina</i>	R	Royal Beaut/M793	Pruning wound on rootstock of a nursery tree	KY312648	MH925318	...	...
STEU 8326	<i>D. eres</i> (A)	K	Golden Delicious/MM109	Bud union of a nursery tree	KY312649	...	...	59
STEU 8327	<i>D. eres</i> (A)	H	Cripps Red	Canker on scion shoot of a 1-year-old tree	KY312650	MH925317	...	...
STEU 8328	<i>D. virgiliae</i>	K	Golden Delicious/MM109	Bud union of a nursery tree	KY312651	MH925316	...	100
STEU 8329	<i>D. virgiliae</i>	L	Golden Delicious/M793	Wound on rootstock of a nursery tree	KY312652	MH925315	...	...
STEU 8330	<i>D. amygdali</i>	K	Royal Beaut/MM109	Bud union of a nursery tree	KY312653	MH925314	...	66
STEU 8331	<i>D. amygdali</i>	K	Royal Beaut/MM110	Wound on rootstock of a nursery tree	KY312654	MH925313	...	...
<i>Diatrypaceae</i>								
STEU 8333	<i>Eutypa lata</i>	K	Golden Delicious	Pruning wound on a mature tree	...	...	MH925330	100
(Continued on next page)								

(Continued on next page)

Table 1. (Continued from previous page)

Group, strain ^a	Species	Area ^v	Cultivar	Plant part	GenBank accession ^t			Support (%) ^w
					ITS	EF	β-Tubulin	
STEU 8334	<i>E. lata</i>	K	Golden Delicious/M793	Bud union of a nursery tree	...	...	MH925329	...
STEU 8335	<i>E. cremea</i>	G	Golden Delicious	Pruning wound on a mature tree	...	...	MH925328	70
STEU 8336	<i>E. cremea</i>	G	Golden Delicious	Pruning wound on a mature tree	...	...	MH925327	...
STEU 8337	<i>Eutypella citricola</i>	R	Royal Beaut/MM109	Wound on scion shoot of a nursery tree	...	...	MH925326	100
STEU 8338	<i>E. citricola</i>	G	Golden Delicious	Canker on scion shoot of a 1-year-old tree	...	...	MH925325	...
Dothideomycetes								
STEU 8339	<i>Didymella pomorum</i>	K	Golden Delicious	Canker on scion shoot of a 1-year-old tree	KY312661	...	...	66
STEU 8340	<i>D. pomorum</i>	K	M793	Rooted rootstock cutting	KY312662	...	...	...
STEU 8341	<i>D. pomorum</i>	K	Golden Delicious/M793	Bud union of a nursery tree	KY312663	...	...	...
STEU 8342	<i>D. pomorum</i>	K	M793	Rooted rootstock cutting	KY312664	...	...	...
STEU 8343	<i>Didymosphaeria variabile</i>	H	Cripps Red	Canker on scion shoot of a 1-year-old tree	KY312665	...	...	94
STEU 8344	<i>D. variabile</i>	G	Golden Delicious	Pruning wound on a mature tree	KY312666	...	...	...
STEU 8345	<i>D. rubi-ulmifolii</i> sensu lato	R	Gale Gala/G222	Bud union of a nursery tree	KY312667	...	...	80
STEU 8346	<i>D. rubi-ulmifolii</i> sensu lato	K	Golden Delicious/M793	Wound on rootstock of a nursery tree	KY312668	...	...	...
Phaeoacremonium								
STEU 8347	<i>Phaeoacremonium australiense</i>	K	Early Red One	Pruning wound on a mature tree	...	...	KY312669	93
STEU 8348	<i>P. australiense</i>	G	Golden Delicious	Pruning wound on a mature tree	...	...	KY312670	...
STEU 8349	<i>P. austroafricanum</i>	L	Golden Delicious/M793	Bud union of a nursery tree	...	...	KY312671	100
STEU 8350	<i>P. austroafricanum</i>	L	Golden Delicious/M793	Bud union of a nursery tree	...	...	KY312672	...
STEU 8351	<i>P. fraxinopenn-sylvanicum</i>	K	Golden Delicious	Pruning wound on a mature tree	...	...	KY312673	99
STEU 8352	<i>P. fraxinopenn-sylvanicum</i>	L	Golden Delicious/MM109	Bud union of a nursery tree	...	...	KY312674	...
STEU 8353	<i>P. inflatipes</i>	K	Early Red One	Pruning wound on a mature tree	...	...	KY312675	100
STEU 8354	<i>P. inflatipes</i>	K	Early Red One	Pruning wound on a mature tree	...	...	KY312676	...
STEU 8355	<i>P. iranianaum</i>	G	Golden Delicious/M793	Pruning wound on a nursery tree	...	...	KY312677	100
STEU 8356	<i>P. minimum</i>	G	Golden Delicious	Pruning wound on a mature tree	...	...	KY312678	100
STEU 8357	<i>P. minimum</i>	G	Golden Delicious	Pruning wound on a mature tree	...	...	KY312679	...
STEU 8358	<i>P. prunicola</i>	K	Early Red One	Pruning wound on a mature tree	...	...	KY312680	100
STEU 8359	<i>P. prunicola</i>	G	Golden Delicious	Pruning wound on a mature tree	...	...	KY312681	...
STEU 8360	<i>P. scolysi</i>	K	Early Red One	Pruning wound on a mature tree	...	...	KY312682	99
STEU 8361	<i>P. subulatum</i>	K	Gale Gala/CG4202	Canker on scion shoot of a 1-year-old tree	...	...	KY312683	100
STEU 8362	<i>P. subulatum</i>	K	Gale Gala/CG4202	Pruning wound on a nursery tree	...	...	KY312684	...
STEU 8363	<i>P. viticola</i>	K	Early Red One	Pruning wound on a mature tree	...	...	KY312685	80
STEU 8364	<i>P. viticola</i>	K	Early Red One	Pruning wound on a mature tree	...	...	KY312686	...
STEU 8402	<i>P. geminum</i>	K	Royal Beaut/M793	Pruning wound on a nursery tree	...	...	KY312689	100
STEU 8401	<i>P. longicollarum</i>	L	Golden Delicious/M793	Bud union of a nursery tree	...	...	KY312688	100
STEU 8400	<i>P. longicollarum</i>	K	Rosy Glow	Pruning wound on a mature tree	...	...	KY312687	...

Table 2. Incidence (number of infected plant parts) of fungal species isolated from apple propagation material, nursery trees, and 1-year-old orchards

Taxonomic group, species	Infected ^v	Incidence for specific material source				
		Orchards ^w	Trees ^x	Scion blocks ^y	Mother blocks ^u	
					Layer	Nursery
Amphisphaeriaceae						
<i>Truncatella angustata</i> ^z	16	1	5	0	10	0
Basidiomycetes						
<i>Bjerkandera</i> sp.	8	0	7	1	0	0
<i>Chondrostereum purpureum</i> ^z	1	0	0	1	0	0
<i>Peniophora</i> sp. 2	4	1	3	0	0	0
<i>Peniophora</i> sp. 1	6	1	3	2	0	0
<i>Schizophyllum commune</i> ^z	16	4	10	1	0	1
<i>Stereum hirsutum</i> ^z	1	1	0	0	0	0
<i>Trametes versicolor</i> ^z	37	2	18	17	0	0
Botryosphaeriaceae						
<i>Botryosphaeria dothidea</i> ^z	9	0	7	0	1	1
<i>Diplodia seriata</i> ^z	78	11	32	17	0	18
<i>Neofusicoccum australe</i> ^z	8	2	6	0	0	0
<i>Neofusicoccum</i> sp. 4 ^z	1	0	1	0	0	0
<i>N. stellenboschiana</i>	2	0	2	0	0	0
<i>N. viticlavatum</i>	1	1	0	0	0	0
<i>Cadophora</i> spp.						
<i>Cadophora luteo-olivacea</i> ^z	17	0	17	0	0	0
<i>Cadophora</i> sp.	6	0	6	0	0	0
<i>Coniochaeta</i> spp.						
<i>Coniochaeta fasciculata</i>	18	3	15	0	0	0
<i>C. velutina</i>	13	1	10	2	0	0
<i>Coniochaeta</i> sp.	1	1	0	0	0	0
<i>Cytospora</i> spp.						
<i>Cytospora malicola/germanica</i> complex ^z	12	0	0	0	0	12
<i>Diaporthe</i> spp.						
<i>Diaporthe ambigua</i> ^z	3	0	0	2	1	0
<i>D. cynaroidis</i>	3	0	3	0	0	0
<i>D. eres</i> (B) ^z	33	2	30	1	0	0
<i>D. foeniculina</i> ^z	7	2	1	3	1	0
<i>D. eres</i> (A) ^z	2	1	1	0	0	0
<i>D. virgiliae</i>	10	0	10	0	0	0
<i>D. amygdali</i>	2	0	2	0	0	0
Diatrypaceae						
<i>Eutypa lata</i> ^z	82	0	5	77	0	0
<i>E. cremea</i>	8	0	0	8	0	0
<i>Eutypella citricola</i>	9	5	1	3	0	0
<i>Didymella</i> spp.						
<i>Didymella pomorum</i> ^z	94	3	34	1	55	1
<i>Didymosphaeria</i> spp.						
<i>Didymosphaeria variabile</i> ^z	29	2	18	8	1	0
<i>D. rubi-ulmifolii</i> sensu lato	276	15	241	13	7	0
<i>Phaeoacremonium</i> spp.						
<i>Phaeoacremonium australiense</i>	1	0	0	1	0	0
<i>P. austroafricanum</i>	18	1	15	2	0	0
<i>P. fraxinopennsylvanicum</i> ^z	19	1	3	15	0	0
<i>P. inflatipes</i>	1	0	0	1	0	0
<i>P. iranianum</i> ^z	7	0	7	0	0	0
<i>P. minimum</i> ^z	13	0	2	11	0	0
<i>P. prunicola</i>	3	0	0	3	0	0
<i>P. scolytii</i>	1	0	0	1	0	0
<i>P. subulatum</i>	5	0	5	0	0	0
<i>P. viticola</i> ^z	16	0	0	15	0	1
<i>P. geminum</i>	2	0	2	0	0	0
<i>P. longicollarum</i>	1	0	0	1	0	0

^u Rootstock mother blocks. Plant parts included rootstock shoots only for 405 asymptomatic layer block shoots and 87 symptomatic 1-year-old rootstock shoots.^v Total number of infected plant parts, including 1-year-old orchards, nursery trees, scion mother blocks, and rootstock mother blocks.^w Plant parts from 130 trees included cankers forming on scion shoots, pruning wounds on rootstocks, bud unions, or rootstocks of 1-year-old diseased orchard trees.^x Plant parts from 480 trees included scion shoots, pruning wounds on rootstocks, bud unions, or rootstocks.^y Plant parts from 310 trees included cankers, pruning wounds, or scion shoots.^z Previously reported on apple trees (Farr and Rossman 2019).

sensu lato, and *Didymella pomorum*, were found on the cankers that formed on the scion shoot. The fruiting structures of *S. commune* were found on one of the dead Royal Beaut trees.

Certified nursery apple trees. In total 480 certified healthy nursery trees were collected of which 312 (65%) harbored possible canker

or wood rot pathogens. The nursery trees did not exhibit any external symptoms; however, a longitudinal section through the bud union and pruning wound often show vascular discoloration and/or soft rot (Fig. 1G, H, I). Thirty-two different fungal taxa were isolated from these nursery trees (Table 2). *D. rubi-ulmifolii* sensu lato was isolated from

Fig. 1. External and internal symptoms exhibited on 1-year-old apple trees (A–F). **A**, Typical canker forming on the scion shoot of a 1-year-old apple tree. **B** and **C**, Sections through the scion shoots and **D**, a pruning wound show vascular discoloration caused by **B**, *Diplodia seriata*; **C**, *Didymosphaeria rubi-ulmifolii* sensu lato; and **D**, *Trametes versicolor*. More than one pathogen was isolated from sections through cankers and pruning wounds. **E**, Causal organisms involved in the symptom expression were *Phaeoacremonium fraxinopennsylvanicum* isolated from the bud union (red arrow) whereas *Coniochaeta fasciculata* (yellow arrow) and *Diplodia seriata* (black arrow) were isolated from the canker that formed on the scion shoot. **F**, *P. austroafricanum* was isolated from the bud union (red arrow) and *Diplodia seriata* isolated from the pruning wound (yellow arrow). Cross sections through the pruning wound and the bud union of external asymptomatic nursery apple trees (**G–I**). Sections through the pruning wound and bud union showing **G**, brown internal discoloration caused by *Didymosphaeria rubi-ulmifolii* sensu lato and white rot symptoms caused by **H**, *Bjerkandera adusta* and **I**, *Trametes versicolor*.

274 plant parts and was the predominant fungus isolated from the nursery material. *D. rubi-ulmifolii* sensu lato was also the dominant pathogen isolated from all four plant parts. *D. seriata*, *Diaporthe eres* (B), *D. pomorum*, *T. versicolor*, *D. variabile*, *Cadophora luteo-olivacea*, *P. austroafricanum*, *Coniochaeta velutina*, *S. commune*, *C. fasciculata*, and *D. virgiliae* were isolated from more than 10 plant parts. The bud union had significantly higher infection levels compared with the rootstocks but was not different from infection levels found in the pruning wounds and scion (Table 3). The infection levels found in the rootstock part of the tree (including pruning wound, bud union, and the rootstock) for the three rootstock cultivars M793 (61.9%), MM109 (50%), and G222 (58.8%) were not significantly different ($P > 0.05$; Supplementary Table S3). High infection levels were found in all four nurseries, with percentage of infections ranging from 51.3 to 71.3% (Table 4).

Scion mother block orchards. Of the 310 scion mother block trees, 146 trees (47%) were infected with possible canker or wood-rotting pathogens. In total, 25 fungal species were identified (Table 2), belonging to seven taxonomic groups. *Eutypa lata* was isolated from 44 pruning wounds and 26 cankers and was the predominant pathogen isolated in the scion mother block trees. Fungal pathogens isolated from more than 10 pruning wounds or cankers include *T. versicolor*, *D. seriata*, *P. fraxinopennsylvanicum*, *P. viticola*, *D. rubi-ulmifolii* sensu lato, and *P. minimum*. The incidence of infected cankers and pruning wounds was similar, with 27.6 and 27.4%, respectively ($P = 0.992$). The same pathogens were isolated from both the pruning wounds and cankers, with the exception of *Didymosphaeria* spp. and *Diaporthe* spp., which were isolated more often from pruning wounds (Supplementary Table S4). *Didymosphaeria* spp. were isolated from 16 pruning wounds and two cankers found on scion mother block apple trees.

In general, fewer fungal isolates were recovered from cankers found on older trees. The scion mother blocks were divided into three

age groups. Orchards of 10 to 20 years had a significantly higher infection (66%) than orchards older than 20 years (41.4%). Orchards younger than 10 years old were significantly less infected (25.2%) than older trees ($P < 0.05$). Golden Delicious was the most infected cultivar (58.9%), followed by Early Red One (44.4%) and, finally, Rosy Glow, with an infection of 25.3%. Infection levels of Early Red One did not differ from Golden Delicious or Rosy Glow; however, the infection in Golden Delicious trees was higher than on Rosy Glow trees ($P = 0.007$). The diversity of fungal taxa isolated from the three different cultivars was similar. *E. lata* was the predominant fungus isolated from the three cultivars Golden Delicious (41 times), Early Red One (30 times), and Rosy Glow (6 times).

Fruiting bodies of Basidiomycete wood-rotting fungi were present on the dead wood in several of the older mother block orchards. *T. versicolor* and other wood rot pathogens were isolated from pruning wounds and cankers collected from these orchards. Of the 310 new shoots investigated, 16 (5%) had possible canker and wood rot pathogens present in either the shoot (disks) or the buds. Of the total number of buds isolated, only 0.32% were infected. The pathogens found in the green shoots were *C. velutina*, *E. lata*, *E. cremea*, *D. rubi-ulmifolii* sensu lato, *D. seriata*, and *T. versicolor* (Table 5). These pathogens were also isolated from the cankers and pruning wounds.

Rootstock mother blocks. *Layer blocks.* Seven fungal species were isolated from asymptomatic layer block shoots (Table 2). All fungal species isolated from the asymptomatic layer block were also found in the nursery trees. The predominant fungal species were *D. pomorum* followed by *Truncatella angustata*. These two were isolated from both the 1-year-old trees and the nursery trees. The mean percentages of infection of the three rootstock cultivars M793 (23.8%), MM109 (23%), and G222 (20.7%) were not statistically different ($P > 0.05$). Latent infections were detected in 12, 24, and 33%, respectively, of symptomless layer blocks (not statistically different from each other, $P > 0.05$) from the three PIOs.

One-year-old rootstock trees. Of the 87 symptomatic shoots that were sampled, 36 (41%) shoots harbored possible canker-causing pathogens. For M793, 21 (47%) shoots and, for MM109, 15 (36%) shoots harbored possible canker pathogens (data not shown). The predominant pathogen was *D. seriata*, followed by the *C. schulzeri* species complex (Table 2). Both fungal taxa are known dieback pathogens on apple trees. *P. viticola*, *B. dothidea*, and *S. commune* are known dieback pathogens and were also isolated from the nursery material. *D. pomorum*, which was the predominant species in the asymptomatic material, was reported once from the symptomatic rootstock material. Typical symptoms observed on the affected 1-year-old rootstock shoots were constricting cankers. A cross section through the cankers exposed dark-brown vascular discoloration, streaking down into the live tissue of the nonbudded tree. Fruiting structures which formed on the cankerous part of the shoots belonged to *D. seriata*, the *C. schulzeri* species complex, and *D. pomorum*.

Pathogenicity trial. There were no significant differences between the two trials ($P = 0.5563$); therefore, the trials were combined. For the combined trials, there was a significant species-isolate interaction ($P = 0.0001$). Therefore, the mean lesion lengths and standard deviations for each isolate are presented separately (Table 6). All 72

Table 3. Percent infection in different plant parts isolated from the nursery apple trees

Plant part	Plant parts infected (%) ^z
Bud union	38.40 a
Pruning wound	30.90 ab
Scion	19.70 ab
Rootstocks	15.90 c

^z Means followed by different letters are significantly different at $P < 0.05$.

Table 4. Incidence of nursery apple trees infected with canker and wood rot pathogens sampled from four nurseries

Nursery	Trees		
	Sampled (n)	Infected (n)	Infection (%)
A	80	41	51.30
B	80	57	71.30
C	160	112	70.00
D	160	100	62.50

Table 5. Canker and wood rot pathogens found in the green shoots of the scion mother blocks

Cultivar	Bud	Number of buds infected		Disk	Number of disks infected	
Golden Delicious	<i>Trametes versicolor</i>	1		...		0
	<i>Eutypa lata</i>	1		...		0
	<i>Didymosphaeria rubi-ulmifolii</i> sensu lato	1		...		0
Rosy Glow	...	0		<i>D. rubi-ulmifolii</i> sensu lato		1
	...	0		<i>Diplodia seriata</i>		1
	...	0		<i>E. cremea</i>		1
Early Red One	<i>D. rubi-ulmifolii</i> sensu lato	1		<i>D. seriata</i>		3
	<i>Coniochaeta velutina</i>	1		<i>E. lata</i>		2
	<i>D. seriata</i>	3		...		0

isolates formed lesions significantly longer than the control (Table 6; Supplementary Figs. 16, 17, 18, and 19). The mean lesion length varied from 15.14 to 95.05 mm. *Neofusicoccum viticlavatum* isolate STEU 8304 formed the longest lesions (95.05 mm). However, its lesions were not significantly different from *D. foeniculina* (isolate STEU 8325) and *D. eres* (B) (isolate STEU 8322). All isolates were reisolated from the discolored tissues of all inoculated shoots, and no inoculated fungi were isolated from the control, fulfilling Koch's postulates. Positive identifications of all species were found when the sequences were compared with the original sequences.

Discussion

This study found that 65% of the certified nursery apple trees investigated yielded fungi associated with canker and wood rot symptoms. All nurseries and rootstocks had similar infection rates, suggesting that the problem is not restricted to a specific nursery or rootstock. Canker and wood rot species found from 1-year-old diseased commercial trees were also found in the nursery trees as latent infections.

Forty-five fungal species belonging to 18 genera were identified in this study. Eighteen of these species have been reported on apple trees in South Africa (Adams et al. 2006; Cloete et al. 2011; Crous et al. 2000; Smit et al. 1996, 1997; Sutton et al. 2015); thus, the other

27 species are first reports on apple trees in South Africa. Except for five species, these are also new reports for apple world-wide. *Neofusicoccum* sp. 4, *C. luteo-olivacea*, *Stereum hirsutum*, *T. angustata*, *D. eres* (A), and *D. eres* (B) has been reported from apple trees elsewhere in the world (Farr and Rossman 2019; Slippers et al. 2007; Spadaro et al. 2011; Sutton et al. 2015). Recently, *D. eres* was divided into two groups A and B (Guarnaccia et al. 2018). Isolates identified as *Diaporthe pernicioso* fall within *D. eres* (B). Further taxonomic studies are needed to resolve *D. eres*. The pathogenicity results confirmed that all of the species identified were pathogenic on apple, causing significant lesions on 2-year-old apple shoots.

The five fungal species causing the highest numbers of cankers or dieback in commercial apple orchards were also found at high levels causing latent infections in certified apple nursery trees. These species were *D. rubi-ulmifolii* sensu lato, *D. seriata*, *S. commune*, *D. pomorum*, and *C. fasciculata*. *D. rubi-ulmifolii* sensu lato was the dominant species in both certified nursery apple trees and 1-year-old commercial apple trees. These species can be considered the most important species found in this study.

D. rubi-ulmifolii sensu lato, which belongs to the class Dothideomycetes, was the most dominant pathogen causing canker symptoms in commercial orchards and causing latent infections in nursery trees.

Table 6. Mean lesion lengths and percent reisolation of fungal species on field-grown Golden Delicious apple shoots 5 months after inoculation

Fungal species and isolate number	Lesion length (mm) ^x	Log-transformed mean ^w		Reisolation (%) ^y
		Mean	SD	
<i>Neofusicoccum viticlavatum</i> STEU 8304	95.05 A	4.56	0.41	67.50
<i>Diaporthe foeniculina</i> STEU 8325	77.14 AB	4.36	0.51	32.24
<i>D. eres</i> (B) STEU 8322	76.93 AB	4.36	0.57	75.00
<i>D. eres</i> (A) STEU 8326	68.33 BC	4.24	0.48	50.63
<i>Neofusicoccum</i> sp. 4 STEU 8284	55 CD	4.03	0.51	73.13
<i>Diplodia seriata</i> STEU 8299	54.55 CD	4.02	0.57	73.13
<i>Diaporthe seriata</i> STEU 8300	53.36 CDE	4.00	0.38	67.50
<i>Phaeoacremonium australiense</i> STEU 8348	50.19 DEF	3.94	0.55	65.00
<i>P. iraniana</i> STEU 8355	46.35 D-G	3.86	0.29	80.63
<i>P. subulatum</i> STEU 8362	45.97 D-G	3.85	0.55	55.63
<i>D. eres</i> (A) STEU 8327	45.42 D-H	3.84	0.50	39.38
<i>P. viticola</i> STEU 8364	45.39 D-H	3.84	0.44	39.38
<i>Coniochaeta fasciculata</i> STEU 8312	44.73 D-H	3.82	0.51	6.88
<i>D. ambigua</i> STEU 8320	44.51 D-H	3.82	0.53	30.00
<i>P. prunicola</i> STEU 8359	43.77 D-I	3.80	0.58	45.63
<i>P. longicollarum</i> STEU 8402	43.11 D-J	3.79	0.48	34.38
<i>D. cynaroidis</i> STEU 8321	42.34 D-K	3.77	0.65	28.95
<i>D. eres</i> (B) STEU 8322	42.22 D-K	3.77	0.62	56.25
<i>P. inflatipes</i> STEU 8353	41.45 D-L	3.75	0.37	71.25
<i>P. prunicola</i> STEU 8358	41.41 D-L	3.75	0.5	56.25
<i>P. austroafricanum</i> STEU 8349	41.35 D-L	3.75	0.42	56.88
<i>Didymosphaeria rubi-ulmifolii</i> sensu lato STEU 8404	41.23 D-L	3.74	0.44	35.00
<i>P. viticola</i> STEU 8363	40.64 D-M	3.73	0.47	61.88
<i>P. geminum</i> STEU 8400	40.03 E-N	3.71	0.47	65.63
<i>P. austroafricanum</i> STEU 8350	39.67 E-O	3.71	0.60	61.88
<i>P. subulatum</i> STEU 8361	39.56 E-O	3.70	0.45	50.00
<i>P. scolymus</i> STEU 8360	39.55 E-O	3.70	0.51	51.25
<i>D. rubi-ulmifolii</i> sensu lato STEU 8405	38.92 F-P	3.69	0.53	47.5
<i>Cadophora luteo-olivacea</i> STEU 8308	38.75 F-P	3.68	0.59	74.38
<i>Didymella pomorum</i> STEU 8339	38.21 F-Q	3.67	0.43	36.88
<i>Coniochaeta fasciculata</i> STEU 8313	38.21 F-Q	3.67	0.51	21.71
<i>P. australiense</i> STEU 8347	37.03 G-R	3.64	0.5	73.13
<i>P. geminum</i> STEU 8401	36.78 G-R	3.63	0.52	56.25
<i>Coniochaeta velutina</i> STEU 8314	36.69 G-R	3.63	0.59	27.50
<i>C. velutina</i> STEU 8315	36.56 G-R	3.63	0.48	27.50
<i>Truncatella angustata</i> STEU 8284	36.34 G-R	3.62	0.56	45.00

(Continued on next page)

^w Least significant difference of logarithmic transformed lesion lengths = 0.2969. SD = standard deviation.

^x Back-transformed mean lesion length is based on 20 replicates, with 10 replicates per orchard. Means followed by different letters differ significantly ($P < 0.05$) according to Student's *t* test.

^y Mean reisolation.

^z PDA+ = potato dextrose agar amended with streptomycin sulfate.

Members of the class Dothideomycetes are lesser known as canker-causing pathogens. However, a pathogenicity study conducted by Cloete et al. (2011) found that *D. rubi-ulmifolii* sensu lato, previously known as *Paraconiothyrium brasiliense* Verkley, formed significant lesions on apple shoots. Cloete et al. (2011) only isolated the pathogen from canker symptoms collected from diseased pear trees. Recently, *D. rubi-ulmifolii* sensu lato was found to be the causal agent of cankers identified near the base of young apple trees that resulted in a loss of 13,928 trees (57%) from 2012 until 2018 in Ethiopia (A. Yirgu and A. Gezahgne, the Central Ethiopia Environment and Forest Research Center, personal communication). These losses were incurred by one producer who decided to remove their apple orchards in March 2019 due to the high occurrence of cankers. Little is known regarding the biology of *D. rubi-ulmifolii* sensu lato; therefore this occurrence requires further investigation.

Even though infection levels in the nursery trees were high, apple producers do not find a similarly high disease incidence of cankers or wood rot in newly established commercial orchards. Canker and wood rot pathogens are opportunistic and can induce symptoms when the host experiences stress (Menapace et al. 2015). Symptoms of the canker pathogen *Valsa mali* var. *mali* were observed due to stress experienced by the apple trees (Zang et al. 2012). In the present study, one soil sample for each of the 13 orchards was analyzed for physiochemical characteristics (data not shown). In general, the soils with a high phosphorus level indicated that the plant had difficulty

taking up water and a low, acidic pH indicated an aluminum toxicity (Jensen 2010; Marschner et al. 1987). These factors indicate that the trees were planted in suboptimal soil, which will place either toxicity or mineral deficiency stress on the trees (Jensen 2010; Marschner et al. 1987). These findings need to be confirmed with more soil samples per orchard analyzed. In general, stress conditions contribute to a more vulnerable tree for the development of dieback.

The presence of the wood rot pathogens in both the young trees and the nursery trees was surprising because these pathogens usually occur on older trees and dead wood (Matthee and Thomas 1977). The fruiting bodies of Ascomycete and Basidiomycete fungi were found on the diseased or dead 1-year-old apple trees. These fruiting bodies produce spores, contributing to the aerial inoculum present in orchards. The spores can be spread with wind and rain and potentially infect new wounds (Matthee and Thomas 1977; Valiūskaitė and Raudonis 2008). For this reason, it is very important to remove the dead trees from the young orchards.

Most of the cankers formed on the scion shoot of the 1-year-old commercial apple trees. McCracken et al. (2003) also found that cankers caused by *N. ditissima* mostly developed on the scion shoots after establishing apple trees. Infection of the scion shoot can occur during the final stages of propagation when the lateral shoots are removed from the main stem. Alternatively, infections can also occur through wounds made on the scion shoot in newly established orchards.

Table 6. (Continued from previous page)

Fungal species and isolate number	Lesion length (mm) ^x	Log-transformed mean ^w		Reisolation (%) ^y
		Mean	SD	
<i>Cytospora schulzeri</i> species complex STEU 8317	36.25 G–R	3.62	0.63	8.13
<i>Cadophora luteo-olivacea</i> STEU 8309	35.3 G–S	3.59	0.56	62.50
<i>Cadophora</i> sp. STEU 8311	35.1 G–S	3.59	0.65	68.13
<i>Coniochaeta</i> sp. STEU 8316	34.96 G–T	3.58	0.57	16.88
<i>Cadophora</i> sp. STEU 8310	34.7 G–T	3.58	0.49	48.75
<i>Eutypella citricola</i> STEU 8337	33.85 H–U	3.55	0.58	28.75
<i>Didymosphaeria variabile</i> STEU 8344	33.78 H–U	3.55	0.37	49.38
<i>Eutypella citricola</i> STEU 8338	32.68 I–V	3.52	0.61	16.25
<i>Didymella pomorum</i> STEU 8341	32.67 I–V	3.52	0.50	36.88
<i>Didymosphaeria variabile</i> STEU 8343	32.45 I–W	3.51	0.49	65.00
<i>Bjerkandera adusta</i> STEU 8285	31.94 J–W	3.49	0.60	30.63
<i>Truncatella angustata</i> STEU 8283	31.76 K–W	3.49	0.41	43.13
<i>Peniophora</i> sp. 1 STEU 8288	31.28 K–X	3.47	0.46	1.88
<i>Trametes versicolor</i> STEU 8296	30.89 L–X	3.46	0.48	31.25
<i>Diaporthe virgiliae</i> STEU 8328	30.68 L–X	3.46	0.44	34.38
<i>Didymella pomorum</i> STEU 8340	30.6 L–X	3.45	0.51	21.88
<i>Didymosphaeria rubi-ulmifolii</i> sensu lato STEU 8403	30.36 M–X	3.45	0.44	50.63
<i>Didymella pomorum</i> STEU 8342	30.14 M–X	3.44	0.45	26.25
<i>Diaporthe ambigua</i> STEU 8319	30.05 M–X	3.44	0.46	44.38
<i>Cytospora schulzeri</i> species complex STEU 8318	29.93 N–X	3.43	0.61	10.00
<i>Eutypa cremea</i> STEU 8335	29.41 O–Y	3.41	0.47	32.5
<i>D. amygdali</i> STEU 8330	28.81 P–Y	3.39	0.44	58.75
<i>E. cremea</i> STEU 8336	28.67 P–Y	3.39	0.50	22.50
<i>Bjerkandera adusta</i> STEU 8286	28.47 Q–Y	3.38	0.64	25.00
<i>Trametes versicolor</i> STEU 8295	28.07 R–Y	3.37	0.47	28.75
<i>Didymosphaeria rubi-ulmifolii</i> sensu lato STEU 8346	27.95 R–Y	3.37	0.48	48.13
<i>D. rubi-ulmifolii</i> sensu lato STEU 8345	26.44 S–Z	3.31	0.56	16.25
<i>Peniophora</i> sp. 2 STEU 8291	25.81 T–Z	3.29	0.45	23.75
<i>Botryosphaeria dothidea</i> STEU 8298	25.22 U–Z	3.27	0.50	79.61
<i>Diaporthe foeniculina</i> STEU 8324	24.6 V–Z	3.24	0.58	54.38
<i>D. amygdali</i> STEU 8331	23.86 W–Z	3.21	0.41	56.25
<i>Peniophora</i> sp.1 STEU 8289	23.03 XYZ	3.18	0.59	26.25
<i>Stereum hirsutum</i> STEU 8294	21.77 YZ	3.13	0.49	17.50
<i>Botryosphaeria dothidea</i> STEU 8297	21.71 YZ	3.12	0.56	70.63
<i>D. virgiliae</i> STEU 8329	20.05 aZ	3.05	0.62	53.75
<i>Peniophora</i> sp. 2 STEU 8290	15.14 a	2.78	0.43	21.71
Control (PDA+) ^z	8.68 b	2.27	0.49	0

The pruning wound on the rootstock and the bud union had the highest infection in the nursery trees. Canker and wood rot symptoms that developed from these sites were found in 19% of the infected 1-year-old apple trees. These two wound sites are important sites for infection. Infection occurs via aerial inoculum present in the nursery when the wounds are made or via infected bud material. Older apple orchards and other fruit hosts in close vicinity can harbor similar pathogens and can contribute to the aerial inoculum present (Damm et al. 2008; Halleen et al. 2007; Slippers et al. 2007).

Canker and wood rot fungi isolated from pruning wounds and cankers from scion mother block trees were similar to those found in 1-year-old apple trees and nursery trees. *E. lata* was the predominant species found in the scion mother block cultivars; however, it was seldom found in the young nursery trees. This pathogen often occurs on grapevines causing *Eutypa* dieback (Trouillas and Gubler 2010) and is commonly associated with decline on older vines (Moyo et al. 2016; White et al. 2011). Cloete et al. (2011) also isolated this pathogen from older apple orchards, which can suggest that *E. lata* prefers older tissue and is why this pathogen was not commonly found in young nursery trees.

Golden Delicious trees had higher infection levels than Rosy Glow trees. This is probably due to the average ages of the orchards. Rosy Glow is a newer cultivar, with an average age of 8 years, compared with Golden Delicious orchards, which had an average age of 20 years. The younger orchards, which in this case were all Rosy Glow trees, had few to no cankers on the trees. It is expected that older orchards would harbor more canker pathogens because they have been pruned for longer, accumulating more wounds on the trees, which have been exposed to aerial inoculum for longer. High infection levels in the pruning wounds indicate that apple wounds are susceptible to infection by canker and wood rot pathogens that may result in the development of cankers. In some of the older, severely diseased trees with larger cankers, the causal organisms were not isolated easily, resulting in a lower infection percentage obtained for trees older than 20 years versus between 10 and 20 years in age. This can be due to the hardness of the wood after several years, or the pathogen may only be present as fruiting structures on the surface of the canker.

A low infection rate (5%) was found in the new green shoots, however; this study shows that infection of newly grafted plants can occur directly through infected scion buds used during propagation. The fungal pathogens *D. rubi-ulmifolii* sensu lato, *E. lata*, and *T. versicolor* were isolated from infected scion buds and were also found in the nursery trees and 1-year-old diseased orchards.

The rooted rootstock cuttings also proved to be a direct inoculum source for infection of the nursery trees, with an overall infection of 21%. The predominant pathogens found were *D. pomorum*, which have been reported as canker-causing pathogens of apple in South Africa by Crous et al. (2000). This was followed by *T. angustata*, which is found as a weaker pathogen that can cause decline of grapevines in Iran (Arzanlou et al. 2013), Texas (United States) (Úrbez-Torres et al. 2009), and Australia (Sergeeva et al. 2005). Latent infections of the rootstock layer blocks by *D. pomorum*, *T. angustata*, *B. dothidea*, and *D. foeniculina* were also found in the nursery trees. This latent infection in the rootstock layer blocks can be expressed when shoots experience stress similar to what was seen in the 1-year-old nursery rootstock blocks. Fruiting structures were found on the cankers of the 1-year-old nursery shoots that can provide aerial inoculum during budding.

The incidence of dieback in 1-year-old commercial orchards due to canker and wood rot can be reduced by managing abiotic and biotic stress factors placed on the apple tree. This should be done throughout the propagation process, during storage, and during the establishment of young trees. Abiotic stresses responsible for low productivity in apple orchards include fluctuation in temperature, nutrient deficiencies and toxicities, low-temperature injuries such as frost, water logging, insect damage, and improper horticultural practices (Arzanlou and Bakhshi 2012; Krishna et al. 2010; Marek et al. 2013; Slippers et al. 2007; Valiushkaitė and Raudonis 2008).

To reduce infection levels in nursery trees, aerial inoculum in the nurseries as well as in commercial orchards can be reduced by applying sanitation practices. Sanitation practices include the removal of dead and cankered material, especially when fruiting bodies are present. The importance of sanitation has been shown for the management of *E. lata* and *Valsa leucostoma* in stone fruit orchards (Bertrand and English 1976; Carter 1983). Sanitation is important in all phases of propagation as well as in established orchards. When the canker forms on the scion shoot of a 1-year-old tree, the diseased part of the tree can be removed. The tree will produce a new scion shoot in the next growing season; this will allow the tree to recover from the infection.

The cankered rooted rootstocks in the 1-year-old nursery blocks should be removed before budding as well as before the rootstock shoot is pruned back. This will reduce infection during budding. A pruning wound protectant containing a fungicide should be used throughout the propagation process to reduce infections in nurseries. Younger scion orchards were less infected than older orchards; thus, PIOs should renew mother block orchards at a younger age. This will reduce the infection of the scion buds used during propagation.

Certified nursery apple trees were infected with canker and wood rot pathogens, which resulted in the distribution of seemingly healthy trees to farmers. Nursery trees get infected during the propagation process via aerial inoculum, which is present at the time of budding and pruning back. The plant material used during propagation also plays a role in infecting the nursery apple trees because a direct infection was found via the scion bud and rooted rootstock cutting. The young trees expressed canker and wood rot symptoms shortly after experiencing stress conditions during the establishment of the orchards. The postnursery handling of nursery trees is very important; to what extent storage conditions of trees and optimal planting and postplanting practices would contribute to the expression of latent infections of canker and wood rot pathogens needs to be further investigated. Future work should aim to lessen infections via propagation material and provide efficient protection of the pruning wound.

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