POME FRUIT TREES AS ALTERNATIVE HOSTS OF GRAPEVINE TRUNK DISEASE PATHOGENS

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Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Agriculture at the University of Stellenbosch

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March 2010

Declaration

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SUMMARY

A survey was undertaken on apple and pear trees in the Western Cape Province to determine the aetiology of trunk diseases with reference to trunk diseases occurring on grapevine. Grapevine trunk diseases cause the gradual decline and dieback of vines resulting in a decrease in the vine's capability to carry and ripen fruit. In recent years, viticulture has been expanding into several of the well established pome fruit growing areas. The presence of trunk pathogens in pome fruit orchards may affect the health of the pome fruit trees as well as cause a threat to young vineyards planted in close proximity to these potential sources of viable inoculum.

Several genera containing species known to be involved in trunk disease on pome fruit and grapevine were found, including *Diplodia*, *Neofusicoccum*, *Eutypa*, *Phaeoacremonium* and *Phomopsis*. *Diplodia seriata* and *D. pyricolum*, were isolated along with *N. australe* and *N. vitifusiforme*. Four *Phaeoacremonium* species, *P. aleophilum*, *P. iranianum*, *P. mortoniae* and *P. viticola*, two *Phomopsis* species linked to clades identified in former studies as *Phomopsis* sp. 1 and *Phomopsis* sp. 7, and *Eutypa lata* were found. In addition, *Paraconiothyrium brasiliense* and *Pa. variabile*, and an unidentified *Pyrenochaeta*-like species were found. Of these the *Phaeoacremonium* species have not been found on pear wood and it is a first report of *P. aleophilum* occurring on apple. This is also a first report of the *Phomopsis* species and *Eutypa lata* found occurring on pome trees in South Africa

Two new coelomycetous fungi were also found including a *Diplodia* species, *Diplodia pyricolum* sp. nov., and a new genus, *Pyrenochaetoides* gen. nov. with the type species, *Pyrenochaetoides mali* sp. nov., were described from necrotic pear and apple wood. The combined ITS and EF1-α phylogeny supported the new *Diplodia* species, which is closely related to *D. mutila* and *D. africana*. The new species is characterised by conidia that become pigmented and 1-septate within the pycnidium, and that are intermediate in size between the latter two *Diplodia* species. Phylogenetic inference of the SSU of the unknown coelomycete provided bootstrap support (100%) for a monophyletic clade unrelated to known genera, and basal to *Phoma* and its relatives. Morphologically the new genus is characterised by pycnidial with elongated necks that lack setae, cylindrical conidiophores that are seldomly branched at the base, and *Phoma*-like conidia. The phylogenetic results combined with its dissimilarity from genera allied to *Phoma*, lead to the conclusion that this species represents a new genus.

A pathogenicity trial was undertaken to examine the role of these species on apple, pear and grapevine shoots. *N. australe* caused the longest lesions on grapevine shoots, while *Pyrenochaetoides mali*, *Pa. variabile*, *D. seriata* and *P. mortoniae* caused lesions that were significantly longer than the control inoculations. On pears, *D. pyricolum* and *N. australe* caused the longest lesions, followed by *D. seriata* and *E. lata*. On apples, the longest lesions were caused by *N. australe* and *P. iranianum*. *D. seriata*, *D. pyricolum*, *E. lata*, *N. vitifusiforme*, *Pa. brasiliense*, *P. aleophilum* and *P. mortoniae* also caused lesions on apple that were significantly longer than the control.

The study demonstrated that close cultivation of grapevine to apple and pear orchards may have inherent risks in terms of the free availability of viable inoculum of trunk disease pathogens.

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1. A REVIEW OF ASCOMYCETOUS TRUNK PATHOGENS OCCURRING ON POME FRUIT TREES AND GRAPEVINES

INTRODUCTION

The production of pome fruit, most notably apples (*Malus domestica* Borkh.) and pears (*Pyrus communis* L.), for export has played an important role in the development of the agricultural sector in the Western Cape, especially in areas such as the Overberg and Ceres. In 2007, South Africa was the 9th ranked producer of pears in the world, with a total production of 325 000 metric tons (OABS, 2009; Belrose, 2008a). South Africa was ranked the 17th largest producer of apples in the world, having produced 650 000 metric tons in 2007, but remains in a favourable position for export due to its location in the Southern Hemisphere (Belrose, 2008b).

In apple production, with a national netto production area of 20 736 ha in 2008, Elgin is the most important area with a total of 6 062 ha planted, followed by Ceres with 5 048 ha and the Villiersdorp / Vyeboom area as the fourth largest producer with 3 475 ha planted. The most important apple cultivar in terms of the total area under cultivation is Granny Smith, with a total of 5 050 ha (25%) cultivated in 2008 (OABS, 2009).

In 2008, Ceres was the most important pear cultivation area for pears with 4 355 ha from a total of 11 425 ha under cultivation. Elgin (1 452 ha) and Villiersdorp / Vyeboom (945 ha) were the third- and fifth-most important areas respectively. Packham's Triumph is the most important pear cultivar with a total of 3 278 ha under cultivation, making up 29% of the total pear cultivation in South Africa in 2008 (OABS, 2009).

The cultivation of grapevine (*Vitis vinifera* L.) for the production of table grapes, wine, brandy and other grape related products, such as concentrate, grape juice and wine for distillation, forms an integral part of the agricultural economy of the Western Cape and South Africa as a whole. In 2008, the industry produced some 1089 million litres of wine, brandy and grape juice. Wine grape production currently takes up a total of 96 296 hectares of arable land in the Western Cape province, creating a total income of R 3 319 million for producers (SAWIS, 2009)

As one of several "new-world" countries with a healthy wine-industry, winemakers in South Africa have been under increasing pressure to produce an export product acceptable to an international market. In 2008, South Africa exported 412 million litres of wine, almost 35% more than during 2006 (SAWIS, 2009). The development of "terroir" as a marketable

concept has led to wine-farmers increasingly moving into new areas, thought to be more suited to certain wine-styles and cultivars.

Terroir is traditionally defined as “an area or terrain, usually rather small, whose soil and microclimate impart distinctive qualities to food products” (Barham, 2003). Although the precise notion of terroir has been contentious in the past (Carey, 2005), terroir can roughly be divided into two groups of factors, namely natural and human factors (Morlat, 2001). Natural factors include soil and climate, while human factors consist of viticultural and oenological practices. Cooler temperatures during ripening, associated with the relatively mild summers of traditional pome-fruit growing areas such as Elgin, can make a significant difference in sought-after flavour aspects, good colour development and the prevention of delayed budding in temperature sensitive cultivars such as Sauvignon blanc, Pinot noir and Chardonnay (Archer, 2005).

In the Western Cape, the establishment of viticulture in traditional pome-fruit growing areas to take advantage of unique terroir aspects has been on the increase since the 1990’s. From a phytopathological point of view, this practice is not without risks as some of the most common grapevine trunk pathogens such as *Eutypa lata*, certain species of *Phomopsis* and the *Botryosphaeriaceae* are relatively cosmopolitan (Carter *et al.*, 1983; Moleleki *et al.*, 2002; Slippers and Wingfield, 2007). In viticulture, these organisms cause the gradual blockage and death of vascular tissue in the permanent structures of the vine. This phenomenon leads to a decrease in the conductivity of the xylem vessels and may cause the death of entire arms and, eventually, entire vines (Goussard, 2005). Siebert (2001) found four losses associated with trunk disease during a study of damage caused by both Eutypa die-back and Bot canker in viticulture; grouped because the symptoms caused by both are often indistinguishable. The so-called four losses were yield loss due to the decline of the vine’s ability to carry and ripen bunches, the cost of preventative measures such as wound protectants, labour and material costs for corrective pruning, top working and replanting as necessary and the eventual replacement of the entire vineyard.

Since these trunk pathogens are mainly spread to fresh infection sites such as pruning and desuckering wounds via air- and waterborne inoculum (Trese *et al.*, 1980; Pseidt and Pearson, 1989), having a low pre-existing inoculum pressure is a logical part of disease prevention. Sanitation by way of removing debris from the vineyard or orchard is one such mechanism that has been shown to be more or less effective depending on the organism involved (Starkey and Hendrix, 1980; Uddin and Stevenson, 1998).

Given the propensity for organisms such as *Eutypa lata* and the *Botryosphaeriaceae* to have wide host ranges, including crops such as pome and stone fruit trees, it may be complex to manage inoculum pressure within a multi-crop system. The existence of a vast inoculum source in areas where new vines are planted leaves young plants in peril of early infection during the first few seasons after establishment, drastically reducing the quality and quantity of grape yields from the very start of the vineyard's productive life-time. Further knowledge regarding the identity, epidemiology and aetiology of trunk diseases already occurring in these areas is therefore needed and is the focus of this study.

This chapter seeks to examine the pathogens known to be involved in trunk diseases on the most commonly cultivated pome fruit, the apple tree and the European pear tree, with specific reference to pathogens also known to occur on grapevine.

THE *BOTRYOSPHAERIACEAE* AS CAUSAL ORGANISMS OF DECLINE AND DIE-BACK ON POME FRUIT TREES AND GRAPEVINE

The family *Botryosphaeriaceae* Theiss. & P. Syd contains many species known to cause various manifestations of disease on a wide range of hosts. The genus *Botryosphaeria* Ces. & De Not (Ascomycota, Dothideomycetidae, Dothideales, *Botyrosphaeriaceae*) was first introduced in 1863, with *Botryosphaeria dothidea* (Moug. Fr.) Ces. & De Not. as type species and consists of many species with a cosmopolitan distribution (Crous *et al.,* 2006).

Members of the *Botryosphaeriaceae* have been isolated from various hosts (Parker and Sutton, 1993; Brown-Ritlewski and McManus, 2000; Ntahimpera *et al*, 2002; Phillips, 2002). Manifestations on grapevine and fruit trees include wood symptoms such as gummosis, cankers, sectorial vascular necroses, brown vascular streaking, graft union failure and other symptoms such as bud blight, shoot blight, frog-eye leaf-spot and white and black fruit rots (McGlohon, 1982; Smith *et al.,* 1984; Brown and Britton, 1986; Milholland, 1991; Pusey and Bertrand, 1993; Mila *et al.,* 2005). In grapevine, *Botryosphaeria* spp. have often been regarded as weak pathogens (Phillips, 2002; van Niekerk *et al.*, 2006) but many species have been shown to cause severe symptoms on hosts, especially since many of the *Botryosphaeriaceae* live as endophytes within plants (Slippers and Wingfield, 2007) and may only cause disease once infected plants are under stress (Schoeneweiss, 1981). Conversely certain species such as *Neofusicoccum australe* Crous, Slippers and A.J.L Phillips have been shown to be both pathogenic and extremely virulent on hosts such as grapevine and *Eucalyptus* (Van Niekerk, 2004, Taylor *et al.,* 2005).

Sexual structures of the teleomorph, *Botryosphaeria*, are rare in nature and do not commonly form on artificial media. Eighteen different anamorph genera have, in the past, been linked to *Botryosphaeria*. Most of these were eventually grouped under the genera *Diplodia* and *Fusicoccum*, based on conidial characteristics and internal transcribed spacer (ITS) region sequences (Denman *et al.*, 2000). In 2006, after a survey of DNA sequence data from the 28s rDNA region (large subunit) in an attempt to impose a "genus for genus concept" (Seifert *et al.*, 2000), Crous *et al.* (2006) recognised ten clades within the *Botryosphaeriaceae* and introduced new anamorph genera.

The morphological identification of *Botryosphaeriaceae* is difficult due to a certain degree of uniformity within both teleomorph and anamorph species (Jacobs and Rehmer, 1998; Slippers *et al.*, 2007), especially in genera with a *Diplodia*-like anamorph such as *Diplodia*, *Lasiodiplodia* and *Dothiorella* since their conidia are difficult to distinguish (De Wet *et al.*, 2008). For example, although *Botryosphaeria dothidea* has been reported to be one of the most important and widespread causal organisms of peach gummosis (Pusey, 1993), Slippers *et al.* (2004a) recently proved that what was previously considered to be *B. dothidea* can now be distinguished as *N. ribis*, *N. parvum* and *B. dothidea*. Molecular identification methods have therefore been useful to determine species identification and to elucidate the taxonomy of the family.

Jacobs and Rehmer (1998) and Denman *et al.* (2000) used ITS region phylogenies in combination with morphological details and were able to distinguish between several anamorph species. The non-coding ITS region in combination with the introns of coding genes such as the translation elongation factor 1-alpha and β-tubulin have also been used successfully to reliably distinguish between species (Slippers *et al.*, 2004a, b; Van Niekerk *et al.*, 2004), but sequencing is expensive and time-consuming when dealing with large numbers of isolates. Alves *et al.* (2005) used amplified ribosomal DNA restriction analysis (ARDRA), which was inexpensive, fast and useful in distinguishing between ten *Botryosphaeria* species using two restriction enzymes in various combinations. In 2007, Alves *et al.* suggested the use of microsatellite-primed polymerase chain reaction (MSP-PCR) and repetitive-sequence-based polymerase chain reaction (rep-PCR) to rapidly distinguish between "*Botryosphaeria*" species using only one primer or a primer set. These tools may be helpful in rapid identification of *Botryosphaeria* spp. which will assist in furthering the understanding of the epidemiology of the *Botryosphaeriaceae*.

During a review of reported plant pathogens in South Africa, Crous *et al.* (2000) listed "*Botryosphaeria" obtusa* (Schwein.) Shoemaker, *B. dothidea* (Moug. Fr.) Ces. & De Not and

"B". ribis Grossenb. & Duggar (N*eofusicoccum ribis*) as previously reported on *Malus* and *Vitis* and "*Botryosphaeria" obtusa* and *Botryosphaeria dothidea* as previously reported on *Pyrus*. Carstens (2006) also listed "*B". parva* Pennycook & Samuels (*Neofusicoccum ribis*), "*B". rhodina* (Berk. & Curtis) Arx (*Lasiodiplodia theobromae*), in addition to the species Crous *et al.* listed, as occurring on *Malus* in South Africa. During a survey of species of *Botryosphaeria* occurring on grapevines, Van Niekerk *et al.* (2004) found *B. australis, B. lutea, B. obtusa, B. parva, B. rhodina* and an unknown *Diplodia* sp. in addition to describing *Diplodia porosum*, *Fusicoccum viticlavatum* (*Neofusicoccum viticlavatum*) and *F. vitifusiforme* (*Neofusicoccum vitifusiforme*) on South African grapevines. Van Niekerk *et al* (2004) did not find any *Botryosphaeria dothidea* during the survey, but Bester (2006) isolated it from symptomatic table grapevine from Mpumalanga.

According to a recent phylogenetic study of the Botryosphaeriaceae on pome and stone fruit in South Africa, six species were found on fruit trees namely *Neofusicoccum ribis , N. parvum, N. australe, Botryosphaeria dothidea, Diplodia mutila* (Fr.) Mont. and "*Botryosphaeria*" *obtusa* (*Diplodia seriata*) (Slippers *et al*., 2007). Most recently, Damm *et al.* (2007) isolated eight different species from stone fruit in South Africa, including *Diplodia seriata, D. pinea, D. mutila, Dothiorella viticola, Neofusicoccum australe, N. vitifusiforme* and two previously unknown species, *Diplodia africana* Damm & Crous and *Lasiodiplodia plurivora* Damm & Crous.

"*Botryosphaeria" obtusa (*synonym: *Physalospora obtusa*) was found to be the most frequently isolated species on fruit trees, representing 90% of the species isolated by Slippers *et al*. (2007), and has been found to be the dominant species isolated from Bot canker in grapevine in South Africa (Van Niekerk *et al*., 2010). "*Botryosphaeria" obtusa* has been found to be an illegitimate moniker for the species since the teleomorph is hardly ever seen. The names *Diplodia malorum* Fuckel, *Sphaeropsis malorum* Peck and *Sphaeropsis malorum* (Berk.) Berk. have been used for this anamorph in the past. *Sphaeropsis malorum* Peck was declared illegitimate since *Sphaeropsis malorum* (Berk.) Berk. is the older name and the latter has since been found to be a synonym of *Diplodia mutila* (Stevens, 1933; Alves *et al*., 2004). Phillips *et al.* (2007) have since conclusively named the anamorph *Diplodia seriata.*

A few studies have been done on the epidemiology of *Botryosphaeria* species on apple and peach, but comparatively little have been undertaken on grapevine until recently. The variety of species that have been associated with manifestations of disease on various hosts further complicates the epidemiological study of the *Botryosphaeriaceae* because results have shown that there are differences between species in terms of factors such as the

conditions required for sporulation, germination and host infection (Sutton, 1981; Arauz and Sutton, 1989a; Arauz and Sutton, 1989b; Copes and Hendrix, 2004), as well as differences between cultivars within host species (Biggs and Miller, 2003; Latorre and Toledo, 1984) and variation in virulence within species (Parker and Sutton, 1993; Brown-Rytlewski and McManus, 2000, Van Niekerk *et al.*, 2004, Damm *et al.*, 2007).

Temperature has been shown to have an effect on *in vitro* sporulation in *B. dothidea, Diplodia seriata* and *Lasiodiplodia theobromae* in that the three species have different requirements for sporulation and conidial maturation and that higher temperatures are required for germination and mycelial growth than for sporulation (Copes and Hendrix, 2004).

Holmes and Rich (1970) investigated factors contributing towards the release and dissemination of ascospores and conidia of *"B". obtusa* in apple orchards and found that a temperature of between 6 and 16°C coupled with rainfall events was needed for optimum spore release to take place. Van Niekerk (2007) linked higher levels of airborne spores of various *Botryosphaeriaceae* with rainfall as little as 0.25 mm and found high levels occurring during years with a higher mean rainfall. Holmes and Rich (1970) found that there are three modes of dissemination for ascospores and conidia within an orchard, namely water splash from rainfall, wind and the insect vector *Hippodamia convergens* (the convergent lady beetle). It has since been reported that while *Botryosphaeria dothidea* discharges its ascospores immediately after the start of or during a rainfall event, *"B". obtusa* will only discharge its ascospores during the later part of a rainy period. Ascospores are only found during and immediately after rainfall events and both ascospores and conidia of both species are most abundant during late spring and early summer. Rain splash is the most important method of dissemination of conidia and ascospores, but ascospores may also be airborne during windy periods (Sutton, 1981). Pusey (1989) found that conidia of *B. dothidea* makes up the greatest proportion of waterborne spores from diseased trees in peach orchards whereas conidia of *"B". obtusa* were found to be dominant in dead prunings. Airborne ascospores of *B. dothidea* were found at high levels during spring but airborne ascospores of *"B". obtusa* and *B. rhodina* were found at high levels for the duration of the season after periods of wetness (Pusey, 1989; Van Niekerk *et al.*, 2010).

Arauz and Sutton (1989b) demonstrated that both conidia and ascospores of *"B". obtusa* need a 100% relative humidity during a period of at least four hours at 16 – 32° C for optimum germination and that no germination would take place at a relative humidity of less than 88.5%. Infection is another aspect of the life-cycle of *Botryosphaeria* where

temperature and moisture plays an important role and it has been demonstrated that *"B". obtusa* conidia and ascospores need temperatures of around 26.6°C with a wetting period of between 4.5 and 13 hours for optimal leaf infection and 20 – 24°C for 9 hours for optimal fruit infections to take place (Arauz and Sutton, 1989a). The *Botryosphaeriaceae* are notorious wound pathogens and infection may take place through natural or man-made wounds, or natural openings such as lenticels (Pusey and Bertrand, 1993; Brown-Rytlewski and McManus, 2000).

As with other trunk disease pathogens the recent developments in molecular identification and detection methods will surely assist in the further unraveling of the taxonomy and epidemiology of the family *Botryosphaeriaceae* in pome fruit, grapevine and other hosts.

DIAPORTHE AND *PHOMOPSIS* SPECIES AS CAUSAL AGENTS OF DIE-BACK ON POME FRUIT TREES AND GRAPEVINE

The genus *Diaporthe* Nitschke (Ascomycota, Sordariomycetidae, Diaporthales, *Diaporthaceae*) consists of more than 800 named taxa with a coelomycete anamorph, *Phomopsis* (Sacc.) Bubák, consisting of more than 900 species (Uecker, 1988); many of which are pathogenic on a variety of hosts including apple, pear, asian pear, peach, plum and grapevine (Rehner and Uecker, 1994; Smit *et al.,* 1996; Uddin and Stevenson, 1998; Kanematsu *et al.,* 2000; Van Niekerk *et al.,* 2005; Van Rensburg *et al.,* 2006).

Differentiating between species within *Diaporthe* and *Phomopsis* has been fraught with difficulty due to a large amount of variability in morphological characteristics between species (Wehmeyer, 1933; Rehner and Uecker, 1994). Based on this problem, species have been characterised by host specificity which has led to a great proliferation of species, especially in *Phomopsis* (Wehmeyer, 1933; Rehner and Uecker, 1994; Rossman *et al.*, 2007). This has proved problematic since many species of *Phomopsis* have been shown to be pathogenic on various hosts and since certain crops such as grapevine have been proven to host a variety of distinct *Phomopsis* species (Rehner and Uecker, 1994; Uddin and Stevenson, 1998; Kajitani and Kanematsu, 2000; Mostert *et al.*, 2001; Van Niekerk *et al.,* 2005). Most recently Van Niekerk *et al.* (2005) found fifteen distinct species of *Phomopsis* occurring on grapevine in South Africa.

The emergence of PCR, sequencing and phylogenetics has shed some light on the problem of species proliferation and Rehner and Uecker (1994) was able to distinguish three

clades based on ITS phylogeny using isolates of which the species identity was purposely not included. The three clades, consisting of 43 North American and Caribbean strains of *Phomopsis* corresponded in origin, host affiliation and morphology. In the light of those results, more recent studies on the topic have focused on combining morphological, sequence and pathological data to elucidate the taxonomy of the *Diaporthaceae* (Uddin and Stevenson, 1998; Kanematsu, 2002).

Phomopsis disease on grapevine takes on a general form known as Phomopsis cane and leaf spot, cane and leaf blight or grapevine swelling arm. The condition is mainly caused by *Phomopsis viticola* (Sacc.) Sacc., but *P. vitimegaspora* Kuo & Leu (teleomorph *Diaporthe kyushuensis* Kajitani and Kanem.), *P. amygdali* (Delacr.) J.J Tuset & M.T Portilla and a species referred to as *Diaporthe perjuncta* Niessl have also been associated with similar manifestations of disease (Pine, 1959; Kuo and Leu, 1998; Kajitani and Kanematsu, 2000; Mostert *et al.*, 2001). Rawnsley *et al.* (2004) demonstrated that *D. perjuncta* does not cause these symptoms in grapevine in Australia, though the organism has been isolated from diseased vines elsewhere.

Phomopsis cane and leaf spot is a well-studied disease of *Vitis vinifera* and *Vitis labrusca* L. occurring in all grape-growing regions of the world. It is characterised by spotting and necrosis of leaves on the basal nodes of shoots, corky abrasions on infected shoots, longitudinal dark lesions with pycnidia on shoots, petioles, tendrils and rachises, the splitting of infected parts, death of shoots and the bleaching of dormant canes and vines often take on a bushy appearance caused by suckering around dead spurs (Pine, 1959) and after a period of two years or more following infection, nodes on infected canes may appear hypertrophied, hence the name “swelling arm disease” (Kuo and Leu, 1998; Kajitani and Kanematsu, 2000). Typical cankers may be observed after a period of around 4 years on older arms of infected vines (Kajitani and Kanematsu, 2000).

When rainfall occurs during bloom and before harvest, grape berries may become infected and fruit rot occurs (Pine, 1959; Pscheidt and Pearson, 1989; Rawnsley *et al.*, 2004). Erincik *et al.* (2003) reported that on *V. labrusca* an optimum temperature and wetness-duration required for leaf infection would be between 16 and 20°C and 8.2 to 12.4 hours of wetness. They also referred to Bugaret (1984), who reported significantly higher optimum temperatures (23 – 25°C) required for leaf infection in *V. vinifera* in France. The difference in optimum temperatures could be due to the different *Vitis* species or differences between *P. viticola* isolates.

The primary source of inoculum of *P. viticola* is pycnidia on infected spurs and clusters left in the vineyard and the secondary source is mycelial growth from diseased parts of the vine (Pine, 1959). Berries remain susceptible throughout the growing period, regardless of growth stage, though infections may remain latent until the fruit ripens (Erincik *et al.*, 2001; Pscheidt and Pearson, 1989). Pscheidt and Pearson (1989) found a 37.7% yield loss estimate associated with *Phomopsis* infection of Concord (*V. labrusca*) grapes. They also found rachis infection to be the most important phase of the disease in terms of yield losses incurred because infected rachises become brittle and may cause the entire cluster to drop from the vine.

Species of *Phomopsis* are important pathogens of *Prunus* species, especially peach, almond and plum. Constriction canker, shoot blight and fruit rot of peach (*Prunus persica* L.) caused by, amongst others, *Phomopsis amygdali*, is one of the most serious diseases affecting peach in Japan and the south-eastern United States (Uddin and Stevenson, 1997; Farr *et al*., 1999; Kanematsu *et al*., 1999a, b). Symptoms are difficult to distinguish from those caused by other shoot canker pathogens such as the *Botryosphaeriaceae* and are characterised by the development of necrosis from a node to the current season's shoot with cankers expanding around buds to eventually constrict the shoot and disrupt the flow of water, causing wilting and shoot death (Uddin and Stevenson, 1997). Uddin *et al*. (1998) found the highest amount of inoculum to occur during spring when temperatures are between 18 and 24°C but pycnidia were found to produce copious amounts of alpha conidia throughout the entire year (Lalancette and Robison, 2001). There is a wetness requirement for inoculum to spread since pycnidia are produced within a gelatinous matrix, which has to be dissolved before conidia can be released. Inoculum is spread within trees through the movement of water and the dispersal of conidia from debris on the orchard floor was found unlikely to be a source of further infection (Uddin and Stevenson, 1998). Breaking buds, wounded buds and leaf scars were found to be the most susceptible sites of infection (Uddin and Stevenson, 1997).

Despite the many different *Phomopsis* species found on South African grapevines and other crops such as *Aspalathus linearis* (Mostert *et al.*, 2001; Van Niekerk *et al.*, 2004; Janse van Rensburg *et al.*, 2006), not many instances of *Phomopsis* occurring on pome fruit trees have been reported in South Africa. Certain species have been known to occur on apple, pear and Asian pear (*Pyrus pyrifolia* Nakai) worldwide. Diaporthe canker and Phomopsis canker caused by *Diaporthe tanakae* Kobayashi & Sakuma and *D. perniciosa* Em. Marchal.

respectively, are reported to be serious diseases of pear and apple wood in Japan, North America and Europe (Jones and Aldwinkle, 1990).

Uddin *et al.* (1998) found several different isolates of *Phomopsis* on peach, plum and Asian pear to be pathogenic on apple and European pear, suggesting that *Phomopsis* might become problematic in areas where pome and stone fruit are planted in close proximity. During a Japanese survey of *Phomopsis* species on fruit trees, all isolates taken from peach, Asian pear and apple were found to be pathogenic on twigs from those three hosts, while *D. tanakae* taken from European pear was found to be non-pathogenic on all hosts. The pathogenic isolates from apple were identified as *P. mali* Roberts, *P. oblonga* (Desmazieres) Höhnel and *D. perniciosa* (anamorph: *P. mali* Roberts) and all the isolates from Asian pear were identified as *P. fukushiii* Endo & Tanaka (Kanematsu *et al.*, 1999a).

In a recent review of the quarantine status of fungal pathogens on *Malus*, Carstens (2006) found no evidence of any known *Phomopsis* species in South Africa and only one *Diaporthe* species, *Diaporthe ambigua*. Smit *et al.* (1996) identified *Diaporthe ambigua* as the cause of a canker disease of apple, pear and plum rootstocks in South Africa. *D. ambigua* was found to cause longitudinally cracked, sunken lesions with perithecia developing on dead wood, killing nursery infected material within a short period by girdling the shoot. Mature rootstocks have been found to take longer to display symptoms associated with infection by *D. ambigua*. Smit *et al.* (1997) observed a large amount of vegetative compatibility groups occurring in *D. ambigua* from apples, pears and plums and tentatively concluded that the fungus might be indigenous to the Western Cape on account of this diversity.

Isolates of *D. ambigua* from South African fruit trees have been found to vary in terms of both virulence and morphology (Smit *et al.*, 1996) and Moleleki *et al.* (2002) used PCR-RFLP to delineate three species occurring on stone and pome fruit namely *D. ambigua*, *D. perjuncta* and an unknown *Phomopsis* species.

Due to the proliferation of species in the past, the taxonomy of the *Diaporthaceae* needs to be reviewed taking into account the latest research on molecular methods of identification since these methods should be helpful in clearing up confusion caused by the large degree of plasticity in terms of morphological characteristics between species of *Phomopsis.*

THE *DIATRYPACEAE* AS CAUSAL AGENTS OF DIE-BACK ON POME FRUIT TREES AND GRAPEVINE

The genus *Eutypa* (Ascomycota, Sordariomycetidae, Xylariales, *Diatrypaceae*) has been in existence since 1863 (Tulasne and Tulasne, 1863) and has become one of the major vascular diseases of grapevine worldwide (Carter *et al.*, 1983). The first reported incidence of pathogenicity of a *Eutypa* species was when *Eutypa armeniacae* Hansf. & Carter was linked to die-back on apricot (*Prunus armeniaca* L.) in Australia (Samuel, 1933). For a long time, *Eutypa armeniacae* was regarded as a pathogen specific to apricot (Carter, 1957), causing gummosis characterised by longitudinal cracks in the bark, brittle limbs and the occasional occurrence of a gum-like exudate (Samuel, 1933); however, it was soon isolated from a variety of cultivated and wild hosts internationally (Carter *et al.*, 1985).

Eutypa armeniacae was considered to be a pathogenic strain of *Eutypa lata* (Pers.) Tul. & C. Tul. (anamorph: *Libertella blepharis* A.L Smith) since distinguishing between the species morphologically was considered problematic (McKemy *et al.*, 1993). Glawe and Rogers (1982) found many similarities within the anamorphs in terms of conidial morphology, conidial ontogeny and proliferation and cultural characteristics and could only tentatively identify isolates to species level. During a much later study, DeScenzo *et al.* (1999) separated the two species using amplified fragment length polymorphism (AFLP) and sequence data of the internal transcribed spacer (ITS) region of the ribosomal DNA. Based on the existence of a genetically distinct cluster in both the AFLP and ITS data, DeScenzo *et al.* (1999) concluded that there was a difference between *E. lata* and *E. armeniacae* despite both species being able to infect various hosts. During an exhaustive reassessment of *E. lata, E. armeniacae* was conclusively confirmed as synonymous to *E. lata* by Rolshausen *et al.* (2006) following morphological and biochemical studies and phylogenetic analysis of the β-tubulin gene and the ITS region.

Eutypa lata was first reported as being associated with die-back and canker symptoms on *Malus domestica* Borkh. by Carter in 1960 and on *Pyrus communis* L. by Carter in 1982 (Carter, 1960; Carter, 1982). In 1981, Messner and Jähnl reported die-back associated with a *Libertella* sp. on the apple cultivar McIntosh causing severe losses in Austria. Glawe *et al.* reported *E. lata* from *M. domestica* in Washington State in the United States in 1983.

By 1985, the confirmed host range of *E. lata* extended to 80 plant species from 27 families, including *M. domestica*, *Pyrus communis*, several *Prunus* species and four *Vitis* species. Pathogenicity had been confirmed on 13 commercially cultivated crops (Carter *et*

al., 1983; Carter *et al*., 1985). Unfortunately there has been a dearth of published research on Eutypa die-back on rosaceous crops since the 1980's.

Several other members of the *Diatrypaceae* have been isolated from diseased grapevine. Trouillas and Gubler (2004) have identified *Eutypa leptoplaca* (Mont.) Rappaz as a distinct disease-causing species occurring on grapevine in California, previously thought to be *E. lata.* Two species of *Cryptovalsa* Ces. & De Not., namely *C. ampelina* (Nitschke) Fuckel and *C. protracta* (Pers.) De Not., have been associated with grapevine decline in the past, though only the species *C. ampelina* has been found in South Africa (Mostert *et al.,* 2004). During a molecular survey of symptomatic grapevines in South Africa, Safodien (2007) found *E. lata*, *E. leptoplaca*, *Eutypella vitis* (Schwein.:Fr.) Ellis and Everhart and *Cryptovalsa ampelina*, but no species of *Diatrype* and *Diatrypella* occurring in South African vineyards. Most recently, Trouillas *et al.* (2009) were able to identify 11 diatrypaceous species from symptomatic grapevine in California, including *Cryptosphaeria pullmanensis* Glawe, *Cryptovalsa ampelina*, *Diatrype oregonensis* (Wehm.) Rappaz, *D. stigma* (Hoffm.:Fr.), *D. whitmanensis* J.D Rogers and Glawe, an unidentified *Diatrype* species, *Diatrypella verrucaeformis* (Ehrh.:Fr.) Nitschke and four putative *Eutypella* species. Interestingly, the authors of the latter study suggest that the greater diversity in *Diatrypaceae* in California might be ascribed to the introduction of pathogenic species to grapevine from native trees such as the California bay laurel (*Umbellularia californica*).

Eutypa lata causes one of the most economically relevant grapevine trunk diseases with annual losses of up to $260 million having been ascribed to a combination of Bot canker and Eutypa die-back in California (Siebert, 2001). As such, the disease known as Eutypa die-back has been studied more thoroughly on grapevine than on any other host. Munkvold *et al.* (1994) found that yields in a susceptible vineyard will start decreasing from its twelfth year and estimated a yield loss of between 30.1% and 61.9% depending on disease severity.

Eutypa lata generally produces its ascospores in perithecial stromata on dead host wood and has been shown to occur widely in the 330 – 762 mm rainfall area of South Australia (Ramos, 1975a). It has been demonstrated that ascospore release starts between 5 to 10 minutes after wetting in the laboratory and 3 hours in the field after the start of a rainfall event and will continue until the stromata dries out (Carter, 1957; Pearson, 1980). Ascospore release occurs throughout the year but the highest rate will coincide with rainy periods in winter and spring (Pearson, 1980). Upon release, windborne ascospores may travel as far as 50 kilometres to germinate in wounds on the surface of susceptible hosts (Moller and Carter, 1965; Ramos *et al.,* 1975b). Ascospores may stay viable for several weeks after release

(Carter, 1957) and Trese *et al.* (1980) showed that ascospores will germinate even during periods of alternating temperatures and that a period of temperatures well below freezing (-20°C) will only delay germination until temperatures rise above 0°C. A study by Ju *et al.* (1991) revealed very low rates of conidial germination and it was concluded that the conidial state may only play a small role in infection over very small distances or in dry conditions where the sexual state can not occur due to a lack of moisture.

After germination, *Eutypa lata* slowly colonises woody host-tissue, producing characteristic internal necroses eventually resulting in the girdling and death of affected parts after which stromata forms on dead tissue (Carter, 1957). Symptoms of infection of trees and vines include external cankers and sectorial internal necrosis developing over many years. Pruning wounds are considered to be the primary infection site and the fungus infects and colonises the xylem tissue of the vascular system, followed by the cambium and bark, resulting in cankers forming externally (English and Davis, 1965). Cankers from which *Libertella* was isolated have been found to increase in size during summer months while remaining static during colder months on the apple cultivar McIntosh (Messner and Jähnl, 1981), presumably due to decreased growth of mycelium within the vascular system during colder temperatures.

Foliar symptoms occur on vines and consist of stunted shoots with shortened internodes and dwarfed, cup-shaped leaves (Goussard, 2005) and are associated with the production of the toxin eutypine produced by *E. lata* in the plant (Tey-Rulh *et al.*, 1991). Eutypine has been found to cause ultrastructural changes in grapevine leaves, brought about by cytoplasm lysis followed by chloroplast swelling (Deswarte *et al.*, 1994). Foliar symptoms do not always occur and occurrence may vary between seasons, though symptom expression will be similar in the same geographic region, which suggests the involvement of climate in symptom expression. Sosnowski *et al.* (2007) made several observations regarding this phenomenon over several seasons. It was found that disease incidence in terms of visible symptoms decreased during periods of high temperature, high available moisture and very low available moisture. Possible reasons were given for each scenario. In the case of high temperatures, it may be that vines grow more vigorously during this time which may decrease the ability of toxins to reach foliage or result in a decrease of toxin concentration. It may also simply be that the ability of *E. lata* to produce toxins is reduced under higher temperatures. During periods with high available moisture there might be an actual dilution of toxins within the vascular system during improved transport of water to foliage. Conversely, during periods with very little available water, there is very little water transport to foliage to

conserve moisture. Water stress on the fungus may also reduce its ability to produce toxins (Sosnowski *et al.*, 2007).

More work is needed to elucidate the relationship between the *Diatrypaceae* and its hosts.

CONCLUSION

There is a clear indication in the literature that certain trunk disease causing organisms such as the *Botryosphaeriaceae*, various *Phomopsis* species and *E. lata* have the potential to infect vines, apple trees and pear trees. The host range of other trunk pathogens common to grapevine in South Africa, such as the various *Phaeoacremonium* species and *P. chlamydospora* linked with esca and Petri disease, remains unknown. No reports of *Phaeomoniella* occurring on pome fruit have been found. With the exception of a single instance where *Phaeoacremonium angustius* and *Phaeoacremonium mortoniae* have been reported from *Malus* in California, the organism has been unknown on pome fruit trees (Rooney-Latham *et al.*, 2006). A recent study undertaken by Damm *et al.* (2008a) in South Africa revealed several *Phaeoacremonium* species occurring on *Prunus* species, an indication that the organism may be present in woody agricultural crops other than vines. Moreover, Damm and co-workers identified several other fungal species in various genera, including *Aplosporella, Lasiodiplodia, Paraconiothyrium, Jattaea and Calosphaeria* from wood decay symptoms on *Prunus*, indicating the species diversity and potential complexity of trunk disease aetiology in these hosts (Damm *et al.* 2007a, 2007b, 2008b, 2008c). The possibility of these fungi occurring on pome fruit, and its role in this host in South Africa has yet to be explored.

The ever-increasing practice of planting grapevine in close proximity to commercial pome fruit orchards bears inherent risks with regards to the free and unrestrained availability of trunk disease inoculum. The possible presence of fungal inoculum in existing orchards should have an effect on cultural practices and disease control measures taken in young vineyards. It is important to note that vines may also pose a risk to the pome fruit industry and care should be taken in providing adequate protection to orchards in close proximity to vineyards. To this end, this study aims to elucidate the incidence and aetiology of die-back causing fungi in pome fruit orchards in the Western Cape.

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2. FUNGI ASSOCIATED WITH DIE-BACK SYMPTOMS OF APPLE AND PEAR TREES WITH A SPECIAL REFERENCE TO GRAPEVINE TRUNK DISEASE PATHOGENS

ABSTRACT

A survey was undertaken on apple and pear trees in the main pome fruit growing areas of the Western Cape to determine the aetiology of trunk diseases with specific reference to pathogens occurring on grapevine, which are cultivated in close proximity of these orchards in many cases. Several genera containing known trunk disease pathogens were found, including *Diplodia*, *Neofusicoccum*, *Eutypa*, *Phaeoacremonium* and *Phomopsis*. Two *Diplodia* species, *D. seriata* and *Diplodia* sp., were isolated along with *Neofusicoccum australe* and *N. vitifusiforme*. Four *Phaeoacremonium* species, *Phaeoacremonium aleophilum*, *P. iranianum*, *P. mortoniae* and *P. viticola*, two *Phomopsis* species linked to clades identified in former studies as *Phomopsis* sp. 1 and *Phomopsis* sp. 7, and *Eutypa lata* were found. In addition, *Paraconiothyrium brasiliense* and *Pa. variabile* and an unidentified *Pyrenochaeta*-like species were found. *D. seriata* (56% of total isolates) and *P. aleophilum* (22%) were isolated most frequently. Of these, the *Phaeoacremonium* species have not been found on pear wood and it is a first report of *P. aleophilum* occurring on apple. This is also a first report of these *Phomopsis* species on pome trees. *Paraconothyrium brasiliense* has not previously been found on pear and *Pa. variabile* not on apple. *Eutypa lata* is also reported here for the first time on pome trees in South Africa. 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 the *Pyrenochaeta*-like sp., *Pa. variabile*, *D. seriata* and *P. mortoniae* caused lesions that were significantly longer than the control inoculations. On pears, *Diplodia* sp.and *N. australe* caused the longest lesions, followed by *D. seriata* and *E. lata*. On apples, the longest lesions were caused by *N. australe* and *P. iranianum*. *D. seriata*, *Diplodia* sp., *E. lata*, *N. vitifusiforme*, *Pa. brasiliense*, *P. aleophilum* and *P. mortoniae* also caused lesions on apple that were significantly longer than the control. The results of this study demonstrated that apple and pear orchards in the Western Cape are host to many known grapevine trunk pathogens along with possible new trunk disease causing fungi.

INTRODUCTION

Trunk disease is a broad term used when describing various abnormalities of the woody parts of perennials such as vines, plantation trees and fruit trees. Its various external and internal manifestations are mainly caused through invasion by various fungal organisms, directly and indirectly causing damage and blockage to the vascular system. Many of these organisms invade the plant through wounds such as pruning wounds, scars, and stomata. This gives rise to various symptoms such as cankers, twig blights and wood rot, 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 an entire plant (Mugnai *et al.*, 1999; Brown-Ritlewski and McManus, 2000; Lalancette and Robinson, 2001; Slippers *et al.*, 2007; Van Niekerk *et al.*, 2010).

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 al.*, 1983; Jones, 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*., 2006; 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 summary of apple and pear diseases compiled by Crous *et al*. (2000) relies mostly on records that date back to 1950 and earlier. The extent and cause of trunk diseases in the pome fruit growing regions of the Western Cape is unknown, though symptoms may commonly be observed in orchards.

Grapevine trunk diseases cause the gradual decline and die-back of vines resulting in a decrease in the vine's capability to carry and ripen fruit. The organisms involved in the different manifestations vary, as do the symptoms themselves. Van Niekerk *et al.* (2010) identified six different symptom types 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 disease (Pine, 1959; Galet, 1995; Crous *et al*. 1996; Mugnai *et al*., 1996;

Larignon and Dubos, 1997; Phillips, 2002; van Niekerk *et al.*, 2004; van Niekerk *et al*., 2005; Mostert *et al.*, 2006).

The exact extent of losses caused by trunk diseases is unknown, but a study examining quantifiable losses caused by Eutypa die-back and Botryosphaeria canker in California on grapevines 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 infection in susceptible vineyards. Apart from the inevitable yield loss, 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 of the well established pome fruit growing areas especially in the Elgin, Grabouw and Villiersdorp regions of the Western Cape province of South Africa. In these areas, unprofitable orchards are often replaced with vineyards. Several trunk pathogens such as *Eutypa lata*, *Phomopsis* species and the *Botryopshaeriaceae* are known to be cosmopolitan (Carter *et al.*, 1983; Murali *et al.*, 2006; Slippers *et al.*, 2007), while the exact host range of certain of the involved organisms such as *Phaeomoniella chlamydospora* and *Phaeoacremonium* species is unknown. During an investigation by Damm *et al.* (2008), 14 different species of *Phaeoacremonium* were found to be present in stone fruit trees, which suggest that *Phaeoacremonium* spp. may be present on other fruit trees. The presence of trunk pathogen populations in old pome fruit orchards may cause a long term threat to young vineyards planted in close proximity to these potential sources of viable inoculum.

The purpose of the present study is to investigate the nature of trunk diseases in aging pome fruit orchards in the areas with the largest 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 in South Africa.

MATERIALS AND METHODS

Sampling

Symptomatic wood from trees showing die-back symptoms was collected in September and October over two 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. The cultivars selected for sampling were the green apple cultivar Granny Smith and the green pear cultivar Packham's Triumph. Samples of living symptomatic wood were taken from trees in orchards older than 15 yrs and stored at 4°C for up to 2 weeks until dissection.

Isolations

Samples were taken from storage and 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 seconds, in a 1% NaOCl solution for 1 minute and in 70% ethanol for a further 30 seconds. Following sterilisation, wood pieces were air-dried in the laminar flow cabinet 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) amended with streptomycin sulphate (40 mg/L, Calbiochem, Merck). 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 similar conditions.

In cases where sporulation had not taken place, isolates were placed on divided plates containing unamended PDA and water agar (WA, Biolab, Midrand), with a portion of carnation leaf placed on the WA to enhance sporulation. Following the latter method, most isolates were 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 refrained from sporulating 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 Leica WILD microscope and slides were made by mounting fungal material in lactic acid. Slides were examined under a Zeiss MC80 microscope and identified based on structures formed. 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 Lee and Taylor (1990) with chloroform:isoamylalcohol instead of chloroform:phenol and using sterile water as a suspension medium for the DNA. Products were visualised using electrophoresis. Primers for amplification were selected according to genus. For the *Botryosphaeriaceae, Phomopsis, Eutypa* and unidentified genera, the internal transcribed spacers (ITS1 and ITS2) and the 5.8S ribosomal gene was 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) with an increase in the $MgCl_2$ concentration to 2 – 3 mM depending on the difficulty of amplification. There has been some doubt regarding the robustness of the ITS gene region with regards to the identification of *Phaeoacremonium* (Groenewald *et al.*, 2001; Mostert *et al.*, 2005), and accordingly the β-tubulin gene was amplified in these isolates 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.* (2005, 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). 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.) 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* (Essakhi *et al*., 2008; Mostert *et al*., 2006), *Phomopsis* (Mostert *et al*., 2000; 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 was 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 or not using PAUP's bootstrap search option set to 1000 bootstrap replications. The measures 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.

Pathogenicity trial

A pathogenicity trial was conducted on detached woody shoots of grapevine (cv. Sauvignon blanc), pear (cv. Packham's Triumph) and apple (cv. Granny Smith) and was based on the protocol described in Van Niekerk *et al.* (2004) and Damm *et al.* (2007; 2008a). For each host, the trial layout was a randomised block design consisting of three blocks, or incubation chambers. The treatments (listed in Table 2) included 32 fungal isolates and two negative controls, *Acremonium strictum* and an uncolonised agar plug. Agar plugs measuring 4 mm in diameter were made 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 by soaking in a 70% ethanol solution for 30 seconds, in a 1% NaOCL solution for 1 minute and in 70% ethanol for a further 30 seconds. 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 conditions in moisture chambers (RH >93%). After 14 days, shoots were removed from moisture chambers. The bark surrounding each wound site was stripped off and lesions were measured using digital callipers. Isolations were made from the margin 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 measurements were normalised by the removal of outliers and Student's t-test for least significant difference was calculated to compare the differences between taxa on the three hosts. A correlation between lesion length and re-isolation frequency was determined by calculating the correlation coefficient for every host to determine whether there was an interaction between the lesion lengths obtained and their establishment within the host.

RESULTS

Isolations

Following the work of Van Niekerk *et al.* (2010), six different internal symptom types similar to those occurring in grapevine were identified in pear and apple and are depicted in Figure 1. These were brown vascular streaking, black vascular streaking, wedge-shaped necrosis, watery necrosis, brown internal necrosis and soft rot. Isolations could be traced back to the specific sample and symptom type they were made from.

Morphological identification

Fungi were identified on cultural and morphological characters as *Botryosphaeriaceae* (van Niekerk *et al*., 2004), *Phomopsis* species (van Niekerk *et al*., 2005), *Phaeoacremonium* species (Mostert *et al*., 2006; Damm *et al*. 2008a), *Eutypa lata* (Rumbos, 1988) and *Paraconiothyrium* (Damm *et al*. 2008b). Aside from suspected genera of plant pathogens, a large variety of other taxa such as *Alternaria*, *Penicillium*, *Ulocladium*, *Epicoccum*, *Fusarium*, *Cladosporium*, *Aureobasidium* and *Trichoderma* were also identified, but were considered to be saprophytic and therefore not included in further analyses.

Molecular characterisation and phylogeny

Isolates identified as species of the *Botryosphaeriaceae*, *Phaeoacremonium*, *Phomopsis*, *Eutypa* as well as several recurring unidentified isolates were sequenced. Phylogenetic inference of the ITS were done for the *Botryosphaeriaceae* (Fig. 2) and *Phomopsis* (Fig. 3).

A β-tubulin phylogeny for the *Phaeoacremonium* species is shown in Fig. 4. Table 1 lists the different isolates, their origin and identity.

Four species of *Botryosphaeriaceae* were identified based on their ITS phylogeny. The greatest majority of isolates belonged to *D. seriata* (77% bootstrap support). Three isolates grouped with *N. australe* (bootstrap support of 97%) and two isolates with *N. vitifusiforme* (bootstrap support of 79%). Six isolates formed a strongly supported monophyletic clade (99% bootstrap support) that was identified as a seperate *Diplodia* species closely related to *D. mutila* and *D. africana*. These isolates were described as a new species of *Diplodia* in Chapter 3.

Three isolates were identified as *Phomopsis* species. *Phomopsis* is a problematic genus since the precise identities of many species are uncertain (Rehner and Uecker, 1994). This is due to a large amount of morphological plasticity between species resulting in a proliferation of species which still has to be resolved (Rehner and Uecker, 1994; Rossman *et al.*, 2007). Of the three isolates found during this study, two were identical and grouped with *Phomopsis* species 7, while the third isolate grouped with *Phomopsis* species 1, identified by Van Niekerk *et al.* (2004) from grapevine. The bootstrap value for the latter 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 *P. aleophilum.* A single isolate was identified as *P. iranianum*, two isolates as *P. viticola* and two isolates as *P. mortoniae*. 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 similarity of 99.6% between the sequences.

Sequences identified as *Pa. brasiliense* were aligned to five reference sequences (AY642531, EU295638, EU295635, EU295637, EU295636) with a percentage similarity of 99.3% between the sequences. Sequences identified as *Pa. variabile* were aligned to four reference sequences (EU295649, EU295646, EU295647, EU295648) with a 94.8% similarity between sequences.

Several isolates were identified as a *Pyrenochaeta*-like species and were described in Chapter 3.

Symptomatology

Wood pieces taken from orchards generally had one symptom type present and no instances of more than one symptom type occurring on a single sample were observed. The isolation of more than one fungal taxon from a single symptomatic sample occurred only once, where *Phomopsis* sp. 7 and *P. aleophilum* was isolated from the same sample.

The occurrence of various species in association with the six symptom types described in Van Niekerk *et al.* (2010) is given in Fig. 5. Most species were isolated too infrequently to be conclusively linked to a certain symptom, but it is interesting to note that the wedge-shaped necrotic symptom normally associated with *Eutypa* and the *Botryosphaeriaceae* was the dominant symptom associated with the frequently isolated *D. seriata* and *P. aleophilum*; both these species displayed a wide symptom profile. Other species in *Phaeoacremonium* and *Botryosphaeriaceae* were also mostly isolated from wedge-shaped and brown internal necrotic lesions. These symptom types also yielded the *Phomopsis*, *Pyrenochaeta*-like and *Paraconiothyrium* species, as well as *E. lata*. *E. lata* was also isolated from watery necrosis and brown streak on single occasions.

Pathogenicity trial

The results (Table 2) showed a large variation in length of lesions formed and re-isolation frequencies between hosts. These factors were generally poorly correlated. On grapevine, the correlation between lesion length and re-isolation was 9.8%, while on pear and apple it was 18 and 23% respectively.

On grapevine, *N. australe* was the most virulent species with a mean lesion length of 19.99 mm. The *Pyrenochaeta*-like sp. (8.57 mm), *Pa. variabile* (8.16 mm), *D. seriata* (6.55 mm) and *P. mortoniae* (6.23 mm), could also be considered pathogenic, since their lesion lengths were significantly longer than the negative controls. There was no significant difference between the remaining species and the negative controls, *Acremonium strictum* (2.47 mm) and a non-colonised PDA plug (1.84 mm). Re-isolation percentages were relatively high (29.1% to 77.1%), except for *Pa. brasiliense* (16.6%), which did not cause lesions significantly different from *A. strictum*.

On pear, the most virulent species were the *Diplodia* sp. (55.03 mm) and *N. australe* (52.28 mm). The lesion lengths of *D. seriata* (43.04 mm) and *E. lata* (43.71 mm) were also significantly different from the negative controls (17.69 mm and 4.36 mm for *A. strictum* and

the PDA plug, respectively), indicative of their pathogenic nature. *A. strictum* had a re-isolation rate of 100%, while most species were re-isolated at a frequency of between 25% and 65% on pear. *Phomopsis* sp. 7 (10.4%) and *E. lata* (4.2%) had low re-isolation frequencies and *Phomopsis* sp. 1 were not successfully re-isolated.

On apple, *N. australe* (40.19 mm) and *P. iranianum* (41.21 mm) gave the longest lesions and could be considered the most virulent species on this host. The *Diplodia* sp. (27.34 mm), *P. aleophilum* (25.21 mm), *N. vitifusiforme* (23.76 mm), *D. seriata* (20.14 mm), *P. mortoniae* (19.73 mm), *E. lata* (19.19 mm) and *Pa. brasiliense* (18.14 mm) could be considered pathogenic with lesion lengths significantly longer than the negative controls (10.08 mm and 5.64 mm for *A. strictum* and the PDA plug, respectively). On apple, all of the *Botryosphaeriaceae*, all of the *Phaeoacremonium* species except for *P. viticola*, *E. lata* and *Pa. brasiliense* caused statistically significant lesions. This was a wider array of pathogenicity than on the other two hosts, and might suggest that apple is more sensitive to invasion than pear or grapevine. All isolates were frequently re-isolated (37.5% to 87.5%).

DISCUSSION

The results of the isolations made during this study confirmed that apple and pear orchards in the Western Cape are host to many known grapevine trunk pathogens along with various other fungal species that might be considered pathogenic to apple and pear trees as well as grapevines.

The symptom types found to occur in symptomatic apple and pear wood were similar to the symptomatology described by Van Niekerk *et al*. (2010) for grapevine trunk diseases. The species associated with one symptom type only, namely *P. iranianum*, *Phomopsis* sp.1, *Phomopsis* sp.7, *Pa. brasiliense* and *Pa. variabile* did not occur frequently enough to conclusively associate these species solely with these specific symptom types.

The overwhelming presence of the wedge-shaped necrotic symptom suggested the presence of a large number of either *Botryosphaeriaceae* or *E. lata*, since this symptom type has commonly been associated with these pathogens in the past (Moller and Kasimatis, 1978; Van Niekerk *et al.*, 2004, 2010). A large number of *Botryosphaeriaceae* were indeed isolated from this symptom type. The majority (84%) of the *Botryosphaeriaceae* associated with symptomatic wood were *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). This was not without precedent as its prevalence corresponds to the findings of

Slippers *et al.* (2007), who found *D. seriata* to make up 90% of *Botryosphaeriaceae* found on pome fruit in their study. *Diplodia seriata* has also been found to be the dominant species on stone fruit (Damm *et al.*, 2007) and grapevine (van Niekerk *et al*., 2004, 2010) 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 lesions showing various symptoms, 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); however, in a study conducted by Van Niekerk et al. (2010), *E. lata* was isolated from esca-like soft brown wood rot symptoms in winter-rainfall areas.

Four *Phaeoacremonium* species were found during the study, namely *P. aleophilum*, *P. iranianum*, *P. mortoniae* and *P. viticola*. *P. aleophilum* comprised 85% of *Phaeoacremonium* isolates, while the other three species had a very limited occurrence. This is a first report of any *Phaeoacremonium* species occurring on pear wood and a first report of *P. aleophilum* occurring on apple. Of the different *Phaeoacremonium* species, only *P. angustius* and *P. mortoniae* have been reported from *Malus* in California (Rooney-Latham *et al.*, 2006). Interestingly, *P. aleophilum* is also the most common species of *Phaeoacremonium* found associated with Petri disease in grapevines (Mostert *et al*., 2005).

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 may indicate a difference in a certain host characteristics, which make certain species more suited to the conditions on certain hosts. However, the re-isolation frequencies indicate that all species had become established within host tissue to some extent, with the exception of *Phomopsis* sp.1 on pear. This may suggest that these particular *Phomopsis* isolates were unable to establish growth within the host, *Pyrus*, for some reason. Higher rates of re-isolation were not associated with an increase in virulence, measured by the lesion lengths. What the re-isolation may suggest instead is a preliminary indication of how easily various species can occur endophytically within the host. The variation in pathogenicity results 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, Urbez-Torres *et al.*, 2008), these results should be considered as indicative of potential pathogenicity only.

On grapevine shoots the lesions lengths were generally shorter than on pear and apple shoots, indicating that the grapevine shoots would need a longer time to obtain more conclusive results. *E. 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. *E. lata* did, however, cause a pathogenic reaction on apple and pear.

The *Phomopsis* species could not be considered pathogenic on any hosts. In a similar pathogenicity test, Van Niekerk (2005) also found no significant different lesions of *Phomopsis* sp.1 and 7 in comparison with the negative control when tested on the grapevine cultivars Pinotage and Chenin Blanc.

The lesions caused by *N. australe* on all hosts were significantly longer than those caused by other species, a finding that is in accordance with that of Van Niekerk *et al.* (2004), who found the same species to cause severe lesions on grapevine. *N. vitifusiforme* only caused lesions statistically different from the negative controls on apple wood. *D. seriata* caused lesions statistically different from the negative controls on all hosts, and considering the profusion with which it is found in Western Cape orchards, this warrants further investigation. Damm *et al.* (2007) found similar results when testing the pathogenicity of *D. seriata* on *Prunus* shoots. Although Van Niekerk *et al.* (2004) found *D. seriata* to be weakly pathogenic on mature grapevine canes and non-pathogenic on green shoots, Urbez-Torres *et al.* (2008) found *D. seriata* pathogenic on rooted cuttings and green shoots. The results found in this study suggest that *D. seriata* can be considered pathogenic on pome fruit. 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.*, 2008), kiwifruit vines (Di Marco *et al.*, 2004) and olive trees (Hawksworth *et al.*, 1976). This study is the first to report on the pathological relevance of *Phaeoacremonium* species on apples and pear trees. Of the four *Phaeoacremonium* species found during the study, *P. iranianum* caused statistically significant lesions on apple, while *P. aleophilum* and *P. mortoniae* could be considered pathogenic on the same host. None of the species formed significant lesions on either of the other hosts. This is particularly surprising as *P. 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 be recorded on this host.

P. aleophilum also failed to produce significantly longer lesions than the negative controls in a similar detached shoot assay (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 species 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 esca-like wood symptoms in grapevine, but Van Niekerk *et al.* (2010) found *Phaeoacremonium* species commonly associated with wedge-shaped necrotic symptoms 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 symptom. It is interesting to note that *Phaeoacremonium* species were commonly associated with 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 that the latter may naturally occur after the former in a progressive cycle of symptom development (Van Niekerk *et al.*, 2010), a suggestion that certainly deserves 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 *Pa. brasiliense* on pear and *Pa. variabile* on apple. The genus *Paraconiothyrium* is not known to be pathogenic on the hosts tested. The species *Pa. brasiliense* proved to be potentially pathogenic on apple, while *Pa. variabile* caused statistically significant lesions on grapevine. Damm *et al.* (2008b) recently reported *Pa. brasiliense* occurring on necrotic wood of *Prunus* spp. and described *Pa. variabile* from the same hosts in South Africa.

The unidentified *Pyrenochaeta*-like species isolated from *Malus* caused statistically significant lesions on grapevine, which might be an indication that this organism could be a possible future grapevine trunk pathogen.

The results of the isolations have proved the presence of several major grapevine trunk pathogens on pome fruit in the Western Cape 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 all hosts involved.

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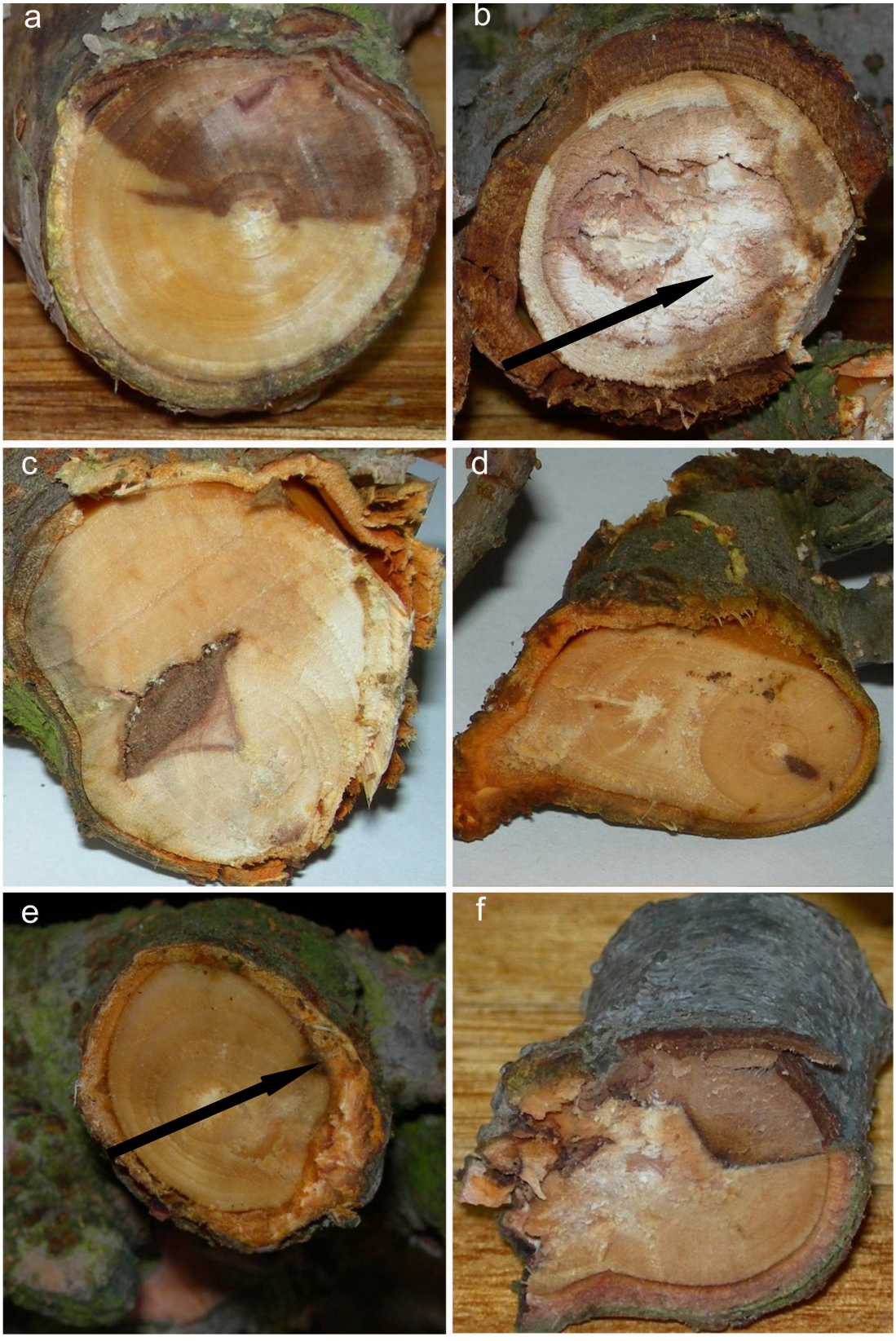

Fig. 1. Symptom types associated with trunk disease on pome fruit trees, a) watery necrosis, b) soft rot indicated by arrow, c) brown internal necrosis, d) black/brown streaking, e) brown streaking, f) wedge-shaped necrosis.

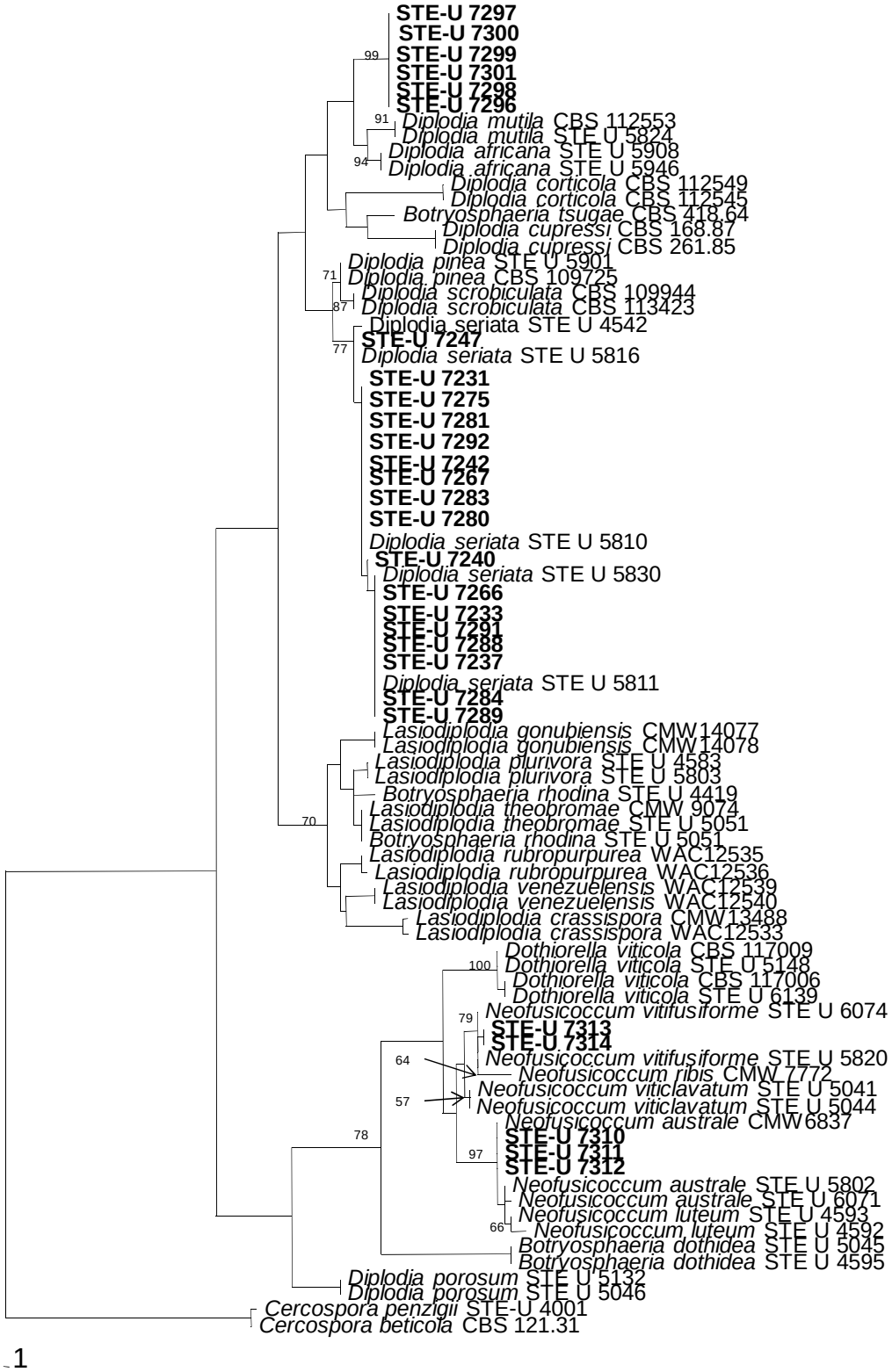

Fig. 2. One of 840 most parsimonious trees obtained from ITS sequences of the *Botryosphaeriaceae*. Bootstrap support values above 50% from 1000 replicates are shown at the nodes. Tree scores include Length = 294, CI = 0.707, RI = 0.931, RC = 0.658, HI = 0.293. Isolates from the present study in bold.

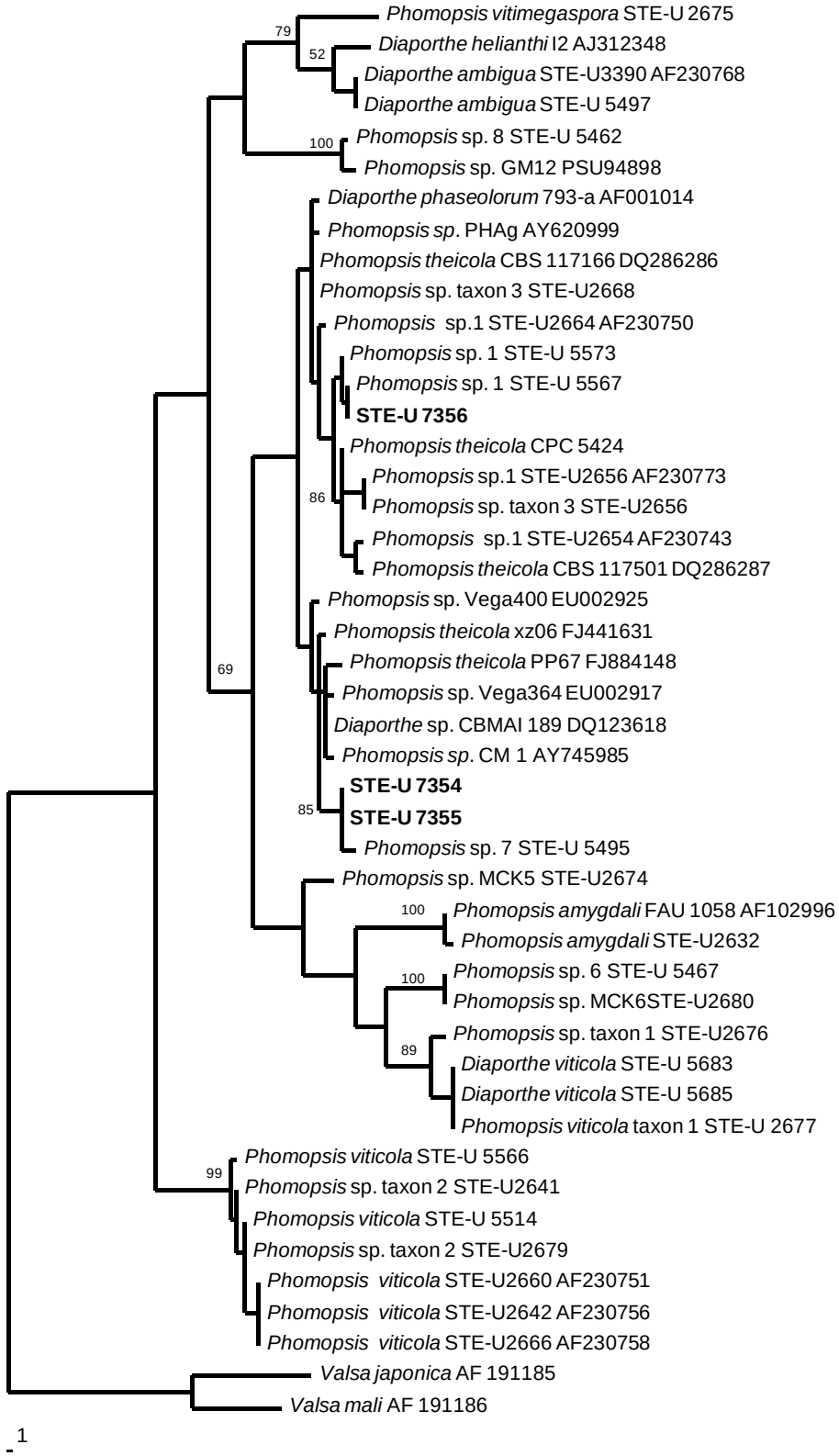

Fig. 3. One of 150 most parsimonious trees obtained from ITS sequences of the *Phomopsis* isolates. Bootstrap support values above 50% from 1000 replicates are shown at the nodes. Tree scores include TL = 246, CI = 0.675, RI = 0.841, RC = 0.568, HI = 0.325. Isolates from the present study in bold.

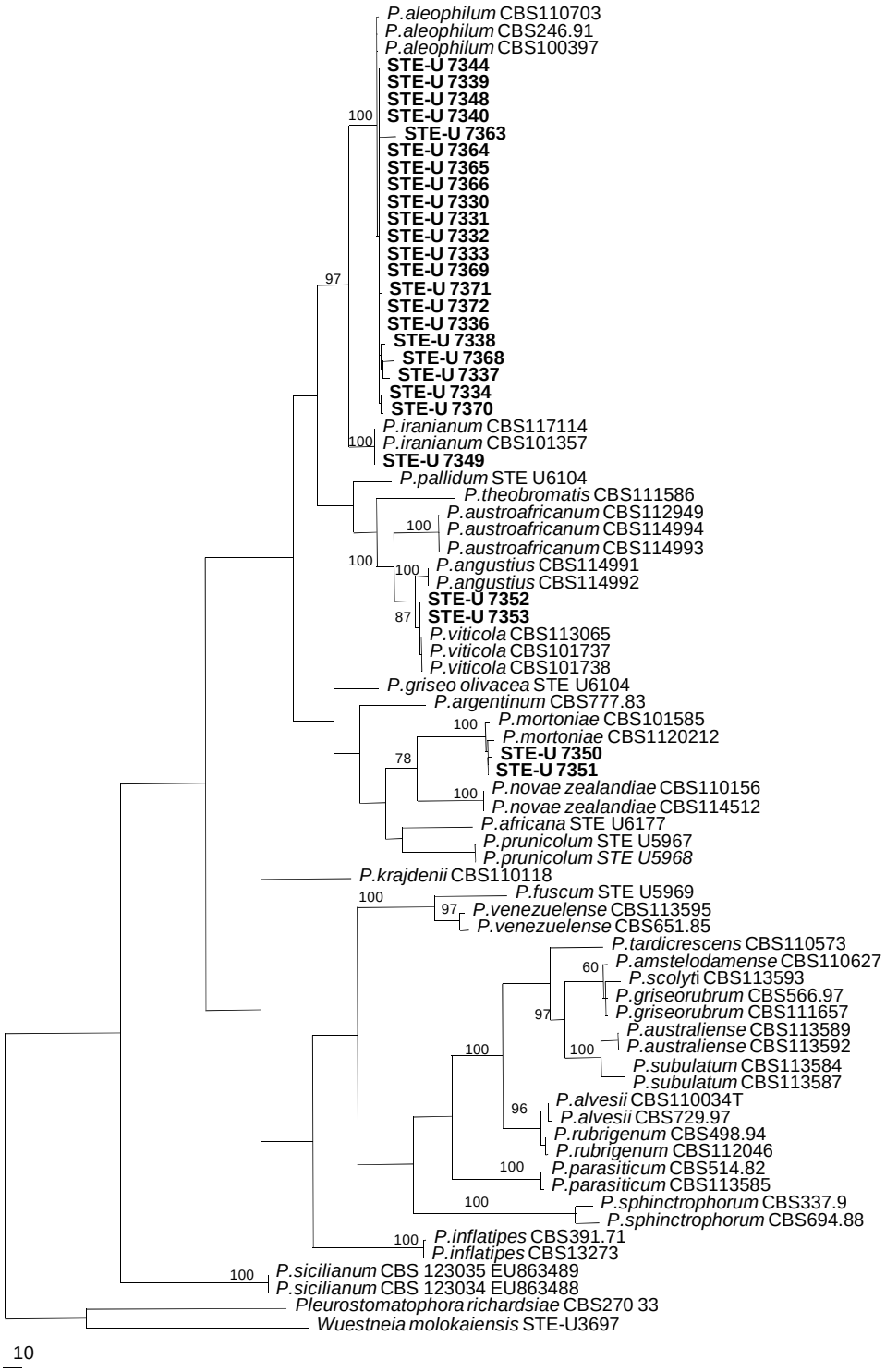

Fig. 4. One of 190 most parsimonious trees obtained from β-tubulin region sequences of the *Phaeoacremonium* isolates. Bootstrap support values above 50% from 1000 replicates are shown at the nodes. Tree scores include TL = 1512, CI = 0.506, RI = 0.823, RC = 0.416, HI = 0.494. Isolates from the present study in bold.

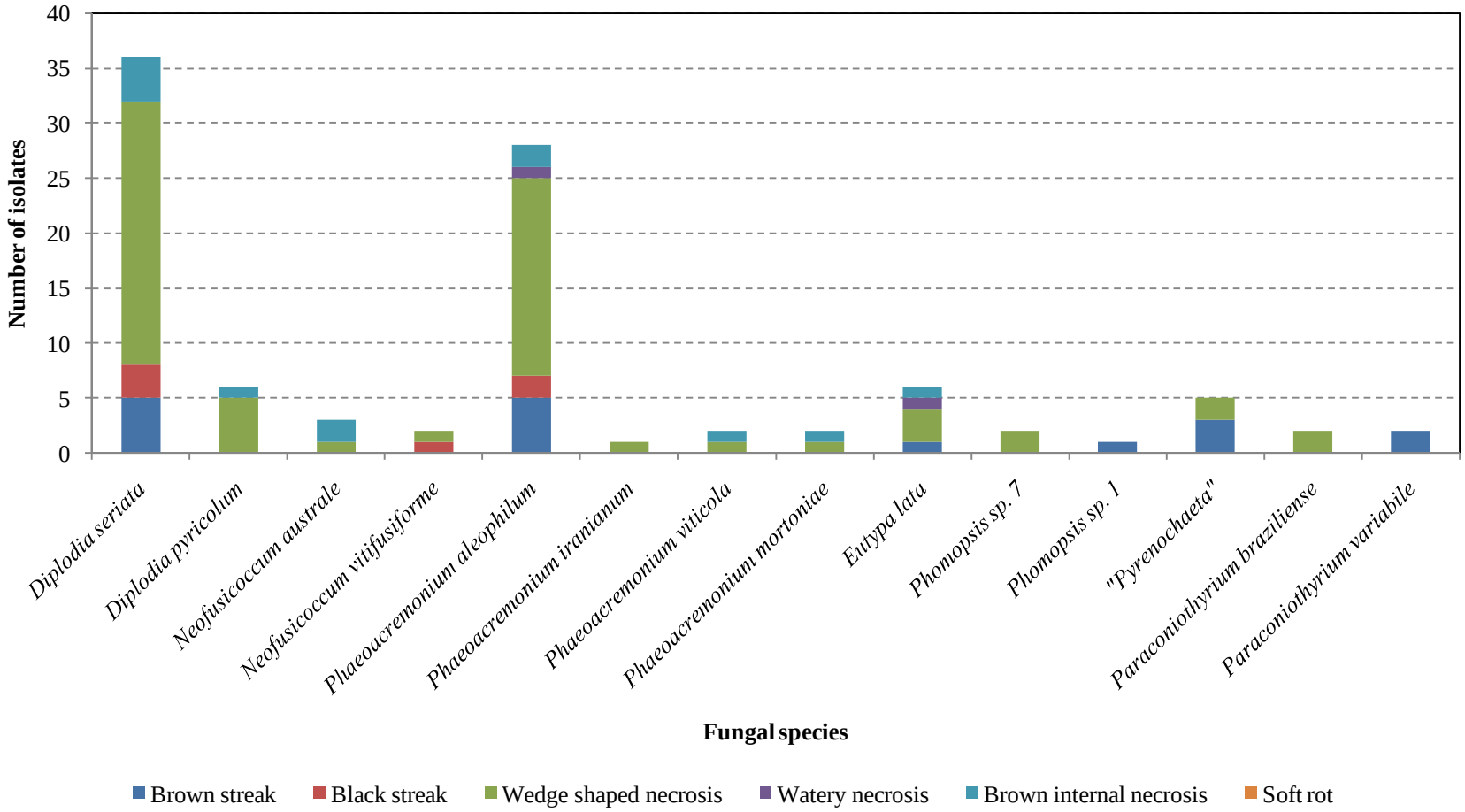

Fig. 5. Frequency of isolation of fungal species from the six symptom types found on apple and pear trees with die-back symptoms.

Table 1. Isolates obtained from internal wood necrosis symptoms of pear and apple trees in the Western Cape of South Africa.

Fungal species	STE-U number	Isolation number	Host	Location	Orchard age
Diplodia seriata	STE-U 7229	CPA 9-1	*Pyrus*	Ceres	20
	STE-U 7230	CPA 14-1	*Pyrus*	Ceres	20
	STE-U 7231	CPA 14-2	*Pyrus*	Ceres	20
	STE-U 7232	CPA 13-1	*Pyrus*	Ceres	20
	STE-U 7233	CPA 15-1	*Pyrus*	Ceres	20
	STE-U 7234	CPA 1-1	*Pyrus*	Ceres	20
	STE-U 7235	CPA 4-2	*Pyrus*	Ceres	20
	STE-U 7236	CPA 4-1	*Pyrus*	Ceres	20
	STE-U 7237	CPB 10-1	*Pyrus*	Ceres	20
	STE-U 7238	CPB 3-1	*Pyrus*	Ceres	20
	STE-U 7239	CPB 3-2	*Pyrus*	Ceres	20
	STE-U 7240	CPB 12-1	*Pyrus*	Ceres	20
	STE-U 7241	CPB 14-2	*Pyrus*	Ceres	20
	STE-U 7242	CPB 2-2	*Pyrus*	Ceres	20
	STE-U 7243	CPB 2-7	*Pyrus*	Ceres	20
	STE-U 7244	CPB 8-1	*Pyrus*	Ceres	20
	STE-U 7245	CPB 4-1	*Pyrus*	Ceres	20
	STE-U 7246	PV 9-1	*Pyrus*	Wolseley	30
	STE-U 7247	PV 16-2	*Pyrus*	Wolseley	30
	STE-U 7248	PV 14-1	*Pyrus*	Wolseley	30
	STE-U 7249	PV 15-1	*Pyrus*	Wolseley	30
	STE-U 7250	PV 16-3	*Pyrus*	Wolseley	30
	STE-U 7251	PV 9-2	*Pyrus*	Wolseley	30
	STE-U 7252	PV 15-3	*Pyrus*	Wolseley	30
	STE-U 7253	PV 8-2	*Pyrus*	Wolseley	30
	STE-U 7254	PV 8-1	*Pyrus*	Wolseley	30
	STE-U 7255	PV 1-3	*Pyrus*	Wolseley	30
	STE-U 7256	PV 2-4-3	*Pyrus*	Wolseley	30
	STE-U 7257	PV 2-2-1	*Pyrus*	Wolseley	30
	STE-U 7258	PV 2-14-1	*Pyrus*	Wolseley	30

STE-U 7259	PV 2-7-1	*Pyrus*	Wolseley	30
STE-U 7260	PV 2-11-2	*Pyrus*	Wolseley	30
STE-U 7261	PV 2-11-1	*Pyrus*	Wolseley	30
STE-U 7262	W 5-1	*Pyrus*	Wolseley	25
STE-U 7263	W 3-1	*Pyrus*	Wolseley	25
STE-U 7264	W 3-2	*Pyrus*	Wolseley	25
STE-U 7265	CB 9-1	*Malus*	Ceres	20
STE-U 7266	CB 9-2	*Malus*	Ceres	20
STE-U 7267	CB 2-2	*Malus*	Ceres	20
STE-U 7268	GP 3-1	*Pyrus*	Grabouw	52
STE-U 7269	GP 8-1	*Pyrus*	Grabouw	52
STE-U 7270	GP 10-1	*Pyrus*	Grabouw	52
STE-U 7271	GP 10-2	*Pyrus*	Grabouw	52
STE-U 7272	VyG 1-8-1	*Malus*	Vyeboom	22
STE-U 7273	VyG 1-8-2	*Malus*	Vyeboom	22
STE-U 7274	VyG 1-8-3	*Malus*	Vyeboom	22
STE-U 7275	VyG 1-8-4	*Malus*	Vyeboom	22
STE-U 7276	VG 1-5-1	*Malus*	Villiersdorp	25
STE-U 7277	VG 2-6-2	*Malus*	Villiersdorp	25
STE-U 7278	VG 2-8-1	*Malus*	Villiersdorp	25
STE-U 7279	VG 2-8-2	*Malus*	Villiersdorp	25
STE-U 7280	VG 2-14-1	*Malus*	Villiersdorp	25
STE-U 7281	VP 14-1-1	*Pyrus*	Villiersdorp	22
STE-U 7282	VP 14-2-1	*Pyrus*	Villiersdorp	22
STE-U 7283	VP 14-8-1	*Pyrus*	Villiersdorp	22
STE-U 7284	VP 14-9-1	*Pyrus*	Villiersdorp	22
STE-U 7285	VP 14-11-1	*Pyrus*	Villiersdorp	22
STE-U 7286	VP 15-6-1	*Pyrus*	Villiersdorp	24
STE-U 7287	VP 15-9-1	*Pyrus*	Villiersdorp	24
STE-U 7288	VP 15-10-1	*Pyrus*	Villiersdorp	24
STE-U 7289	VP 15-12-1	*Pyrus*	Villiersdorp	24
STE-U 7290	VP 15-14-1	*Pyrus*	Villiersdorp	24
STE-U 7291	VP 15-16-1	*Pyrus*	Villiersdorp	24

	STE-U 7292	VP 15-16-2	*Pyrus*	Villiersdorp	24
	STE-U 7293	VyP 1-10-1	*Pyrus*	Vyeboom	28
	STE-U 7294	VyP 2-4-1	*Pyrus*	Vyeboom	28
	STE-U 7295	VyP 2-14-2	*Pyrus*	Vyeboom	28
Diplodia sp.	STE-U 7296	GP 10-1	*Pyrus*	Grabouw	52
	STE-U 7297	PV 2-1-1	*Pyrus*	Wolseley	30
	STE-U 7298	PV 8-3 (?)	*Pyrus*	Wolseley	30
	STE-U 7299	PV 11-1	*Pyrus*	Wolseley	30
	STE-U 7300	PV 15-2	*Pyrus*	Wolseley	30
	STE-U 7301	PV 16-1	*Pyrus*	Wolseley	30
Eutypa lata	STE-U 7304	VyG 1-4-1	*Malus*	Vyeboom	22
	STE-U 7305	VyG 1-5-2	*Malus*	Vyeboom	22
	STE-U 7306	PV 2-9-1	*Pyrus*	Wolseley	30
	STE-U 7307	PV 2-12-1	*Pyrus*	Wolseley	30
	STE-U 7308	W 5-2	*Pyrus*	Wolseley	25
	STE-U 7309	GAB 5-3	*Malus*	Grabouw	40
Neofusicoccum australe	STE-U 7310	VyP 2-6-1	*Pyrus*	Vyeboom	28
	STE-U 7311	VyP 2-14-1	*Pyrus*	Vyeboom	28
	STE-U 7312	VyP 2-15-1	*Pyrus*	Vyeboom	28
Neofusicoccum vitifusiforme	STE-U 7313	CPB 12-2	*Pyrus*	Ceres	20
	STE-U 7314	CA 6-1	*Malus*	Ceres	20
Paraconiothyrium brasiliense	STE-U 7315	CPB 11-1	*Pyrus*	Ceres	20
	STE-U 7316	PV 5-1	*Pyrus*	Wolseley	30
Paraconiothyrium variabile	STE-U 7317	GAB 3-2	*Malus*	Grabouw	40
	STE-U 7318	GAB 12-1	*Malus*	Grabouw	40
Phaeoacremonium aleophilum	STE-U 7319	CPA 5-3	*Pyrus*	Ceres	20
	STE-U 7320	CPA 10-1	*Pyrus*	Ceres	20
	STE-U 7321	CPA 12-2	*Pyrus*	Ceres	20
	STE-U 7322	PV 1-1	*Pyrus*	Wolseley	30
	STE-U 7323	PV 2A-1	*Pyrus*	Wolseley	30
	STE-U 7324	PV 12-2	*Pyrus*	Wolseley	30
	STE-U 7325	PV 7-1	*Pyrus*	Wolseley	30
	STE-U 7326	PV 2-6-2	*Pyrus*	Wolseley	30
	STE-U 7327	GAA 1-1	*Malus*	Grabouw	40

	STE-U 7328	GAA 5-1	*Malus*	Grabouw	40
	STE-U 7329	GAA 9-1	*Malus*	Grabouw	40
	STE-U 7330	GAA 9-2	*Malus*	Grabouw	40
	STE-U 7331	GAA 13-1	*Malus*	Grabouw	40
	STE-U 7332	GAB 6-1A	*Malus*	Grabouw	40
	STE-U 7333	GAB 6-1B	*Malus*	Grabouw	40
	STE-U 7334	GAB 11-2	*Malus*	Grabouw	40
	STE-U 7335	GAB 13-2	*Malus*	Grabouw	40
	STE-U 7336	CB 3-1	*Malus*	Ceres	20
	STE-U 7337	W 6-1	*Pyrus*	Wolseley	25
	STE-U 7338	W 6-2	*Pyrus*	Wolseley	25
	STE-U 7339	VP 14-6-2	*Pyrus*	Villiersdorp	22
	STE-U 7340	VP 15-15-1	*Pyrus*	Villiersdorp	24
	STE-U 7341	GP 1-6-1	*Pyrus*	Grabouw	52
	STE-U 7342	VyP 1-1-1	*Pyrus*	Vyeboom	28
	STE-U 7343	VyP 1-1-2	*Pyrus*	Vyeboom	28
	STE-U 7344	VyP 1-7-1	*Pyrus*	Vyeboom	28
	STE-U 7345	VyP 1-13-1	*Pyrus*	Vyeboom	28
	STE-U 7346	VyP 1-5-2	*Pyrus*	Vyeboom	28
	STE-U 7347	VyP 1-5-1	*Pyrus*	Vyeboom	28
	STE-U 7348	VG 2-7-1	*Malus*	Villiersdorp	28
	STE-U 7363	CPA 5-3	*Pyrus*	Ceres	20
	STE-U 7364	CPA 10-1	*Pyrus*	Ceres	20
	STE-U 7365	CPA 12-2	*Pyrus*	Ceres	20
	STE-U 7366	GAA 1-1	*Malus*	Grabouw	40
	STE-U 7367	GAA 5-1	*Malus*	Grabouw	40
	STE-U 7368	PV 1-1-1	*Pyrus*	Wolseley	30
	STE-U 7369	PV 1-2A-1	*Pyrus*	Wolseley	30
	STE-U 7370	PV 1-7-1	*Pyrus*	Wolseley	30
	STE-U 7371	PV 1-12-2	*Pyrus*	Wolseley	30
	STE-U 7372	PV 2-6-2	*Pyrus*	Wolseley	30
Phaeoacremonium iranianum	STE-U 7349	PV 2B-3	*Pyrus*	Wolseley	30
Phaeoacremonium mortoniae	STE-U 7350	VP 15-11-1	*Pyrus*	Villiersdorp	24
	STE-U 7351	CPB 5-1	*Pyrus*	Ceres	20

Phaeoacremonium viticola	STE-U 7352	VP 15-5-1	*Pyrus*	Villiersdorp	24
	STE-U 7353	VyP 1-12-2	*Pyrus*	Vyeboom	28
Phomopsis sp.7	STE-U 7354	VP 14-6-1	*Pyrus*	Villiersdorp	22
	STE-U 7355	VyP 8-1	*Pyrus*	Vyeboom	28
Phomopsis sp.1	STE-U 7356	GAB 3-3	*Malus*	Grabouw	40
Pyrenochaeta-like sp.	STE-U 7357	GAA 2-2	*Malus*	Grabouw	40
	STE-U 7358	GAA 6-1	*Malus*	Grabouw	40
	STE-U 7359	GAA 10-1	*Malus*	Grabouw	40
	STE-U 7360	GAA 10-2	*Malus*	Grabouw	40
	STE-U 7361	GAA 12-3	*Malus*	Grabouw	40
	STE-U 7362	GAA 13-2	*Malus*	Grabouw	40

Table 2. Mean lesion length and re-isolation frequencies of fungal species inoculated onto detached grapevine, pear and apple shoots in a pathogenicity trial.

		Mean lesion length (mm) and t-grouping						Re-isolation frequency %		
Fungal species	**Treatments (STE-U)**	**Grapevine**		**Pear**		**Apple**		**Grapevine**	**Pear**	**Apple**
Phaeocremonium aleophilum	7334, 7337, 7348	5.06	*b,c,d,e,f*	14.46	*d*	25.21	*c,d*	77.1	41.6	76.4
Phaeoacremonium iranianum	7349	2.78	*c,d,e,f*	9.66	*d,e*	41.21	*a*	66.7	62.5	41.6
Phaeoacremonium viticola	7352, 7353	3.72	*c,d,e,f*	10.55	*d,e*	11.79	*e,f*	50.0	56.3	77.0
Phaeoacremonium mortoniae	7350, 7351	6.23	*b,c,d*	15.94	*d*	19.73	*c,d*	29.1	47.9	85.4
Neofusicoccum vitifusiforme	7313, 7314	3.32	*c,d,e,f*	25.63	*c*	23.76	*b,c,d*	93.8	47.9	50.0
Neofusicoccum australe	7310, 7311, 7312	19.99	*a*	52.28	*a,b*	40.19	*a*	93.1	31.9	55.5
Diplodia seriata	7229, 7269, 7279	6.55	*b,c*	43.04	*b*	20.14	*c,d*	73.6	50.0	48.6
Diplodia sp.	7296, 7297, 7299	5.35	*b,c,d,e*	55.03	*a*	27.34	*b*	79.2	26.4	52.7
Eutypa lata	7304, 7306, 7309	1.53	*e,f*	43.71	*b*	19.19	*c,d*	58.3	4.2	68.0
Phomopsis sp. 7	7354, 7355	1.34	*e,f*	13.08	*d,e*	11.78	*e,f*	77.1	10.4	70.8
Phomopsis sp. 1	7356	1.49	*f*	4.21	*e*	10.22	*f*	91.6	0.0	45.8
Pyrenochaeta-like sp.	7358, 7359, 7362	8.57	*b*	8.72	*d,e*	11.42	*f*	38.8	29.2	55.5
Paraconiothyrium brasiliense	7315, 7316	2.89	*c,d,e,f*	10.41	*d,e*	18.14	*d,e*	16.6	47.9	87.5
Paraconiothyrium variabile	7317, 7318	8.16	*b*	13.39	*d,e*	10.47	*f*	39.6	52.1	37.5
Acremonium strictum	6926	2.47	*d,e,f*	17.69	*c,d*	10.08	*f*	75.0	100.0	75.0
PDA plug		1.84	*e,f*	4.36	*e*	5.64	*f*			
LSD (P< 0.05)		3.97		9.25		6.52				

3. NEW COELOMYCETOUS SPECIES ASSOCIATED WITH DIE-BACK SYMPTOMS ON APPLE AND PEAR TREES IN SOUTH AFRICA

ABSTRACT

A survey was undertaken on apple and pear trees in the main pome fruit growing areas of the Western Cape province to determine the aetiology of trunk diseases with occurring on these hosts, which are commonly cultivated in close proximity to these orchards. During the survey, two previously unidentified fungi, a *Diplodia* species and a new genus within the *Pleosporales,* were isolated from symptomatic *Pyrus* and *Malus* wood. The combined ITS and EF1-α phylogeny supported the new *Diplodia* species, *Diplodia pyricolum* sp. nov., forming a group with a bootstrap value of 100% within *Diplodia,* closely related to *D. mutila* and *D. africana*. The new species is characterised by conidia that become pigmented and 1-septate within the pycnidium, and that are intermediate in size between the latter two *Diplodia* species. Phylogenetic inference of the SSU of the unknown coelomycete provided bootstrap support (100%) for a monophyletic clade unrelated to known genera, and basal to *Phoma* and its relatives. Morphologically, the new genus is characterised by pycnidia with elongated necks that lack setae, cylindrical conidiophores that are seldomly branched at the base, and *Phoma*-like conidia. The phylogenetic results combined with its dissimilarity from genera allied to *Phoma,* lead to the conclusion that it represents a new genus *Pyrenochaetoides* gen. nov. with the type species *Pyrenochatoides mali* sp. nov.

INTRODUCTION

The current extent of trunk diseases in the pome fruit growing regions of the Western Cape province is largely unknown, though symptoms may commonly be observed in orchards. Coelemycetous fungi have been isolated from die-back or canker symptoms of pome trees in South Africa, and include *Neofusicoccum ribis* (previously known as *Botryosphaeria ribis*), *Diaporthe ambigua* and *Leucostoma persoonii* (Smit *et al*., 1996; Crous *et al*., 2000). Based on the paucity of current knowledge, the assumption was

made that these hosts may contain previously undescribed fungal species along with well-documented trunk pathogens. During the survey undertaken on diseased pome fruit described in Chapter 2, a previously undescribed *Diplodia* species and a previously undescribed genus appearing to be related to *Phoma* were isolated from symptomatic wood.

Diplodia species have been associated with disease ranging from die-back and cankers to fruit rots on various hosts (Stevens, 1933; Jones and Sutton, 1984). Like many of the *Botryosphaeriaceae, Diplodia* has been found to be cosmopolitan in its host range and distribution. The type species, *Diplodia mutila* Fr. has been associated with cankers and die-back on *Malus* and *Vitis* species (Slippers *et al.*, 2007; Phillips, 1998), but has not been reported to be present in South Africa on either host or on *Pyrus*. Several *Diplodia* species have also been reported from these hosts, including *D. malorum* Fuckel, *D. sarmentorum* (Fr.) Fr. and *D. seriata* De Not. and *D. porosum* Van Niekerk & Crous on grapevine (Crous *et al.*, 2000; Van Niekerk *et al.*, 2004; Carstens, 2006). *Diplodia sarmentorum* has since been reclassified as *Dothiorella sarmentorum* (Fr.) Phillips, Alves and Luque (Phillips *et al.*, 2005). *Diplodia seriata* has been found to be the most common species associated with the *Botryosphaeriaceae* on pome and stone fruit (Slippers *et al.*, 2007; Damm *et al.*, 2008) in South Africa.

During a long-term study of the genus, Boerema *et al.* (2004) created nine sections to house various *Phoma* species on the basis of *in vitro* morphology and identified eight allied genera that have been identified as *Phoma* species in the past. Phylogenetic studies undertaken recently have revealed evidence supporting reclassification of various *Phoma* species in the section *Plenodomus* (Reddy *et al.*, 1998; Torres *et al.*, 2005). A large-scale phylogenetic study was undertaken by De Gruyter *et al.* (2009) to circumscribe *Phoma* and the allied genera using sequence data of the 18S (SSU) and 28S (LSU) nrDNA region. The resulting phylogeny was able to link five out of the nine *Phoma* sections to the teleomorph genus *Didymella* and suggested further work to reclassify the remaining sections in a satisfactory manner. Additionally, *Phoma* has been confused with the genera *Ascochyta*, *Asteromella*, *Microsphaeropsis*, *Pleurophoma* and *Pyrenochaeta* (de Gruyter *et al*., 2009). Commenting on the precise

identity of species thought to be *Pyrenochaeta*- or *Phoma*-like is difficult in the face of the ongoing reclassification of this diverse genus.

There are several species traditionally known as *Phoma* that are involved with disease on pome fruit and grapevine. Carstens (2006) lists *Phoma coonsii* Boerema & Loer., *P. fuliginea* Kidd & Beaumont, *P. glomerata* (Corda) Wollenw. & Hochapf., *P. pomorum* Thüm. var. *pomorum, P. pyrina* (Fr.) Cooke and *P. radicina* (McAlpine) Boerema as occurring on *Malus* causing an array of symptoms from fruit and leaf spot to twig blight and bark irregularities. Of those species, *P. glomerata, P. pomorum* and *P. pyrina* are known to occur on *Pyrus,* but *P. glomerata* is the only *Phoma* species listed as occurring in South Africa. Crous *et al.* (2000) also lists *P. macrostoma* Mont. as occurring on *Malus* in the north-eastern parts of South Africa, but lists no reports of any *Phoma* species occurring on *Pyrus* or *Vitis.* Massee (1915) reported *P. mali* Sacc. & Schulzer to occur in conjunction with blistering and cracking on the shoots of *Malus* and *Pyrus,* though this fungus has now been reclassified as *P. macrostoma* Mont. On grapevine, *P. herbarum* Westend. has been associated with bark necrosis and *P. negriana* Thüm. has been associated with various symptoms on leaves, fruit and stems (Machowicz-Stefaniak and Król, 2007).

The taxonomic novelties, *i.e*. a new species of *Diplodia* was found as well as a Coelomycete species that represents a new genus within the *Pleosporales,* that were observed in the above-mentioned survey, will be characterised in this chapter.

MATERIALS AND METHODS

Sampling

Symptomatic wood from trees showing die-back symptoms was collected in September and October over 2 years, 2006 and 2007. A total of five areas representing the oldest established pome fruit producing areas in the Western Cape were sampled over this period. These areas consisted of Grabouw, Vyeboom, Villiersdorp, Wolseley and Ceres. The cultivars selected for sampling were the green apple cultivar Granny Smith and the

green pear cultivar Packham's Triumph. Samples of living symptomatic wood were taken from trees in orchards older than 15 years and stored at 4°C until dissection.

Isolations

The samples were taken from storage and dissected. Symptoms were described and photographed. Symptomatic wood was cut into pieces measuring approximately 3 × 3 cm and surface sterilised by soaking in a 70% ethanol solution for 30 seconds, in a 1% NaOCl solution for 1 minute and in 70% ethanol for a further 30 seconds. Following sterilisation, wood pieces were air-dried in the laminar flow cabinet and halved using sterilised pruning shears. Pieces of wood measuring approximately 2 × 2 mm were excised from the margins between necrotic and healthy tissue and placed on 2% potato-dextrose agar (PDA; Biolab, Midrand) amended with streptomycin sulphate (40 mg/L, Calbiochem, Merck). 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 similar conditions.

To stimulate sporulation, isolates were placed on divided plates containing unamended PDA and water agar (WA, Biolab, Midrand), with a portion of carnation leaf placed on the WA to enhance sporulation. *Botryosphaeriaceae* isolates were 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 (pine needle agar: PNA; Damm *et al.*, 2007a). Single-conidium isolates were made from all sporulating isolates to obtain pure cultures.

Morphological identification and description

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 Leica WILD microscope and slides were made by mounting fungal material in lactic acid. Slides were examined under a Zeiss MC80 microscope and identified based on

structures formed. Isolates 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.

Measurements and photographs were taken from structures mounted in lactic acid. The 95% confidence intervals, minimum and maximum measurements were calculated for conidia, conidiophores and conidiomata, based on at least 30 observations per structure. A Nikon DXM 1200 digital camera were used to capture photographs. The growth rates, culture characteristics and cardinal temperatures for growth of selected isolates were determined after on PDA between 5–35°C in 5°C intervals. Rayner's (1970) colour rating system was used to describe isolates incubated at 25°C for 7 days under near-ultraviolet light.

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 Lee and Taylor (1990) with chloroform:isoamylalcohol instead of chloroform:phenol and using sterile water as a suspension medium for the DNA. Products were visualised via electrophoresis. The internal transcribed spacers (ITS1 and ITS2) and the 5.8S ribosomal gene was 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) with an increase in the $MgCl_2$ concentration to 3 mM. Following sequencing of the ITS-region, an unidentified *Diplodia* species and an unidentified *Pyrenochaeta*-like species were selected for further characterisation. A part of the translation elongation factor 1-α gene was amplified for the unidentified *Diplodia* using the primer pair EF1-728F and EF1-986R (Carbone and Kohn, 1999), also using the conditions described in White *et al.* (1990) with a modified $MgCl_2$ concentration of 3 mM. The primers NS1 and NS4 (White *et al.*, 1990) were used with the PCR protocol and conditions described in De Gruyter *et al.* (2009) to amplify the 18S (SSU) nrDNA region of the *Pyrenochaeta*-like species.

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). 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.) 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.

Reference sequences representing the relevant species of the *Botryosphaeriaceae* (Van Niekerk *et al.*, 2004, Damm *et al.*, 2007) and *Pleosporales* (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 or not using PAUP's bootstrap search option set to 1000 bootstrap replications. The measures tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated for the tree resulting from the above-mentioned analysis.

RESULTS

Morphology

Diplodia pyricolum Cloete, L. Mostert & Crous, sp. nov. Fig. 1

Etymology. Named after the host from which it was isolated, *Pyrus*.

Description *in vitro* on PNA. *Pycnidia* formed within 10-21 days, separate or aggregated, sub-globose to ovoid, thick-walled, dark brown, partially submerged, becoming erumpent towards maturity, (190–)252–585(–580) × (200–)220–387(–550) µm; pycnidial necks vary between short and wide to elongated and narrow, (120–)162–511(–600) × (100–)104–120(–130) µm. *Conidiogenous cells* 1–2-celled, cylindrical, sometimes ampulliform, hyaline, thin-walled, producing conidia apically, with 1–2 annelations, 16–20(–23) × (2–)2.5–4.5(–5.5) µm. *Conidia* large, smooth, thick-walled, hyaline and sub-cylindrical with a rounded apex and a truncated base, becoming 1-septate and pigmented with a slightly roughened wall, (24–)25–27(–29) × 10–12(–14) µm.

Culture characteristics. Colonies are fast-growing on PDA, reaching 66 mm after 3 days at 25°C and covering a 90 mm Petri dish in 5 days. After 7 days at 25°C under near-ultraviolet, colonies are pale olivaceous-grey (21""d) with pale mouse-grey mycelium (15""d); greenish black (33""k) in reverse.

Type. South Africa, Western Cape, Wolseley, *Pyrus communis*, (isolated from die-back symptoms), 2007, M. Cloete, dried SNA with pine needles in herb XXX, holotype; culture ex-type STE-U 7299.

Distribution. Wolseley, Elgin, Western Cape, South Africa.

Host. *Pyrus communis* cv. Packham's Triumph.

Additional cultures examined. South Africa, Western Cape, Wolseley, *Pyrus communis*, (isolated from die-back symptoms), 2007, M. Cloete, cultures STE-U 7297 (orchard 2, tree 1), STE-U 7298 (orchard 1, tree 8), STE-U 7300 (orchard 1, tree 15), STE-U 7301 (orchard 1, tree 16); Elgin, *Pyrus communis*, (isolated from die-back symptoms), 2007, M. Cloete, culture STE-U 7296.

Notes. The different isolates were similar in pycnidia, conidiogenous cell, and conidial morphology and size. There was some variation in colony colour on PDA after seven days.

Pyrenochaetoides Cloete, L. Mostert, Crous & De Gruyter, gen. nov.

Mycelium superficial, hyaline to pale brown, septate, branched. *Conidiomata* pycnidial, separate or aggregated, immersed, sub-globose to pyriform, pigmented, glabrous to semi-pilose, thick-walled with elongated necks without setae. *Conidiophores* cylindrical, tapering towards apex, septate, hyaline, seldom branched at base. *Conidiogenous cells* phialidic, mostly discrete or with acropleurogenous conidiogenesis. *Conidia* small, hyaline, aseptate, sub-cylindrical to ellipsoidal, guttulate.

Type species. *Pyrenochaetoides mali* Cloete, L. Mostert, Crous & De Gruyter, sp. nov.

Pyrenochaetoides mali Cloete, Mostert, Crous & De Gruyter, sp. nov. Fig. 2

Etymology. Name given from the host it was isolated, *Malus*.

Description *in vitro*. *Pycnidia* formed after 14–21 days on CLA; sub-globose to pyriform with a flattened base, walls consisting of up to 8 layers of pale to medium brown *textura angularis*, glabrous to semi-pilose, (110–)150–230(–260) × (70–)135–240(–270) µm, submerged within the medium; pycnidia with elongated necks, (40–)125–300(–400) × (20–)50–90(–110) µm, without setae. *Conidiophores* 1(-3)-septate, seldom branched at the base, cylindrical and tapering towards the apex, (6–)6.4–12(–18) × 1–2(–3)µm. *Conidiogenous cells* phialidic, mostly discrete or with acropleurogenous conidiogenesis. *Conidia* small, 2–4 × 1–2 µm, hyaline, with 1–2 inconspicuous guttules, aseptate, sub-cylindrical to ellipsoidal, some slightly curved. Conidial exudate buff coloured.

Culture characteristics. Colonies slow-growing on PDA, reaching 11–17 mm after 7 days at 25°C. Colonies cinnamon-honey (62″b – 64″b) coloured and flat with sparse, white aerial mycelia.

Type. South Africa, Western Cape, Elgin, *Malus domestica* (isolated from die-back symptoms), 2007, M. Cloete, dried WA with carnation leaves in herb XXX, holotype; culture ex-type STE-U 7357.

Distribution. Elgin, Western Cape, South Africa.

Host. *Malus domestica* cv. Granny Smith.

Additional cultures examined. South Africa, Western Cape, Elgin, *Malus domestica* (isolated from die-back symptoms), 2007, M. Cloete, cultures STE-U 7358 (tree 6), STE-U 7359 (tree 10), STE-U 7360 (tree 10), STE-U 7361 (tree 12), STE-U 7362 (tree 13).

Notes. The size of the pycnidial necks varied among the isolates from elongated and narrow to shorter and wide. Although *Pyrenochaetoides mali* resembles the genus *Pyrenochaeta*, no setae were found around the ostiole and the conidiophores were shorter, filiform and seldom branched at the base. It also differs from *Pyrenochaeta* in that the pycnidia of all isolates examined formed necks. The conidiophores resemble those of *Phomopsis*, having elongated, phialidic conidiogenous cells. Conidia are similar to *Phoma*, being small, sub-cylindrical with inconspicuous guttules.

Phylogenetic analysis

The combined ITS and EF 1-α phylogeny (Fig. 3) supported the species grouping of the new *Diplodia* species first found in the ITS phylogeny (Chapter 2). The new species formed a group within *Diplodia* with a bootstrap support of 100%, closely related to, but distinct from *Diplodia mutila* and *D. africana*.

The SSU phylogeny of the *Pyrenochaeta*-like species (Fig. 4) was similar to the combined SSU and LSU phylogeny obtained by De Gruyter *et al.* (2009) for *Phoma*, but one of the groups found in De Gruyter *et al.*, A11, failed to form a monophyletic clade. This could be due to the absence of the LSU region on the alignment. The *Pyrenochaeta*-like species formed a distinct clade (100% bootstrap support) basal to *Phoma* and many of its allied genera. Basal to the *Pyrenochaeta*-like species, *Pyrenochaetoides mali*, lie the following genera allied to *Phoma*, namely *Pleurophoma*, as well as *Asteromella tillae* and *Pyrenochaeta romeroi*, both non-typical species to the respective genera. The strongly supported monophyletic clade that does not group with any of the other known genera supports the naming of a new genus for these isolates.

The ITS sequences obtained from *Pyrenochaetoides mali* were compared to the nucleotide database on GenBank and the closest hit was *Pyrenochaeta romeroi* (DQ836803) with a similarity of 86% over 526 bp.

DISCUSSION

In recent years, multiple studies have been done on the taxonomy of the *Botryosphaeriaceae*, especially regarding the identity of its anamorphs (Jacobs and Rehner, 1998; Denman *et al.*, 2000; Zhou and Stanosz, 2001). These studies concluded that anamorphs can roughly be divided into two clades on the basis of ITS phylogeny, namely *Fusicoccum* with species forming hyaline, thin-walled conidia narrower than 10 µm and *Diplodia* with species forming pigmented, thick-walled conidia broader than 10 µm. Unfortunately, the conidia of species characterised as *Fusicoccum* can also become pigmented with age (Crous *et al.*, 2006) and this method of determining anamorph placement is therefore oversimplified.

Within the clade characterised by pigmented conidia, Denman *et al.* (2000) identified four genera as synonymous to the genus *Diplodia*, namely *Sphaeropsis* Sacc., *Dothiorella* Sacc., *Macrophoma* (Sacc.) Berl & Vogl. and *Lasiodiplodia* Ell. & Everh. Sutton (1980) declared *Macrophoma* synonymous to *Sphaeropsis*. Crous *et al*. (2006) found that *Diplodia*, *Lasiodiplodia* and *Sphaeropsis* form a single clade, although a poorly resolved one, based on LSU phylogeny. *Sphaeropsis* was subsequently clarified in Phillips *et al.* (2008) and was shown to be phylogenetically and morphologically distinct from *Diplodia* and *Lasiodiplodia*.

Based on work done by Pavlic *et al.* (2004) and the LSU phylogeny in Crous *et al.* (2006), *Lasiodiplodia* has not formally been declared synonymous to *Diplodia* and is still considered to be a distinct genus grouping within the *Diplodia* clade. *Lasiodiplodia* is distinguished from *Diplodia* by its conidial striations, which are notably absent in *Diplodia*.

Crous *et al.* (2006) found that *Sphaeropsis visci* (Fr.) Sacc. resides within the *Lasiodiplodia* and also found that conidia of *S. subglobosa* C. Booth are hyaline and thick-walled, often becoming pigmented with age. Mature conidia were found to have

the appearance of striations thought to be formed by having more than one germ slit, leading to the conclusion that the species would also be better suited to *Lasiodiplodia* than to *Diplodia.* Phillips *et al.* (2008) resolved *Sphaeropsis* by using a multigene approach to associate *Sphaeropsis* with the dark-spored teleomorphs, *Neodeightonia* and *Phaeobotryosphaeria*.

Dothiorella was found to reside in a separate clade by Crous *et al.* (2006), a finding that is consistent with the finding by Phillips *et al.* (2005), seperating *Dothiorella* from *Diplodia* based on conidial morphology and combined ITS and EF1-α phylogeny. Conidia of *Dothiorella* species are different from those of *Diplodia* by the fact that they often become pigmented and 1-sepate before discharge from the pycnidium, often while still attached to the conidiophore. Percurrent proliferation has been found to be rare within *Dothiorella*, whereas within *Diplodia* it is common (Phillips *et al.*, 2005).

In a recent study, Damm *et al.* (2007b) identified a further anamorph genus with dark-spored conidia, namely *Aplosporella*, which grouped separately from both the *Diplodia*/*Lasiodiplodia* and the *Dothiorella* clades. The genus is characterised by verrucose, brown conidia, prominent paraphyses and multilocular pycnidia with a single ostiole.

In this study, the species *Diplodia pyricolum* sp. nov., was found to be closely related to *D. mutila* and *D. africana* Damm & Crous, residing within the *Diplodia* clade on the basis of the combined ITS and EF1-α phylogeny. Another apparently related species of interest within the context of the study is *D. seriata*, which is a common pathogen of pome fruit trees and vines. These species mainly differ in terms of conidial morphology. *D. mutila* is characterised by smooth, aseptate and hyaline, thick-walled, straight conidia with rounded ends, (23.5–)25.1–25.7(–27.4) × (12.4–)13.2–13.5(–14.3) µm in size. Conidia sometimes become lightly pigmented and septate with age, but this is rare (Sutton, 1980; Alves *et al.*, 2004). *D. africana* differs from *D. mutila* mainly in terms of conidial size, (17–)25.5–33(–34) × (10–)12–14(–15) µm, but the shape of the conidia is also sometimes slightly curved in *D. africana*, and the ends may be less broadly rounded (Damm *et al.*, 2007a). In comparison, conidia of *D. pyricolum* are intermediate in size, (24–)25–27(–29) × 10–12(–14) µm, and, though many conidia

remain hyaline, some become pigmented and septate before discharge from the pycnidium and many become pigmented and septate later.

D. seriata has been found to be more closely related to *D. pinea* and *D. scrobiculata*, and is charaterised by hyaline conidia with an obtuse apex and rounded base, (21.5–)22–27(–28) × (11–)11.5–14.5(–15.5) µm, becoming dark brown with a roughened inner surface and a smooth external wall. The latter feature is also sometimes found in *D. pyricolum*, though not to such an extent as in *D. seriata*. *D. pinea* and *D. scrobiculata* vary rather dramatically from *D. seriata* in terms of conidial size. *D. pinea* forms conidia that are rarely septate, paler in colour than *D. seriata*, and that are between 30–45 µm long and 10–16 µm wide (Sutton, 1980). *D. scrobiculata* forms large ((37.5–)39.5(–41.5) × (13–)14(–15.5) µm) aseptate to 3-septate conidia (De Wet *et al*., 2003). An important difference between the three species is that while the latter two species are mainly found on coniferous hosts, *D. seriata* is found on angiosperms and gymnosperms (De Wet *et al.*, 2008). De Wet *et al*.(2008) also found that *D. mutila* only occurred on angiosperms.

The results of the phylogeny combined with comparison of the morphological data of *D. pyricolum* to closely related *Diplodia* species has led to the conclusion that this is a species distinct from other related species.

The generic name, *Phoma* Sacc., has been used in the past to describe Coelomycetes producing small, hyaline conidia with no septation on monophialidic, flask-shaped conidiogenous cells within thin-walled pycnidia (Sutton, 1980; Boerema and Bollen, 1975). As such, more than 2000 species had been described within the genus by 1980 according to Sutton (1980), vexing mycologists and pathologists alike. Attempts at morphological identification were further complicated by the plasticity of certain morphological features such as the shape and size of pycnidia and conidia when *Phoma* isolates are cultured (Aveskamp *et al.*, 2009).

There are several species considered to be *Phoma* within three sections, *Phoma*, *Pilosa* and *Paraphoma*, out of the nine sections created to house various *Phoma* species that resemble other pycnidial genera with similar characteristics in terms of conidial formation and conidial shape. These are known as allied genera and are characterised by having septate conidiophores with integrated conidiogenous cells bearing unicellular,

hyaline conidia (Boerema *et al.*, 2004). The allied genera include several genera with similar, though not identical, characteristics to the *Pyrenochaeta*-like fungus found in this study, including *Pleurophoma* Höhn., *Pyrenochaeta* De Not., *Asteromella* Pass. & Thüm. and *Phomopsis* (Sacc.) Sacc.

There are several clear morphological differences between the new fungus and the allied genera of *Phoma*. *Pleurophoma* is characterised by having conspicuous filiform, multi-septate conidiophores on which the conidia are produced below the transverse septa and at the apex. *Asteromella* also produces pycnidia with papillate ostioles and conidia at the apex and below the transverse septa. *Pyrenochaeta* is characterised by having plenty of setae around the ostiole and by producing long filiform conidiophores which are multiseptate and are branched at the base. *Pyrenochaetoides* has elongated phialidic cells, similar to *Phomopsis*, but the conidial morphology typical of *Phomopsis* is absent from *Pyrenochaetoides*. Combined with the phylogenetic results, its dissimilarity from other genera allied to *Phoma* has lead to the conclusion that this fungus represents a new genus in the *Pleosporales*.

A novel *Diplodia* species and an unknown coelomycetous fungus representing a new genus were isolated from apple or pear trees with die-back symptoms. Investigations employing molecular techniques and phenotypic characters enabled the identification of fungi previously overlooked, which could become of greater pathological importance in the future.

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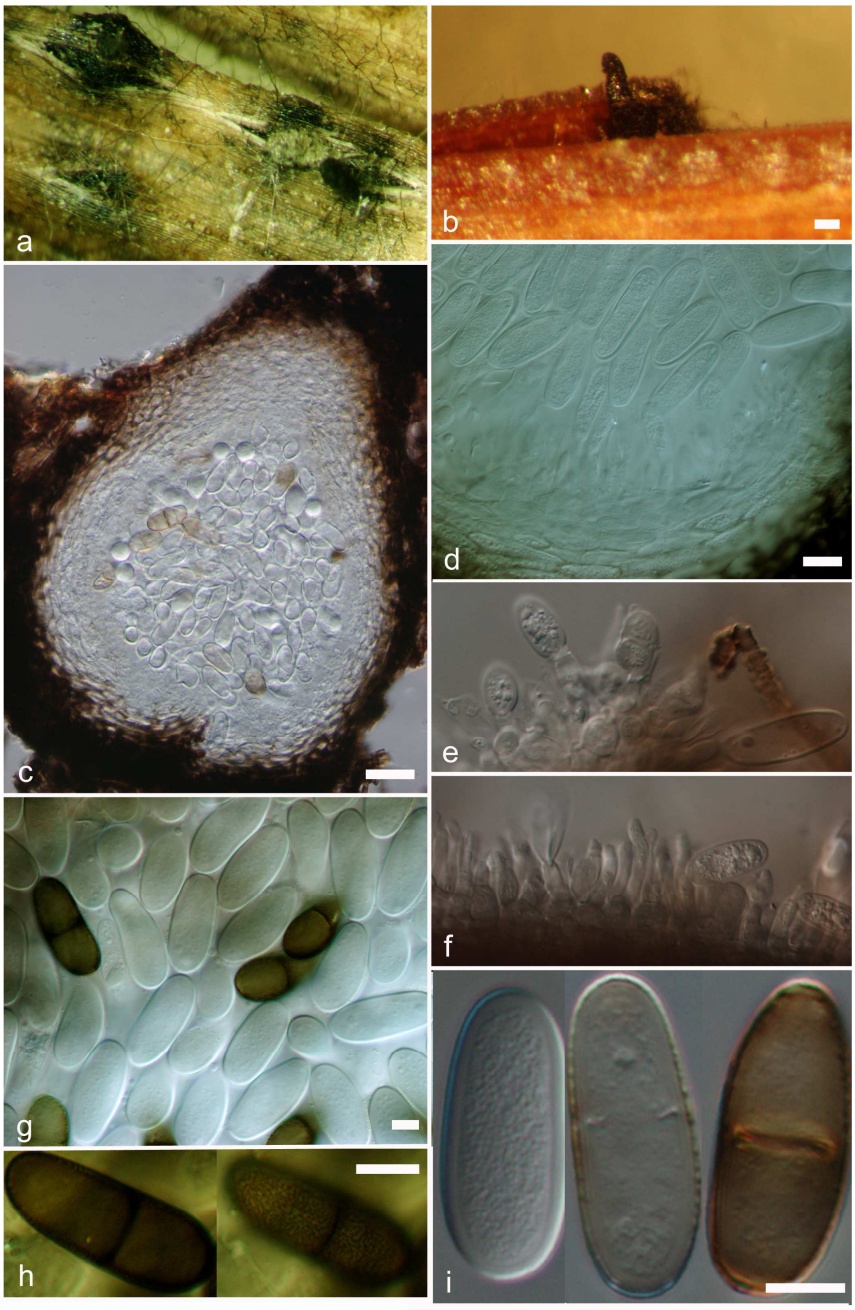

Fig 1. a-b. Pycnidia of *Diplodia pyricolum* emerging from pine needles. c. Section of a pycnidium containing both hyaline and pigmented conidia. d. Part of the interior wall showing conidiophores on the interior layer of a pycnidium filled with hyaline conidia. e. Conidiogenous cells with immature hyaline conidia. f. Cylindrical and ampulliform conidiogenous cells, some with annelations. g. Hyaline and pigmented conidia. h. Mature conidia showing smooth exterior and roughened interior walls. i. A single hyaline conidium, an intermediate conidium showing slight pigmentation and imminent septation and a mature pycnidium, 1-septate and pigmented. Scale bars: b = 100 µm; c = 50 µm, d, g, i = 10 µm. Scale bar of b applies to a; d applies to e and f.

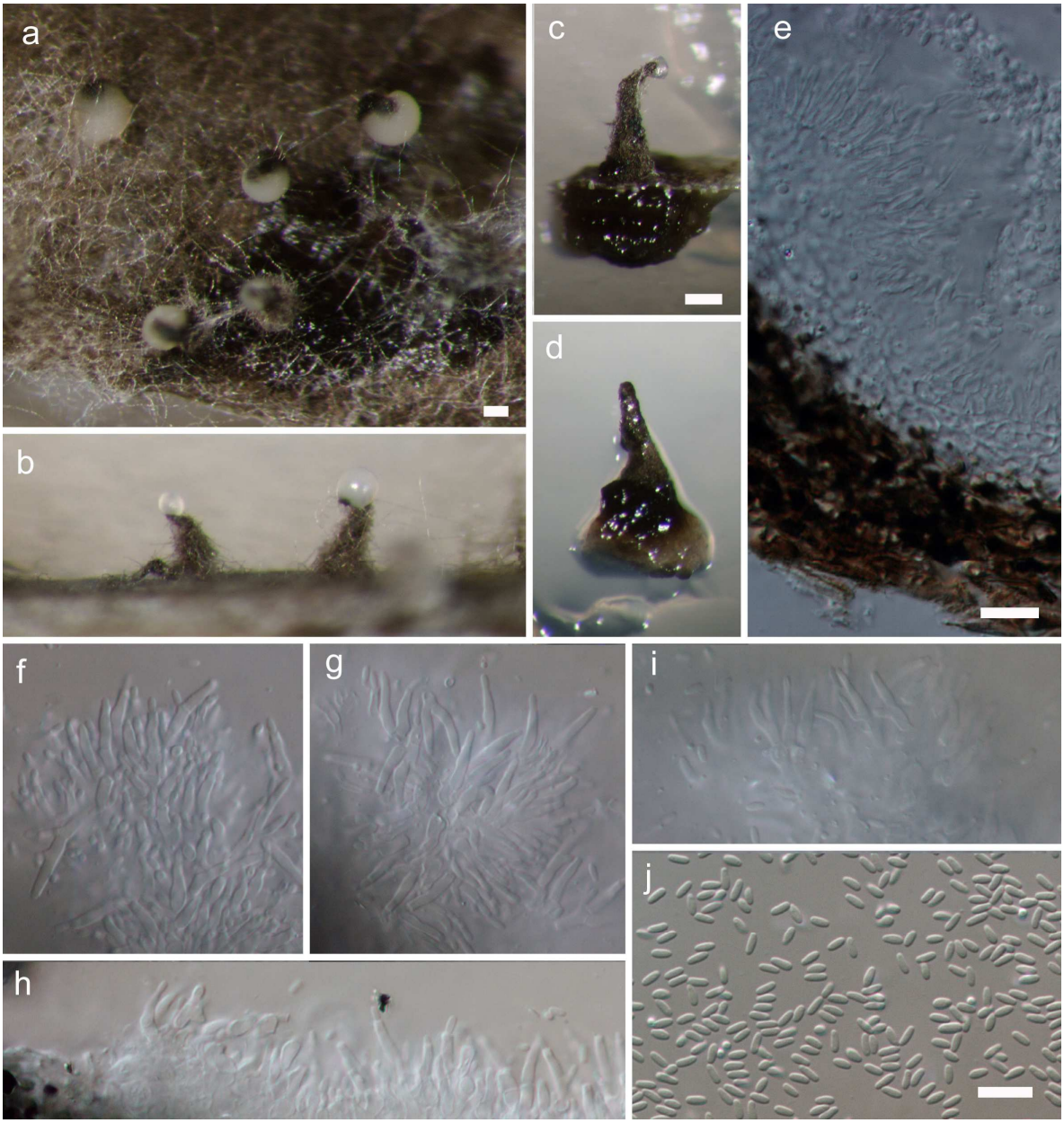

Fig 2. a-b. Pycnidia of *Pyrenochaetoides mali* formed on carnation leaves, exuding buff-coloured conidial droplets. c, d. Sub-globose pycnidia with elongated necks. e. Section of the pycnidial wall showing conidiophores attached to the inner layer. f-i. Cylindrical, hyaline conidiophores tapering towards the apex. j. Hyaline, sub-cylindrical, aseptate conidia with 1-2 inconspicuous guttules. Scale bars: a, c = 100 µm; e, j = 10 µm. Scale bar of a applies to b; c applies to d; e applies to f, g, i and h.

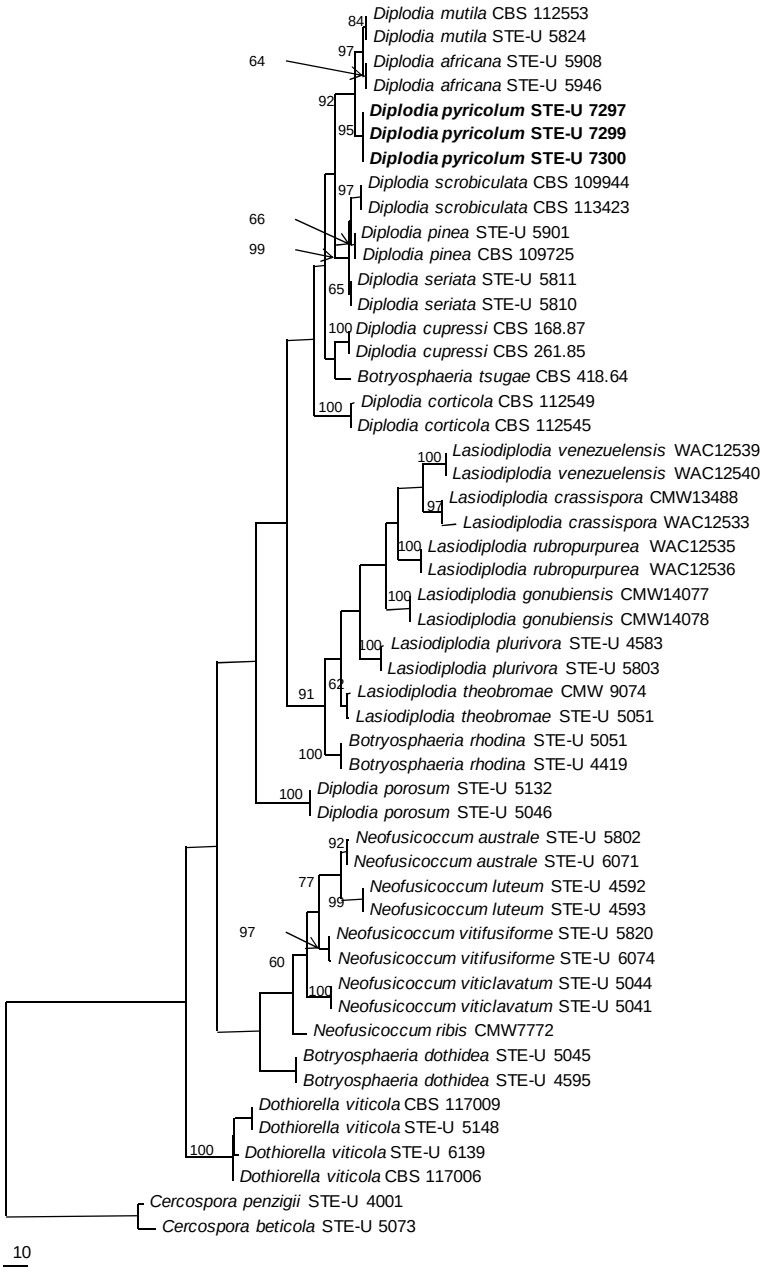

Fig. 3. One of 4 most parsimonious trees obtained from the heuristic search on combined ITS and EF1-α sequences of the *Botryosphaeriaceae*. Bootstrap support values above 50% are shown at the nodes. Tree scores include Length = 1028, CI = 0.724, RI = 0.906, RC = 0.656, HI = 0.276. Isolates sequenced in this study in bold.

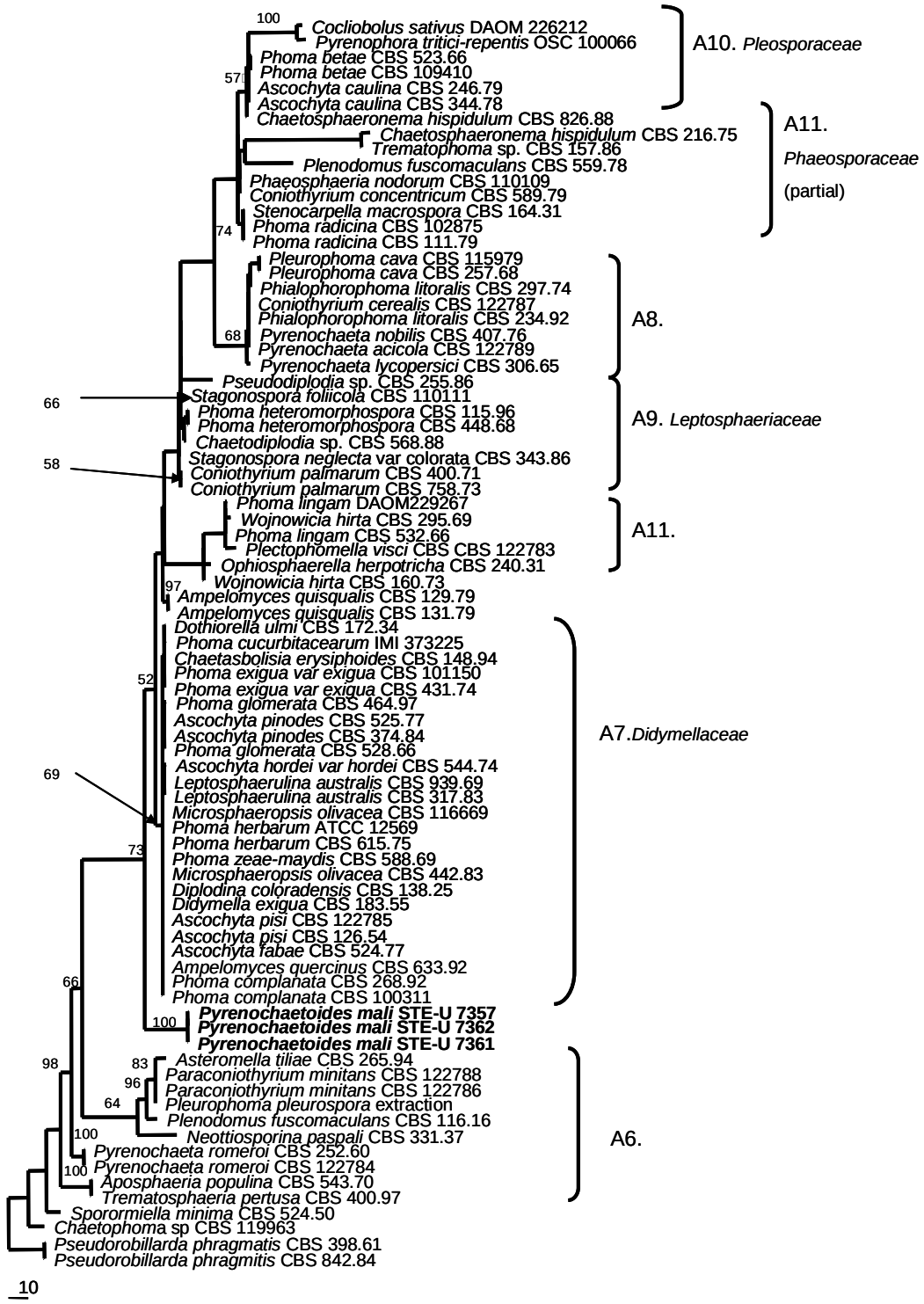

Fig. 4. One of 540 most parsimonious trees obtained from the heuristic search done on SSU sequences of *Phoma* and its allied genera. Bootstrap support values above 50% are shown at the nodes. Tree scores include Length = 394, CI = 0.609, RI = 0.915, RC = 0.558, HI = 0.391. Isolates sequenced in this study in bold.