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Fungi associated with die-back symptoms of apple and pear trees, a possible inoculum source of grapevine trunk disease pathogens

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Summary. A survey was undertaken on apple and pear trees in the main pome fruit growing areas of the Western Cape of South Africa to determine the aetiology of trunk diseases with specific reference to pathogens known to occur on grapevine, which are frequently cultivated in close proximity to these orchards. Several fungal genera containing known trunk disease pathogens were found. Two *Diplodia* species, *D. seriata* and *Diplodia* sp., were isolated along with *Neofusicoccum australe* and *N. vitifusiforme*. Four *Phaeoacremonium* species, *Phaeoacremonium aleophilum*, *Pm. iranianum*, *Pm. mortoniae* and *Pm. viticola*, two *Phomopsis* species linked to clades identified in former studies as *Phomopsis theicola* and *Phomopsis* sp. 7, and *Eutypa lata* were found. In addition, *Paraconiothyrium brasiliense, Pr. variabile* and a *Pyrenochaeta*-like species were also isolated. *Diplodia seriata* (56% of total isolates) and *Pm. aleophilum* (22%) were most frequently isolated. First reports from pear wood include the *Phaeoacremonium* spp. and *Paraconiothyrium brasiliense,* while new reports from apple include *Pm. aleophilum*, *Ph. theicola*, *Phomopsis* sp. 7, *Pr. variabile* and *E. lata*. A pathogenicity trial was undertaken to determine the role of these species on apple, pear and grapevine shoots. *Neofusicoccum australe* caused the longest lesions on grapevine shoots, while *Pr. variabile*, *D. seriata*, *Pm. mortoniae* and the *Pyrenochaeta*-like sp. caused lesions that were longer than non-inoculated and non-pathogen experimental controls. On pear shoots, *Diplodia* sp. and *N. australe* caused the longest lesions, followed by *D. seriata* and *E. lata*. On apple shoots, the longest lesions were caused by *N. australe* and *Pm. iranianum*. These results demonstrate that apple and pear trees in Western Cape orchards are hosts to many known trunk pathogens along with potential new trunk disease-causing fungi.

Key words: β-tubulin, internal transcribed spacers, trunk diseases, *Vitis vinifera*.

Introduction

Trunk disease is a broad term used when describing various abnormalities of the woody parts of perennial plants such as vines and plantation and fruit trees. Its various external and internal manifestations are mainly caused through invasion by various fungal organisms that directly and indirectly cause damage and blockage of the vascular systems. Many of these organisms invade plants through pruning wounds, scars and stomata. This gives rise to various symptoms such as cankers, twig blights and wood rots, which in turn may result in lower yields of decreasing quality. Die-back of affected parts is gradual and it may take years before damage to internal wood is severe enough to kill entire vines or fruit trees (Mugnai *et al.,* 1999; Brown-Rytlewski and McManus, 2000; Lalancette and Robinson, 2001; Slippers *et al.*, 2007; Van Niekerk *et al.*, 2011).

In pome fruit trees, various fungal genera are known to cause trunk diseases worldwide. These include *Botryosphaeria, Chondrostereum*, *Diplodia*, *Eutypa*, *Leucostoma, Neonectria*, *Neofabraea, Neofusicoccum, Phomopsis* and *Valsa* (Glawe *et*

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al., 1983; Jones and Aldwinkle, 1991; Kanematsu, 2002; Slippers *et al*. 2007). The fungi that have been isolated from die-back or canker symptoms of pome trees in South Africa include *Chondrostereum purpureum* and *Diaporthe ambigua* (Smit *et al*., 1996; Crous *et al*., 2000). *Botryosphaeria ribis, Leucostoma persoonii* (from die-back symptoms) and *Schizophyllum commune* (from trunk rot symptoms) have been isolated from apple trees and *Diplodia seriata* from pear trees (Crous *et al*., 2000). The extent and cause of trunk diseases in the pome fruit growing regions of the Western Cape of South Africa are largely unknown, though symptoms may be commonly observed in orchards.

The purpose of the present study was to investigate the cause of trunk diseases in aging pome fruit orchards in the Western Cape. The apple cultivar 'Granny Smith' and pear cultivar 'Packham's Triumph' were selected for sampling, because these cultivars are the oldest and most widely planted in this region, and generally in South Africa.

Materials and methods

Sampling

Symptomatic wood from the green apple cultivar 'Granny Smith' and the green pear cultivar 'Packham's Triumph' showing die-back symptoms was collected in September and October of the years 2006 and 2007. A total of five areas, viz. Grabouw, Vyeboom, Villiersdorp, Wolseley and Ceres, representing the oldest established pome fruit producing areas in the Western Cape, were sampled over this period. Samples of living symptomatic wood were taken from trees in orchards older than 15 years, and stored at 4°C for up to 2 weeks until dissection.

Isolations

Samples were taken from storage and were dissected. Symptoms were described and photographed. Symptomatic wood was cut into pieces measuring approximately 3 by 3 cm and surface-sterilised by soaking in a 70% ethanol solution for 30 s, in a 1% NaOCl solution for 1 min and in 70% ethanol for a further 30 s. Following sterilisation, wood pieces were air-dried and halved using sterilised pruning shears. Pieces of wood measuring approx. 2×2 mm were excised from the margins between necrotic and healthy tissue and placed on 2% potato-dextrose agar (PDA, Biolab, Midrand, South Africa) amended with streptomycin sulphate (40 mg L^{-1}, Calbiochem, Merck, Darmstad, Germany). Plates were incubated at 25°C under natural light until growth could be detected. Subcultures were made from the growing hyphae onto PDA and incubated under the same conditions.

In cases where sporulation had not taken place, isolates were placed on divided plates containing PDA without streptomycin and water agar (WA, Biolab), respectively, with a piece of sterile irradiated carnation leaf placed on the WA to enhance sporulation. Most isolates were then placed on synthetic nutrient agar (SNA; Nirenberg, 1976) amended with 100 mg penicillin G, 50 mg streptomycin sulphate and 10 mg chlortetracycline hydrochloride, to which 3 cm pieces of double-autoclaved pine needles had been added (Damm *et al*., 2007). Single-conidium isolates were made from all sporulating isolates to obtain pure cultures. Isolates that did not sporulate were purified by hyphal-tipping.

Morphological identification

The initial identification of isolates was made based on colony morphology according to visual characteristics such as colony colour and growth. Isolates were examined using a stereo-microscope and slides were made by mounting fungal material in lactic acid. Slides were examined with a compound microscope and morphologically identified. Isolates of the trunk disease genera and other isolates deemed to be of interest were stored in the culture collection of the Department of Plant Pathology of the University of Stellenbosch (STE-U) on PDA slants and in water and maintained at 4°C.

Molecular characterisation and phylogeny

Genomic DNA was extracted from fresh fungal mycelia obtained from PDA plates not older than 14 days, using the extraction protocol of Damm *et al*. (2008a). Products were visualised using electrophoresis. Primers for amplification were selected according to the taxon studied. For the Botryosphaeriaceae, *Phomopsis*, *Eutypa* and unidentified genera, the internal transcribed spacers (ITS1 and ITS2) and the 5.8S ribosomal gene were amplified using the primer pair ITS-1F (Gardes and Bruns, 1993) and ITS4 (White *et al*.,

1990) under the conditions described in White *et al*. (1990). Two to 3 mM $MgCl_2$ was used if the fragment could not be amplified using the standard protocol. The β-tubulin gene was amplified in isolates identified as *Phaeoacremonium* using the primer pair T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995), according to the conditions used by Mostert *et al*. (2003, 2006). Products of amplification were separated through gel-electrophoresis under the conditions described in Van Niekerk *et al*. (2004), and all products were cleaned using a PCR product purification kit (MSB spin PCRapace, Invitek, Berlin, Germany). The amplification products were then sequenced as described in Van Niekerk *et al*. (2004).

Sequences were edited using Geneious Pro 3.5.6 (2007 build, Biomatters Ltd., Auckland, New Zeland), and consensus sequences were run through the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine basic identity. In cases where identity could not be established to a 100% certainty, additional sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank) to build representative alignments for phylogeny. Reference sequences representing the relevant species for Botryosphaeriaceae (Van Niekerk *et al*., 2004; Crous *et al*., 2006; Damm *et al*., 2007; Phillips *et al*., 2008), *Paraconiothyrium* (Damm *et al*., 2008b), *Phaeoacremonium* (Mostert *et al*., 2006; Essakhi *et al*., 2008), *Phomopsis* (Mostert *et al*., 2001; Van Niekerk *et al*. 2005) and *Pyrenochaeta* (de Gruyter *et al*., 2009) were used to build alignments for species identification. Sequences were aligned automatically in Geneious to a global alignment with free end gaps and a 93% similarity cost matrix. Automatic alignments were adjusted manually in Sequence Alignment Editor v. 2.0a11 (Rambaut, 2002) and phylogenetic analyses were performed on alignments in PAUP (Phylogenetic Analysis Using Parsimony) 4.0b10 (Swofford, 2000). Datasets for each region were analysed separately. The heuristic search option was used on all datasets set to 100 random sequence additions and using tree bisection and reconstruction as the branch swopping algorithm. All characters were unordered and of equal weight and gaps in the alignments were treated as missing data. Hillis and Bull's (1993) bootstrapping method was used to determine whether or not trees obtained during the heuristic search could be regarded as robust, using PAUP's bootstrap search option set to 1000 bootstrap replications. The measured tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated for each tree resulting from the above-mentioned analysis. Sequences have been lodged in GenBank for the Botryosphaeriaceae (JF934884-JF934913), *Paraconiothyrium* (JF934920-JF934923), *Phaeoacremonium* (JF934927-JF934952), *Phomopsis* (JF934924-JF934926) and the *Pyrenochaeta*-like sp. (JF934914-JF934919).

Pathogenicity trial

A trial to examine lesion formation on woody shoots was conducted on detached woody shoots of grapevine (cv. Sauvignon blanc), pear (cv. Packham's Triumph) and apple (cv. Granny Smith), using the protocol described in Damm *et al*. (2007; 2008a), which was based on the protocol described in Van Niekerk *et al.,* (2004), also used in Úrbez-Torres *et al*., (2008). For each host, the trial layout was a randomised block design consisting of three blocks, or incubation chambers. The treatments (listed in Table 1) included 32 fungal isolates and two negative controls, *Acremonium strictum* and an uncolonised agar plug. Agar plugs of 4 mm diam. were taken from the margins of the fungal colonies. A maximum of three isolates per species were used according to availability, and each treatment was replicated four times. Shoots were cut into 12 cm pieces and surface-sterilised as described above. Shoots were allowed to air-dry inside a laminar flow cabinet, and were wounded through the phloem and cortex tissue using a 4 mm cork borer. Agar plugs were inserted into wounds immediately after wounding and wounds were wrapped with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA). Shoots were incubated at 25°C under natural light in moist chambers (RH>93%). After 14 days, shoots were removed from moist chambers. The bark surrounding each wound site was stripped off and lesions were measured using digital callipers. Isolations were made from the margins of necrotic lesions onto PDA amended with streptomycin sulphate. Plates were incubated at ambient temperature and under natural light conditions and re-isolation frequencies were determined by calculating the

percentage of isolates retrieved from re-isolations based on colony growth after 14 days.

The data obtained from lesion measurements were normalised by the removal of outliers. Analyses of variance were conducted and Student's t-tests for least significant difference were calculated to compare the differences between fungal taxa on the three hosts.

Results

Morphological identification

Fungi were identified according to cultural and morphological characters as Botryosphaeriaceae (van Niekerk *et al*., 2004), *Phomopsis* spp. (van Niekerk *et al*., 2005; Santos and Phillips, 2009), *Phaeoacremonium* spp. (Mostert *et al*., 2006; Damm *et al*. 2008a), *E. lata* (Rumbos, 1988) and *Paraconiothyrium* (Damm *et al*. 2008b). Several isolates were characterised preliminarily as a *Pyrenochaeta*- or *Phoma*-like species, having morphological features similar to *Pyrenochaeta* and *Phoma* (de Gruyter *et al*., 2009).

Molecular characterisation and phylogeny

Isolates identified as species of the Botryosphaeriaceae, *Phaeoacremonium*, *Phomopsis*, *Eutypa* and several recurring unidentified isolates were sequenced. Phylogenetic inferences of the ITS region were made for the Botryosphaeriaceae (Figure 1) and *Phomopsis* (Figure 2). A β-tubulin phylogeny for the *Phaeoacremonium* species is shown in Figure 3.

Four species of Botryosphaeriaceae were identified according to ITS data. Most isolates belonged to *Diplodia seriata* (77% bootstrap support). Three isolates grouped with *Neofusicoccum australe* (bootstrap support of 97%), which were isolated from *Pyrus,* and two isolates with *N. vitifusiforme* (bootstrap support of 79%), were also isolated from *Pyrus*. Six isolates originating from *Pyrus* formed a strongly supported monophyletic clade (99% bootstrap support) that was identified as a separate *Diplodia* species closely related to *D. mutila* and *D. africana*. A total of 68 *Diplodia seriata* isolates were identified, of which 12 were isolated from *Malus* and 56 from *Pyrus*.

Three isolates were identified as *Phomopsis* species according to the criteria specified by Rehner and Uecker (1994) and Rossman *et al.* (2007). Of the three isolates found during this study, two isolated from *Pyrus* were identical and grouped with *Phomopsis* species 7 of Van Niekerk *et al*. (2004) (with bootstrap support of 85%), while the third isolate, originating from *Malus*, grouped with *Phomopsis* species 1, identified by Mostert *et al*. (2001) from grapevine. *Phomopsis* species 1 has since been named as *Ph. theicola* by Santos and Phillips (2009). The bootstrap value for the *Ph. theicola* group was too low to distinguish a separate species with any certainty. Resolving the taxonomy of the genus *Phomopsis* was outside the scope of this study and the three isolates were treated as two separate species.

The β-tubulin phylogeny revealed four *Phaeoacremonium* species. The majority of isolates were identified as *Pm. aleophilum*. A total of 40 *Pm. aleophilum* isolates were identified, of which 13 were isolated from *Malus* and 27 from *Pyrus*. A single isolate was identified as *Pm. iranianum*, two isolates as *Pm. viticola* and two isolates as *Pm. mortoniae*, all originating from *Pyrus*. The isolates grouped with a bootstrap support of 100%, 100%, 87% and 100%, respectively.

Sequences identified as *E. lata* via BLAST were aligned to eight reference sequences obtained from GenBank (DQ006942, DQ006937, GQ293948, AY684232, DQ006927, DQ006941, AY462541, AY462540) with a percentage pairwise similarity of 99.6% between the sequences. Differences were observed over a length of 529 base pairs which were due to insertions, deletions and substitutions at positions 161, 162, 507, 514 and 521. In total, six isolates were identified as *E. lata*, three from *Malus* and three from *Pyrus*.

Two sequences identified as *Paraconiothyrium brasiliense* were aligned to five reference sequences (AY642531, EU295638, EU295635, EU295637, EU295636) with a percentage pairwise similarity of 99.3% between the sequences. Differences could be observed over a length of 506 base pairs and were due to substitutions at positions 32, 78, 123, 128, 180 and 416, and insertions at 470 and 493. Two sequences identified as *Pr. variabile* were aligned to four reference sequences (EU295649, EU295646, EU295647, EU295648) with a 94.8% similarity between sequences. Differences could be ascribed to a substitution at position 18 and a deletion at 487 over 538 base pairs. *Paraconiothy-*

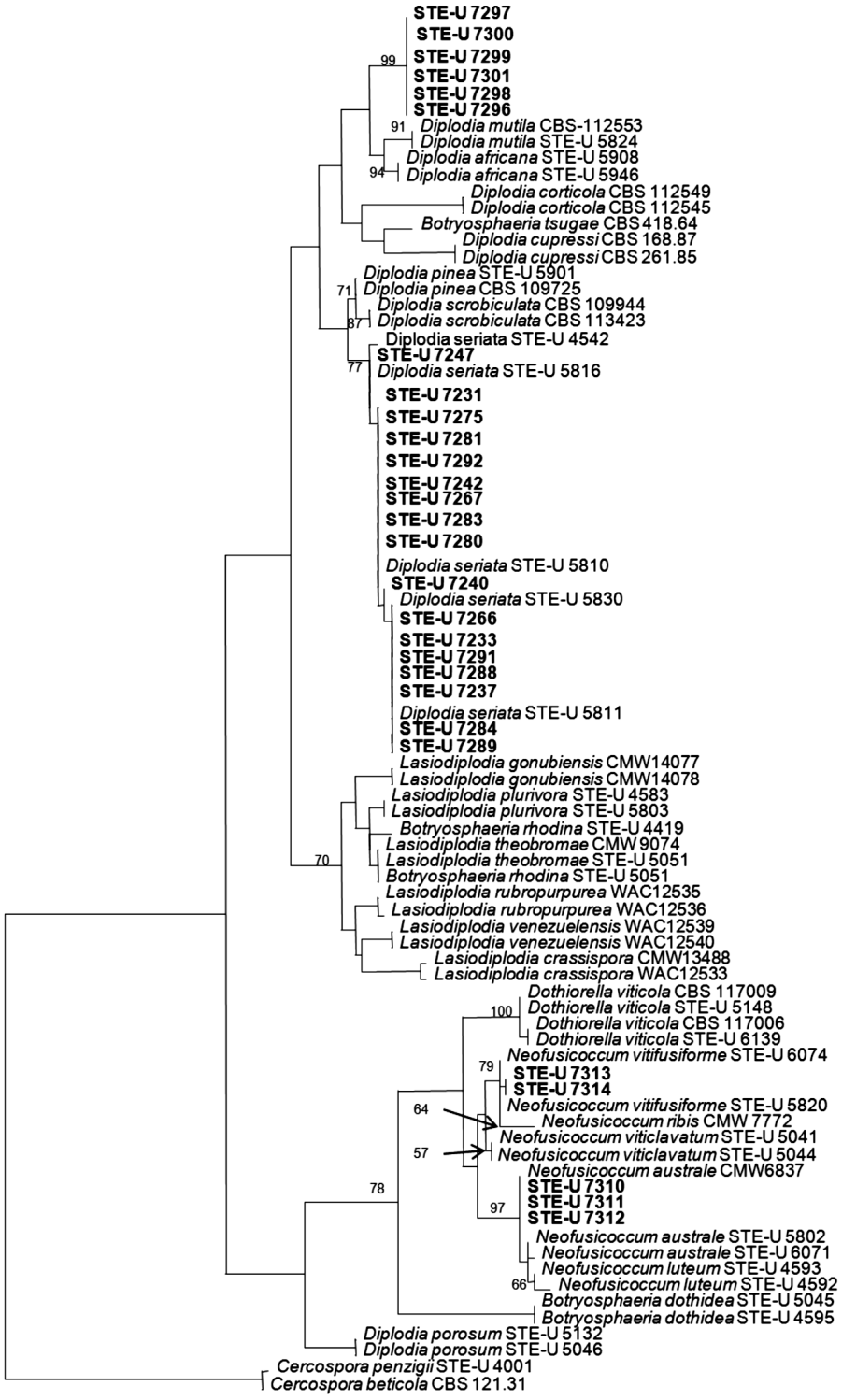

Figure 1. One of 840 most parsimonious trees obtained from ITS sequences of the *Botryosphaeriaceae*. Bootstrap support values above 50% from 1000 replicates are shown at the nodes. Tree scores include Length = 294, CI = 0.707, RI = 0.931, RC = 0.658, HI = 0.293. Isolates from the present study in bold.

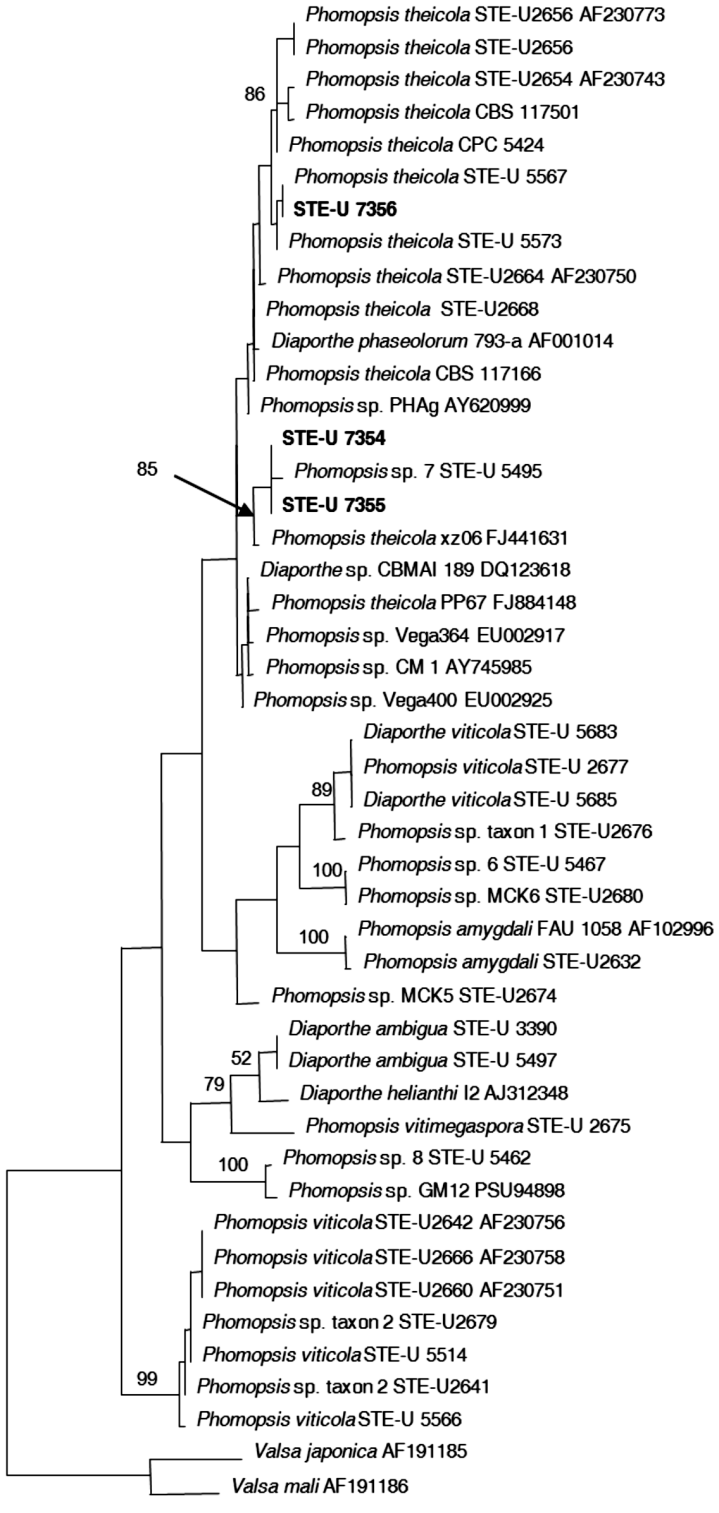

Figure 2. One of 150 most parsimonious trees obtained from ITS sequences of the *Phomopsis* isolates. Bootstrap support values above 50% from 1000 replicates are shown at the nodes. Tree scores include TL = 246, CI = 0.675, RI = 0.841, RC = 0.568, HI = 0.325. Isolates from the present study in bold.

rium brasiliense and *Pr. variabile* isolates originated from *Pyrus*

Symptomatology

Following the work of Van Niekerk *et al*. (2011), six different internal symptom types similar to those occurring in grapevine were identified in pear and apple. These were brown vascular streaking, black vascular streaking, wedge-shaped necrosis, watery necrosis, brown internal necrosis and soft rot (Figure 4). Generally one symptom type was present in each wood piece. The isolation of more

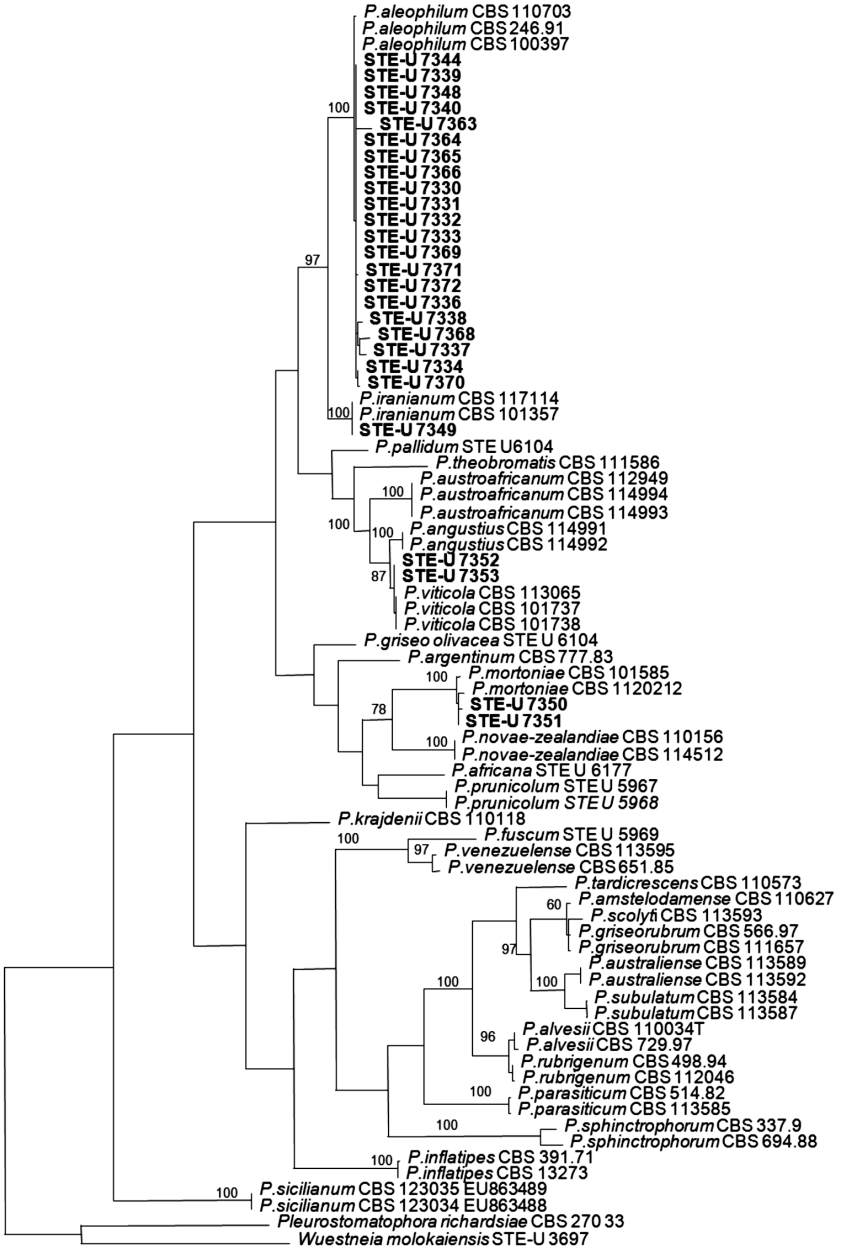

Figure 3. One of 190 most parsimonious trees obtained from β-tubulin region sequences of the *Phaeoacremonium* isolates. Bootstrap support values above 50% from 1000 replicates are shown at the nodes. Tree scores include TL = 1512, CI = 0.506, RI = 0.823, RC = 0.416, HI = 0.494. Isolates from the present study in bold.

than one fungal taxon from a single symptomatic sample occurred only once, where *Phomopsis* sp. 7 and *Pm. aleophilum* were isolated from the same sample. Isolations could be traced back to the specific sample and symptom type from which they were obtained. The occurrence of various species in association with the six symptom types is given in Figure 5.

Most species were isolated too infrequently to be conclusively linked to specific symptoms. Species of *Phaeoacremonium* and Botryosphaeriaceae were mostly isolated from wedge-shaped and brown internal necrotic lesions. These symptom types were also associated with *Phomopsis, Paraconiothyrium* species, *E. lata* and the *Pyrenochaeta*-like species. *Eutypa lata* was also isolated once from watery necrosis and once from brown streaking.

Pathogenicity trial

The results of the pathogenicity trial (Table 1) showed a large variation between hosts, both for length of lesions formed and re-isolation frequencies of fungi.

On grapevine, *N. australe* was the most virulent species with a mean lesion length of 20.0 mm. The *Pyrenochaeta*-like species (8.6 mm), *Pr. variabile* (8.2 mm), *D. seriata* (6.6 mm) and *Pm. mortoniae* (6.2 mm), could also be considered pathogenic, since their lesion lengths were significantly longer than the negative controls. There was no significant difference between length of lesions caused by the remaining species and the negative controls, *Acremonium strictum* (2.5 mm lesion length) and a non-colonised PDA plug (1.8 mm). Re-isolation percentages were between 29.1% and 77.1%, except for *Pr. brasiliense* (16.6%), which did not cause lesions significantly different from *A. strictum*. No correlation was found between lesion lengths and the chance of re-isolation.

On pear, the most virulent species were *Diplodia* sp. (mean lesion length = 55.0 mm) and *N. australe* (52.3 mm). The lesion lengths of *D. seriata* (43.0 mm) and *E. lata* (43.7 mm) were also significantly different from the negative controls (17.7 mm and 4.4 mm for *A. strictum* and the PDA plug, respectively), which may be indicative of their pathogenic nature. *Acremonium strictum* had a re-isolation rate of 100%, while most species were re-isolated at frequencies between 25% and 65% on pear. *Phomopsis* sp. 7 (10.4%) and *E. lata* (4.2%) had low re-isolation frequencies and *Ph. theicola* was not successfully re-isolated.

On apple, *N. australe* (40.2 mm) and *Pm. iranianum* (41.2 mm) gave the longest lesions and were the most virulent species on this host. The *Diplodia* sp. (27.3 mm), *Pm. aleophilum* (25.2 mm), *N. vitifusiforme* (23.8 mm), *D. seriata* (20.1 mm), *Pm. mortoniae* (19.7 mm), *E. lata* (19.2 mm) and *Pr. brasiliense* (18.1 mm) could be considered pathogenic with lesion lengths significantly longer than the negative controls (10.1 mm and 5.6 mm for *A. strictum* and the PDA plug, respectively). This was a wider array of pathogenicity than on the other two hosts, and suggesting that apple is more sensitive to invasion than pear or grapevine. All isolates were frequently re-isolated (37.5 to 87.5%).

Discussion

This study confirms that apple and pear orchards in the Western Cape of South Africa are hosts to many known grapevine trunk pathogens along with various other fungal species that are pathogenic to apple and pear trees as well as grapevines.

Grapevine trunk diseases cause the gradual decline and die-back of vines resulting in decreased capability to carry and ripen fruit. The organisms involved in the different manifestations vary, as do the symptoms themselves. Van Niekerk *et al.* (2011) identified six different types of symptoms associated with trunk diseases, namely brown streaking, black streaking, wedge-shaped necrosis, watery necrosis, brown internal necrosis and soft rot. *Eutypa lata*, *Phaeomoniella chlamydospora*, various species of *Phaeoacremonium* and *Phomopsis*, several members of the Botryosphaeriaceae, and a few basidiomycetes such as *Fomitiporia mediterranea* have been found to be involved in grapevine trunk diseases (Pine, 1958; Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Phillips, 2002; van Niekerk *et al.*, 2004, 2005; Crous *et al.* 2006; Mostert *et al.*, 2006).

A survey of trunk-diseased grapevines in the Stellenbosch area in South Africa revealed an infection level of 31.7% in cv. Cabernet Sauvignon vines older than 10 years that could lead to substantial crop loss (Van Niekerk *et al.*, 2003). A study exami-

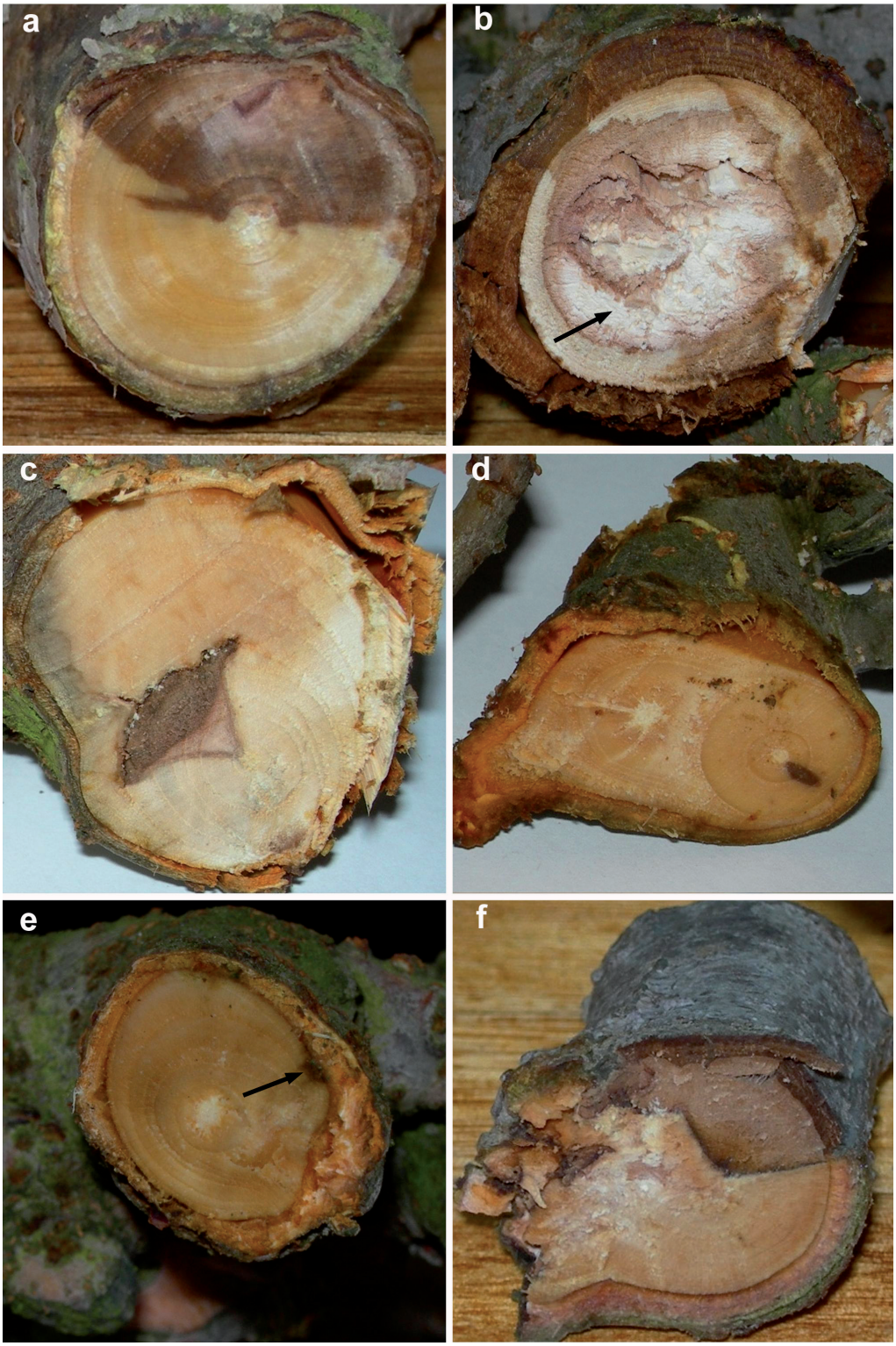

Figure 4. Symptom types associated with trunk disease on pome fruit trees, a) watery necrosis, b) soft rot indicated by arrow, c) brown internal necrosis, d) black/brown streaking, e) brown streaking, f) wedge-shaped necrosis.

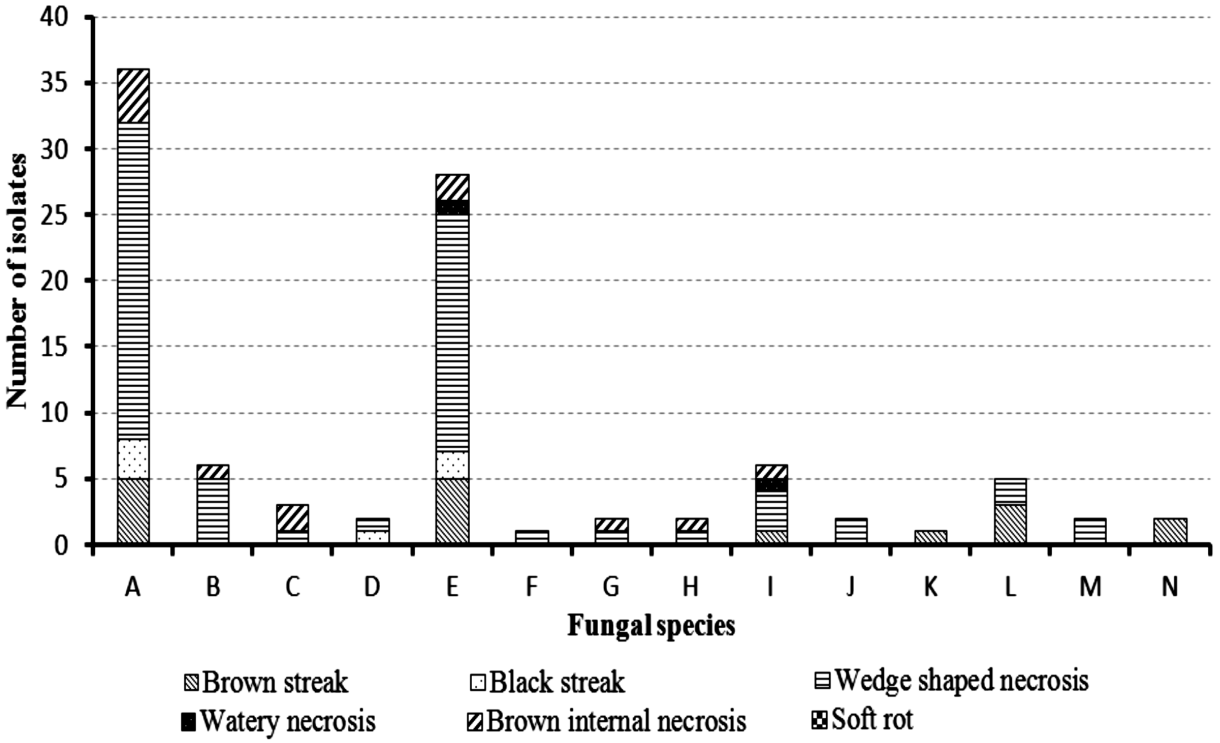

Figure 5. Frequency of isolation of fungal species from the six symptom types found on apple and pear trees with die-back symptoms. The fungi represented by the different bars include: A - *Diplodia seriata*, B - *Diplodia pyricolum*, C - *Neofusicoccum australe*, D - *Neofusicoccum vitifusiforme*, E - *Phaeoacremonium aleophilum*, F - *Phaeoacremonium iranianum*, G - *Phaeoacremonium viticola*, H - *Phaeoacremonium mortoniae*, I - *Eutypa lata*, J - *Phomopsis* sp. 7, K - *Phomopsis theicola*, L - *Pyrenochaeta*-like species, M - *Paraconiothyrium braziliense* and N - *Paraconiothyrium variabile*.

ning quantifiable losses caused by Eutypa die-back and Botryosphaeria canker on grapevines in California calculated an annual loss of $260 million (Siebert, 2001). Munkvold *et al*. (1994) estimated a yield loss of between 30.1 and 69.9% depending on the severity of Eutypa infection in susceptible vineyards. Apart from the inevitable yield losses, trunk disease costs include preventative measures, viticultural practices such as corrective pruning and the eventual loss of vines due to a much decreased lifespan (Siebert, 2001).

In recent years, viticulture has been expanding into several well-established pome fruit growing areas of South Africa, especially in the Elgin, Grabouw and Villiersdorp regions of the Western Cape province. In these areas, unprofitable pome fruit orchards are often replaced with vineyards. Several trunk pathogens such as *E. lata, Phomopsis* species and the Botryosphaeriaceae are known to be cosmopolitan (Carter *et al*., 1983; Murali *et al*., 2006; Slippers *et al*., 2007), while the exact host range of involved organisms such as *Phaeomoniella chlamydospora* and *Phaeoacremonium* species is unknown. During an investigation by Damm *et al*. (2008a, 2010), 14 different species of *Phaeoacremonium* and four species of *Phaeomoniella* were found to be present in stone fruit trees, which suggests that these species may be present on other fruit trees as well. The presence of trunk pathogen populations in old pome fruit orchards may pose long-term threats to young vineyards planted in close proximity to these potential sources of inoculum.

The symptom types found to occur in apple and pear wood were similar to those described by Van Niekerk *et al*. (2011) for grapevine trunk diseases. The species associated with one specific symptom type only, namely *Pr. brasiliense* and *Pr. variabile*,

Table 1. Mean lesion length and re-isolation frequencies of fungal species inoculated onto detached grapevine, pear and apple shoots in a pathogenicity trial.

Fungal species	Strains inoculated (STE-U)	Mean lesion length (mm) and t-grouping[a]			Re-isolation frequency %		
		Grapevine	Pear	Apple	Grapevine	Pear	Apple
Phaeocremonium aleophilum	7334, 7337, 7348	5.1 b,c,d,e,f	14.5 d	25.2 c,d	77.1	41.6	76.4
Phaeoacremonium iranianum	7349	2.8 c,d,e,f	9.7 d,e	41.2 a	66.7	62.5	41.6
Phaeoacremonium viticola	7352, 7353	3.7 c,d,e,f	10.6 d,e	11.8 e,f	50.0	56.3	77.0
Phaeoacremonium mortoniae	7350, 7351	6.2 b,c,d	15.9 d	19.7 c,d	29.1	47.9	85.4
Neofusicoccum vitifusiforme	7313, 7314	3.3 c,d,e,f	25.6c	23.8 b,c,d	93.8	47.9	50.0
Neofusicoccum australe	7310, 7311, 7312	20.0 a	52.3 a,b	40.2 a	93.1	31.9	55.5
Diplodia seriata	7229, 7269, 7279	6.6 b,c	43.0 b	20.1 c,d	73.6	50.0	48.6
Diplodia sp.	7296, 7297, 7299	5.4 b,c,d,e	55.0 a	27.3 b	79.2	26.4	52.7
Eutypa lata	7304, 7306, 7309	1.5 e,f	43.7 b	19.2 c,d	58.3	4.2	68.0
Phomopsis sp. 7	7354, 7355	1.3 e,f	13.1 d,e	11.8 e,f	77.1	10.4	70.8
Phomopsis theicola	7356	1.5 f	4.2 e	10.2 f	91.6	0.0	45.8
Pyrenochaeta-like sp.	7358, 7359, 7362	8.6 b	8.7 d,e	11.4 f	38.8	29.2	55.5
Paraconiothyrium brasiliense	7315, 7316	2.9 c,d,e,f	10.4 d,e	18.1 d,e	16.6	47.9	87.5
Paraconiothyrium variabile	7317, 7318	8.2 b	13.4 d,e	10.5 f	39.6	52.1	37.5
Acremonium strictum	6926	2.5 d,e,f	17.7 c,d	10.1 f	75.0	100.0	75.0
PDA plug		1.8e,f	4.4e	5.6f			
LSD (P<0.05)		3.97	9.25	6.52			

[a]Lesions lengths followed by the same letter were not significantly different.

Ph. theicola, *Phomopsis* sp. 7 and *Pm. iranianum*, did not occur frequently enough to conclusively associate these species with these symptom types.

The overwhelming presence of wedge-shaped necrotic symptoms suggested the presence of a large number of either Botryosphaeriaceae or *E. lata,* since this symptom type has commonly been associated with these pathogens on grapevine in the past (Moller and Kasimatis, 1978; Van Niekerk *et al*., 2004; 2011). A large number of Botryosphaeriaceae were isolated from this symptom type. The most prevalent (84%) of the Botryosphaeriaceae associated with symptomatic wood was *D. seriata*, previously known to be associated with wood rot and black rot of fruit in apples, pears and grapevine (Jones and Aldwinkle, 1991; Phillips, 2002; Phillips *et al*., 2007). This was not without precedent as this prevalence corresponds to the findings of Slippers *et al*. (2007), who found *D. seriata* to make up 90% of the Botryosphaeriaceae found on pome fruit in their study. *Diplodia seriata* has also been found to be the dominant species on stone fru-

it (Damm *et al.*, 2007) and grapevine (van Niekerk *et al*., 2004; 2011) in South Africa.

Eutypa lata was also isolated from pome fruit trees, and this is the first reported occurrence of *E. lata* on *Pyrus* and *Malus* in South Africa, although the pathogen has been reported from these hosts elsewhere in the world (Carter *et al*., 1983). *Eutypa lata* was isolated from different kinds of lesions, including brown streaking, wedge-shaped necrosis, watery necrosis and brown internal necrosis. Usually, *E. lata* is associated with brown, wedge-shaped necrotic sections on grapevine (Moller and Kasimatis, 1978). In a study conducted by Van Niekerk *et al*. (2011), *E. lata* was isolated in five cases from esca-like soft brown wood rot symptoms on grapevines in winter-rainfall areas of South Africa.

Four *Phaeoacremonium* species were found during this study, namely *Pm. aleophilum, Pm. iranianum, Pm. mortoniae* and *Pm. viticola*. *Phaeoacremonium aleophilum* comprised 85% of the *Phaeoacremonium* isolates, while the other three species had a very limited occurrence. This is a first report of *Phaeoacremonium* species occurring on pear wood and a first report of *Pm. aleophilum* occurring on apple. Of the different *Phaeoacremonium* species, only *Pm. angustius* and *Pm. mortoniae* have been reported from *Malus* in California (Rooney-Latham *et al.*, 2006). *Phaeoacremonium aleophilum* is also the most common species of *Phaeoacremonium* found associated with Petri disease in grapevines (Mostert *et al*., 2006).

The pathogenicity test revealed a large variation in lesion lengths between fungal species and between hosts, and also between the profiles of species that could be considered pathogenic on the various hosts. This variation in pathogenicity might be an aberration of the detached shoot assay employed, and although this methodology has been used elsewhere (Van Niekerk *et al.*, 2004; Damm *et al*., 2007; 2008a; Úrbez-Torres *et al.,* 2008), these results should be considered only as indicative of potential pathogenicity.

Variability in pathogenicity may indicate that differences in certain host characteristics cause certain fungal species to favour specific hosts. However, the re-isolation frequencies obtained during this study indicate that all species had become established within host tissue to some extent, with the exception of *Ph. theicola* on pear. This suggests that these particular *Phomopsis* isolates were unable to establish growth within the host, *Pyrus*. Higher rates of re-isolation were not correlated with increased virulence, as measured by the lesion lengths.

On grapevine shoots the lesion lengths were generally shorter than on pear and apple shoots. This is a surprising result which may be an indication that a longer incubation time is needed on grapevine shoots to obtain more conclusive results. *Eutypa lata* is known to develop slowly in host tissue (Munkvold *et al*., 1994), which could also explain the small mean lesion length caused by this fungus on grapevine shoots. *Eutypa lata* did, however, cause significant lesions to develop on apple and pear shoots.

The *Phomopsis* species did not form significant lesions on any of the hosts tested. In a similar pathogenicity test, Van Niekerk *et al.* (2005) also found no significantly different lesions of *Ph. theicola* and *Phomopsis* sp. 7 in comparison with the negative control when tested on the grapevine cultivars 'Pinotage' and 'Chenin Blanc' in a similar test on detached grapevine shoots.

The lesions caused by *N. australe* on all hosts were significantly longer than those caused by other species. This finding is in accordance with that of Van Niekerk *et al*. (2004), who found the same species to cause severe lesions on grapevine. *Neofusiccocum vitifusiforme* only caused statistically significant lesions on apple wood. *Diplodia seriata* caused lesions which were statistically different from the negative controls on all hosts, and the profusion with which it is found in Western Cape orchards certainly warrants further investigation. Damm *et al*. (2007) obtained similar results when testing the pathogenicity of *D. seriata* on detached *Prunus* shoots. Van Niekerk *et al.* (2004) found *D. seriata* to be weakly pathogenic on mature grapevine canes and non-pathogenic on detached green shoots, and Úrbez-Torres *et al.* (2008) found *D. seriata* pathogenic on rooted cuttings and detached green shoots. The results from the present study suggest that *D. seriata* is likely to be pathogenic on pome fruit trees. The *Diplodia* sp. caused statistically significant lesions on apple and pear wood, but not on grapevine.

Phaeoacremonium species are known to cause die-back or decline symptoms on various woody hosts. Economically important crops include

date palms (Hawksworth *et al*., 1976), *Prunus* species (Hawksworth *et al*., 1976; Rumbos, 1986; Damm *et al*., 2008a.), kiwifruit vines (Di Marco *et al*., 2004) and olive trees (Hawksworth *et al*., 1976). The present study is the first to report on the pathological relevance of *Phaeoacremonium* species on apple and pear trees. Of the four *Phaeoacremonium* species found during this study, *Pm. iranianum* caused the longest lesions when compared statistically to the control treatments on apple. *Phaeoacremonium aleophilum* and *Pm. mortoniae* are also likely to be pathogenic on apple as they caused lesions significantly longer than the negative controls. None of these species formed significant lesions on either of the other hosts. This is particularly surprising as *Pm. aleophilum* is considered as one of the main pathogens involved in the esca and Petri disease complex (Mugnai *et al*., 1999; Mostert *et al.,* 2006), which indicates that a longer incubation period and/or stress-predisposition might be required for clear pathogenic reactions to occur. *Phaeoacremonium aleophilum* also failed to produce significantly longer lesions on grapevine than the negative controls in a similar detached shoot assay (U. Damm, unpublished data), which indicates that the assay might not be ideally suited to highlight the pathogenicity of *Phaeoacremonium* species. *Phaeoacremonium iranianum* has not been found on grapevines in South Africa, though it has been found on grapevines in Iran and Italy. Only one isolate of this fungus was found on pears in Wolseley during this study, and its possible occurrence on grapevines in this area should be investigated further. *Phaeoacremonium* species are generally associated with Petri disease and esca-like wood symptoms in grapevine, but Van Niekerk *et al*. (2011) found *Phaeoacremonium* species commonly associated with wedge-shaped necrotic symptoms on this host in winter and summer rainfall areas of South Africa. Although the number of the other *Phaeoacremonium* species isolated was low, these were also found in association with the wedge-shaped and brown necrotic symptoms. It has been postulated that while streaking symptoms are the result of host response to vascular invasion (Atia *et al*., 2003), necroses are more advanced symptoms and these may naturally occur after streaking in a progressive cycle of symptom development (Van Niekerk *et al*., 2011). This suggestion warrants further investigation in pome fruit, especially as necrotic symptoms yielded more fungal species than streaking symptoms in this study.

This is the first reported occurrence of *Pr. brasiliense* on pear and *Pr. variabile* on apple. The genus *Paraconiothyrium* is not known to be pathogenic on the hosts tested, although *Pr. brasiliense* proved to be potentially pathogenic on apple, while *Pr. variabile* caused lesions longer than the control treatments on grapevine. This may be a result of the detached shoot trial and further investigation is warranted. Damm *et al*. (2008b) recently reported *Pr. brasiliense* occurring on necrotic wood of *Prunus* spp. and described *Pr. variabile* from the same hosts in South Africa.

The unidentified *Pyrenochaeta*-like species isolated from *Malus* caused lesions longer than the control treatments on grapevine, which indicates that this organism could be pathogenic on grapevines.

The results of the isolations have revealed the presence of several major grapevine trunk pathogens on pome fruit in the Western Cape, South Africa and, although the pathogenicity trial should only be seen as a preliminary examination of pathogenicity, the results indicate that several of the species found during the study may potentially be pathogenic on pome fruit trees and grapevines.

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