THE CHARACTERIZATION OF THE BASIDIOMYCETES AND OTHER FUNGI ASSOCIATED WITH ESCA OF GRAPEVINES IN SOUTH AFRICA

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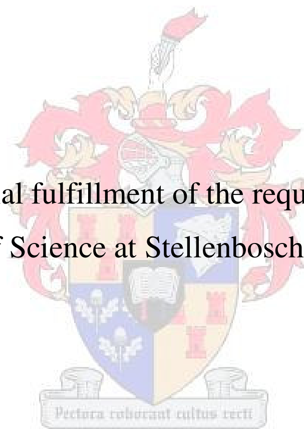

Thesis presented in partial fulfillment of the requirements for the degree of
Master of Science at Stellenbosch University

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December 2010

DECLARATION

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SUMMARY

Esca is a disease affecting grapevines and is potentially devastating as there are economic losses due to a decrease in yield, wine quality and berry quality. Vineyards also need to be replaced earlier and therefore esca has a great impact on the wine, table grape and raisin industries. The disease is known to affect vineyards worldwide and has been studied extensively in Europe, but not in South Africa. Esca diseased grapevines were observed for the first time prior to 1981 in South African vineyards. The disease is primarily caused by *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora* (both causing brown and black wood streaking) and white rot basidiomycete species such as *Fomitiporia mediterranea* which cause wood rot in the trunks and arms of generally older grapevines. Species of the Botryosphaeriaceae and *Phomopsis* (mainly *Phomopsis viticola*) and *Eutypa lata* have also been isolated from esca diseased vines, but their association with esca is unclear.

Some of the symptoms associated with the disease on most grapevine cultivars include 'tiger-stripe' foliar symptoms, apoplexy and berry symptoms such as shriveling, discoloration and 'black measles'. These external symptoms as well as internal symptoms are thought to be a result of toxin and enzyme production by the fungi involved. Symptom expression is erratic and varies from year to year making investigations into the causal fungi and the toxins and enzymes secreted *in planta* difficult.

Vines with internal or external symptoms of esca were sampled in this study from table and wine grape cultivars in 37 towns in the Western Cape, Northern Cape and Limpopo provinces. The majority of sampled vines were over ten years of age, but vines as young as two to three years were also found to be infected. The external symptoms included dieback, tiger striped leaves, berry symptoms (shriveling, insufficient colouring and black spots) and apoplexy. These symptoms resembled those found on grapevines in Europe, Australia and the USA. The internal symptoms found were also similar to European symptoms and included white rot, black and brown wood streaking, brown necrosis within white rot, sectorial brown necrosis and central brown/ red/ black margin. The fungi mostly isolated from the white rot were the basidiomycetes. Black and brown wood streaking was primarily caused by *Phaeomoniella chlamydospora*. Brown necrosis within the white rot was caused by *Phaeomoniella chlamydospora* and less frequently by *Phaeoacremonium* spp., *Eutypa lata,* Botryosphaeriaceae and *Pleurostomophora richardsiae*. The sectorial brown necrosis and the

central/ brown/ red/ black margin were dominated by *Phaeomoniella chlamydospora*. The fruiting bodies of the basidiomycetes were found on only a few grapevines.

The fungal species associated with the internal wood symptoms were characterized on cultural growth patterns, morphology as well as phylogenetic inference. The gene areas sequenced included the internal transcribed spacers and the 5.8S rRNA gene for the basidiomycetes and *Phomopsis* isolates, the partial β-tubulin gene for *Phaeoacremonium* isolates and the partial translation elongation-1α gene for the Botryosphaeriaceae isolates. The basidiomycete isolates fell into ten taxa within the *Hymenochaetales* of which two could be linked to known genera, namely *Fomitiporia* and *Phellinus*. The ten basidiomycete taxa do not correspond to any published sequences. *Eutypa lata*, *Diaporthe ambigua*, *Diplodia seriata*, *Neofusicoccum australe*, *Neofusicoccum parvum*, *Phomopsis viticola*, *Phomopsis* sp. 1, *Phaeomoniella chlamydospora* and six species of *Phaeoacremonium* including *P. aleophilum, P. alvesii, P. parasiticum*, *P. iranianum*, *P. mortoniae* and *P. sicilianum* were also isolated of which the latter three are reported for the first time in South Africa.

To understand the role of the basidiomycetes in the complex, toxin and enzyme analyses was determined for these fungi. Selected basidiomycete isolates were grown up in liquid broth and extractions performed to test for the presence of 4-hydroxy-benzaldehyde. All of the basidiomycete isolates were able to produce this toxin which is known to be phytotoxic. The basidiomycetes were then tested for the presence of certain wood degrading enzymes. All of the taxa were able to produce manganese peroxidase. Laccase was produced by all taxa, except Taxon 8. Lignin peroxidase was produced by Taxa 1, 2, 7, *Fomitiporia* sp. and the *Phellinus* sp. All the basidiomycete isolates were able to produce cellulose and none were able to produce xylanase. These enzyme tests showed that the basidiomycetes produce a wide variety of enzymes which are able to degrade cellulase and lignin which are both structural components of wood.

Given the wide distribution of esca in the grape growing regions investigated in South Africa and the diverse amount of species found, this disease must surely be seen as a limiting factor to the productive lifespan of vineyards and quality of produce. Preventative measures such as sanitation and pruning wound protection contribute to the management of the disease, but many questions still remain about the synergy of the causal fungi, epidemiology and management of esca.

OPSOMMING

Esca is ‘n wingerd siekte wat potensieel skade kan aanrig as gevolg van ekonomiese verliese weens verlaagde opbrengs, wyn kwaliteit en vrug kwaliteit. Wingerde moet ook vroeër vervang word en daarom het esca ’n groot impak op die wyn, tafeldryf en rosyne industrieë. Esca word wêreldwyd gevind op wingerd en is al intensief nagevors in Europa, maar nog nie in Suid-Afrika. Esca is vir die eerste keer in die 1980’s in Suid-Afrikaanse wingerde gerapporteer. Die primêre veroorsaakende organismes van esca is *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora* wat bruin en swart vaatweefsel verkleuring veroorsaak en basidiomycete spesies soos *Fomitiporia mediterranea* wat wit verotting veroorsaak in die stam en arms van ouer wingerd. Spesies van die Botryosphaeriaceae en *Phomopsis* (hoofsaaklik *Phomopsis viticola*) en *Eutypa lata* is ook al vanaf esca simptome geïsoleer, maar hul assosiasie met die siekte is nie duidelik nie.

Algemene simptome wat voorkom op die meeste wingerd kultivars met esca sluit in ‘tiger-stripe’ blaar simptome, apopleksie en vrug simptome soos verdroging, verkleuring en spikkels (black measles). Interne en eksterne simptome kan wees as gevolg van toksiene en ensiem produksie van die swamme wat betrokke is by esca. Eksterne simptoom uitdrukking is wisselvallig en varieer van jaar tot jaar. Dit bemoelik die bestudering van die swamme en die toksiene en ensieme wat afgeskei word *in planta*.

Wingerd monsters met eksterne en interne simptome is versamel van tafel en wyndruif kultivars in 37 dorpe in die Wes-Kaap, Noord-Kaap en Limpopo provinsies. Die meerderheid monsters was ouer as tien jaar maar wingerde wat twee tot drie jaar oud was, was ook gevind. Die eksterne simptome wat op hierdie kultivars gevind is het terugsterwing, ‘tiger striped’ blare, vrug simptome (verkrimping en onvoldoende verkleuring) en apopleksie ingesluit. Hierdie simptome stem ooreen met soortgelyke simptome gevind op wingerd in Europa, Australië en die VSA. Interne simptome was ooreenstemmend met simptome wat gevind word in Europa. Die interne simptome het wit verotting, bruin en swart streepvorming, bruin nekrose met wit verotting, sektoriale bruin nekrose en sentrale bruin/ rooi/ swart kante ingesluit. Basidiomycete swamme is meestal uit die wit verotting gedeeltes geïsoleer. Swart en bruin hout streepvorming was meestal deur *Phaeomoniella chlamydospora* veroorsaak. Bruin nekrose binne die wit verotting was meestal deur *Phaeomoniella chlamydospora* veroorsaak en in ‘n mindere mate deur *Phaeoacremonium* spp., *Eutypa lata,* Botryosphaeriaceae en *Pleurostomophora richardsiae*. *Phaeomoniella chlamydospora* was die hoof veroorsakende organisme van sektoriale bruin nekrose en die

sentrale bruin/ rooi/ swart kante. Vrugliggame van die basiodiomycete is op enkele wingerde gevind.

Swam soorte wat geassosieer word met die interne hout simptome was verder gekarakteriseer op kultuur groei, morfologiese eienskappe, en filogenetiese analise. Die geen areas waarvan die basis paar volgorde bepaal was sluit in die interne getranskribeerde spasies en die 5.8S rRNA geen vir die basidiomycete en *Phomopsis* isolate, die gedeeltelike β-tubulien geen vir *Phaeoacremonium* isolate en die gedeeltelike translasie velenging-1α geen vir die Botryosphaericeae isolate. Die basidiomycete isolate was versprei oor tien taksons binne die *Hymenochaetales* waarvan twee genusse gekoppel kon word aan die genera *Fomitiporia* en *Phellinus*. Die tien basidiomycete taksons kom nie ooreen met enige gepubliseerde DNS volgordes. *Eutypa lata*, *Phomopsis viticola*, *Phomopsis* sp. 1, *Diaporthe ambigua*, *Diplodia seriata*, *Neofusicoccum parvum*, *Neofusicoccum australe*, *Phaeomoniella chlamydospora* en ses spesies van *Phaeoacremonium* insluitend *P. aleophilum, P. alvesii, P. parasiticum*, *P. iranianum*, *P. mortoniae* en *P. sicilianum* is ook geïsoleer. Hierdie is die eerste keer dat *P. iranianum*, *P. mortoniae* en *P. sicilianum* in Suid-Afrika gerapporteer word.

Om die rol wat die basidiomycete in die siekte-kompleks speel beter te verstaan is toksien en ensiem analises uitgevoer. Geselekteerde basidiomycete isolate is gekweek in vloeibare groei medium en ekstraksies uitgevoer om te toets vir die teenwoordigheid van 4-hydroxy-benzaldehyde. Al die basidiomycete isolate kon 4-hydroxy-benzaldehyde, wat bekend is om fitotoksies te wees, produseer. Die basidiomycete isolate was verder getoets vir die produksie van spesifieke hout afbrekende ensieme. Al die basidiomycete taksons kon mangaan-peroksidase produseer. Lakkase was geproduseer deur al die taksons, uitsluitend Takson 8. Lignien-peroksidase was geproduseer deur Taksons 1, 2, 7, *Fomitiporia* sp. en die *Phellinus* sp. Al die basidiomycete isolate kon sellulose produseer, maar geen kon xilanase produseer. Die ensiem analises het gewys dat die basidiomycete wat moontlik betrokke is by esca ‘n wye reeks van ensieme kan produseer wat sellulose en lignien kan degradeer. Sellulose en lignien is beide strukturele komponente van hout.

Weens die wye verspeiding van esca geaffekteerde wingerde in Suid Afrika en die wye reeks van spesies wat betrokke is by die siekte kompleks moet esca sekerlik gesien word as een van die beperkende faktore op die produktiewe leeftyd van wingerde en die kwaliteit van druiwe wat geproduseer word. Sanitasie en snoeiwond beskerming is voorkomende maatreëls wat ingestel kan word om die effek en verspreiding van esca te beperk maar daar is

nog baie vrae wat antwoorde benodig oor die sinergie van die veroorsakende swamme, epidemiologie en bestuur van esca.

ACKNOWLDEGMENTS

I would like to acknowledge the following people for all of their help and advice throughout my Masters degree:

My supervisors, Dr. Lizel Mostert and Dr. Francois Halleen for all their help, patience, guidance and extensive knowledge in the field of trunk diseases;

Members of the Department of Plant Pathology at the University of Stellenbosch for all their advice and friendship;

Staff at ARC- Nietvoorbji for their technical support with the isolations: Zane Sedeman, Linda Nel, Carine Vermeulen, Julia Marais and Addoration Shubane;

The many producers and viticulturists that allowed us access to their vineyards during sampling;

E. Abou-Monsour, Institute of Chemistry, University of Neuchâtel for the toxin standards;

Dr. Marietjie Stander for the toxin analyses;

ARC Infruitec-Nietvoorbij, Department of Plant Pathology, National Research Foundation and Winetech for funding this project;

My family and friends for all their love and support – especially my father, Lester;

My mother, Doreen, who will be sorely missed;

And the Lord who has given me the opportunity to make this all possible.

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CHAPTER ONE

AN OVERVIEW OF ESCA ON GRAPEVINES

ABSTRACT

Esca of grapevines is an economically important disease which takes years to develop. Economic losses occur due to a decrease in yield, wine quality and berry quality. Eventual replacements of vineyards as a result of esca of grapevines are then necessary. This disease, therefore, has great impact on the wine, table grape and raisin industries. The disease is known to affect many vineyards worldwide and has been studied extensively in Europe. However, esca in South Africa has not been researched. Esca is primarily caused by fungal species *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora* (both causing brown and black wood streaking) and white rot basidiomycete species such as *Fomitiporia mediterranea*, which cause wood rot in the trunks and arms of usually older grapevines. Other fungi such as *Eutypa lata, Phomopsis* spp. (mainly *Phomopsis viticola*) and species of the Botryosphaeriaceae have also been isolated from esca diseased vines, but their association with esca is not well understood. Esca is known to affect most grapevine cultivars, although some cultivars may be more susceptible than others. Foliar symptoms (known as 'tiger-stripes'), apoplexy and berry symptoms including shriveling, discoloration and 'black measles' are just a few of the symptoms associated with this disease. The external and internal symptoms are thought to be a result of toxin and enzyme production by the disease-causing fungi. Symptom expression is erratic and varies from year to year, making investigations into the identification of the fungi that secrete toxins and enzymes *in planta* difficult. Preventative measures such as sanitation and pruning wound protection contribute to the management of the disease. However, many questions still remain about the causal organisms, epidemiology and management of esca.

INTRODUCTION

Esca is one of the most destructive diseases affecting grapevine cultivars worldwide (Santos *et al*., 2006a). In Latin, 'esca' means bait, aliment or food and was used to refer to the basidiocarps of *Fomes fomentarius* L. (Fr.) and *Phellinus igniarius* (L.) Quél. that were used in earlier times as tinder for fires (Graniti, 2006). Esca-like symptoms have been associated with vines for centuries and have been reported in ancient Latin and Greek literature (Surico, 2000; Graniti, 2006). It was only in the late 19th century that esca was linked to the basidiomycete fungi, *Phellinus igniarius* and *Stereum hirsutum* (Willd.) Pers. (Ravaz, 1898, 1909; Viala, 1926).

Esca is caused by different fungi, which, according to some theories, infect the plant in succession in different parts of the woody tissue (i.e. in the trunks and arms) as the vine ages (Larignon and Dubos, 1987; Sparapano *et al*., 2000b; Calzarano and Di Marco, 2007). Graniti *et al*. (2000) and Surico (2009) have discussed the theory that esca exists as a complex of diseases or as a disease complex. Research has shown that esca can involve three possible scenarios: firstly, a 'disease complex', whereby a number of fungi and other factors interact and produce an overall syndrome; secondly, a 'complex of at least two diseases', which includes the brown wood streaking and the white rot and, lastly, a 'hardromycosis', which is caused by *Phaeoacremonium* species and *Phaeomoniella chlamydospora* (W. Gams, Crous & M.J. Wingf. & L. Mugnai) Crous & W. Gams, and when present in mature vines is worsened by white rotting *Fomitiporia* spp. Generally speaking, esca is considered to be a disease complex including two diseases, the brown wood streaking and the white rot.

Studies on esca and its aetiology intensified in the 1990's when the disease became more prominent in Germany, Italy and Greece (Mugnai *et al*., 1999; Fischer, 2006). Esca has been studied in various grapevine producing countries including Australia, France, Germany, Greece, Italy, Portugal, Spain and the United States of America (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Pascoe and Cottral, 2000; Armengol *et al*., 2001; Edwards *et al*., 2001b; Redondo *et al*., 2001; Rumbos and Rumbou, 2001; Fischer and Kassemeyer, 2003; Feliciano *et al*., 2004; Gubler *et al*., 2004; Sofia *et al*., 2006; Martin and Cobos, 2007). However, the status of esca and its

causal organisms has not been investigated in South Africa. Only a few esca symptomatic vines were observed prior to 1981 in Rawsonville and Slanghoek in the Western Cape (Marais, 1981). Here, external symptoms were observed on older vines. These included foliar symptoms resembling tiger-stripes, apoplexy, dieback and decline of the vines with internal symptoms showing a yellow rot surrounded by a black zone. Esca is thought to not be a problem in South Africa, possibly due to the lack of foliar symptoms.

In Europe, esca is generally widespread in older vineyards (Surico *et al*., 2006; Sánchez-Torres *et al*., 2008). Higher incidences were found in vineyards 10 years and older (Mugnai *et al*., 1999; Reisenzein *et al*., 2000; Surico, 2001; Romanazzi *et al*., 2009). Vineyards older than 20 years had the highest incidence (Stefanini *et al*., 2000; Péros *et al*., 2008). The higher incidence on older plants is due to the time that the white rot fungi require to infect and colonize the woody tissues of the vines (Sánchez-Torres *et al*., 2008). Esca can occur on vines younger than ten years; however, this is seldomly found in France (Péros *et al*., 2008). In California, young vines (two to six years) infected with esca fungi have been more frequently found (Gubler *et al*., 2004). The incidence on young vines in Italy is also becoming more frequent (Surico *et al*., 2004).

The economic impact of esca, the fungi associated with the disease and the external and internal symptoms will be discussed in this review. The symptom expression and variability, epidemiology, pathogenicity studies, toxins and enzymes produced by the esca fungi and the management of the disease will also be discussed.

ECONOMIC IMPACT

Grapevine and wine production is economically and historically one of the most important agricultural practices in many countries (Redondo *et al*., 2001; Sofia *et al*., 2006). Esca can directly affect the growth of vines and indirectly affect the berry and wine quantity and quality (Mugnai *et al*., 1999; Calzarano *et al*., 2001; Calzarano *et al*., 2004) which can cause considerable economic losses. There is also the cost of replacing diseased vineyards to consider, as the disease poses a threat to the longevity of the vineyards (Edwards *et al*., 2001b; Rumbos and Rumbou, 2001; Aroca *et al*., 2008).

Esca considerably affects grapevine physiology. It causes a decrease in net stomatal conductance, transpiration rates and photosynthetic rates, which in turn cause a reduction in carbohydrate production and therefore sugar production (Calzarano *et al.*, 2004; Petit *et al.*, 2006). Diseased vines are associated with water stress (Calzarano *et al.*, 2004), although nutrient uptake is not hindered by the disease (Calzarano *et al.*, 2009).

The reduced quality of berries can also negatively affect the table grape and wine industries. Infected table grapes can have spots (black measles) or may crack and therefore can not be sold (Chiarappa, 1959b; Mugnai *et al.*, 1999). Berries of infected vines ripen later, have an altered flavor (Mugnai *et al.*, 1999) and a low reducing sugar content due to decreased photosynthetic rates in vines with foliar symptoms, which leads to lower alcoholic content in wines (Calzarano *et al.*, 2001; Calzarano *et al.*, 2009). Wines made from esca diseased vines have a higher total acidity, possibly due to more lactic acid being produced by bacteria when there are residual sugars present for fermentation (Calzarano *et al.*, 2001; Calzarano *et al.*, 2004).

Esca in Italy has an annual incidence increase of approximately 4 to 5 % and is known to affect 90 to 100 % of vineyards (approximately 15 to 25 years old) in many areas of this country (Mugnai *et al.*, 1999). In Spain, grapevine decline increased by 7 % in just six years (Martin and Cobos, 2007). The frequency of esca in Austria has increased from 1.3 % in 1994 to 2.7 % in 2000 (Reisenzein *et al.*, 2000). However, the frequency of esca in Austria ranged from 2 to 20 % in some vineyards and was correlated with vine age (Reisenzein *et al.*, 2000).

Fomitiporia mediterranea M. Fischer has a relative high optimal growth at 30°C (Fischer, 2002). It has been suggested that the species is also adapted to dry conditions (Sánchez-Torres *et al.*, 2008). The increase in temperatures due to climate change could influence the incidence of esca in vineyards where higher temperatures occur more than usual. The incidence could also increase due to the number of aging vines in the grape growing regions.

FUNGI ASSOCIATED WITH ESCA

Brown wood streaking, a decline in young vines caused by Petri disease fungi, *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & L. Mugnai) Crous & W. Gams and *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai can develop into young esca. A combination of these two species with white rot (caused by basidiomycete spp. such as *Fomitiporia mediterranea*) will form esca proper which can start in the nursery and can progress until the plant is mature (Mugnai *et al*., 1999; Surico, 2001).

Five stages of esca development have been defined in relation to the grapevines' age and origin of infection (Mugnai *et al*., 1999; Graniti *et al*., 2000; Surico, 2001; Surico *et al*., 2006; Surico, 2009). Firstly, brown-wood streaking occurs with no external symptoms. Secondly, Petri grapevine decline occurs, which is a decline of young vines. Thirdly, young esca occurs which also involves *Phaeoacremonium* spp. infections and is usually found on vines between eight and ten years old. Black/ brown wood streaking, xylem darkening and vascular gummosis occur in the wood and foliar symptoms may, or may not, be present. Vines can also wilt, develop dieback or die altogether. The fourth stage is white rot, which is mostly found in older vines and is characterized by a white, spongy wood rot and leaf and berry symptoms may or may not be visible. The last stage is esca proper, in which the brown wood streaking occurs at the same time or prior to white rot. The sequence of stages can start in nurseries, then persist in young vineyards and progress to esca proper in mature vines (Mugnai *et al*., 1999; Graniti *et al*., 2000; Surico, 2001; Surico *et al*., 2006; Surico, 2009).

Apart from *F. mediterranea*, *Ph. chlamydospora* and *P. aleophilum,* there are various other trunk disease-causing fungi which have also been isolated from esca diseased vines. These include *Eutypa lata* Tul. & C. Tul (Mugnai *et al*., 1999), Botryosphaeriaceae species such as *Diplodia seriata* De Not. (*Botryosphaeria obtusa* (Schwein.) Shoemaker) (Mugnai *et al*., 1999; Calzarano and Di Marco, 2007) and *Phomopsis viticola* (Sacc.) Sacc. (Mostert *et al*., 2001; van Niekerk *et al*., 2005). The roles of these fungi in esca still need to be determined, as well as the interactions with the other fungi in the complex (Péros *et al*., 2008).

The combination of fungi associated with esca diseased vines varies among countries. The fungi associated, as well as the frequency at which esca occurs, are similar in Italy, France and Spain and these species include *P. aleophilum, Ph. chlamydospora, Phomopsis viticola, F. mediterranea*, species within the Botryosphaeriaceae (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Serra *et al*., 2000; Péros *et al*., 2008; Sánchez-Torres *et al*., 2008) and *E. lata* (Serra *et al*., 2000; Péros *et al*., 2008). The same species were found in Germany and Greece, as well as species of *Phomopsis* and *Cylindrocarpon* (Rumbos and Rumbou, 2001; Fischer and Kassemeyer, 2003). In a study in Castilla y León, Spain, *F. mediterranea, Stereum hirsutum* and *Phomopsis viticola* were found on a few occasions (Martin and Cobos, 2007), but in another study in Spain, in the Comunidad Valenciana, *F. mediterranea* and *S. hirsutum* were found to be the most frequently isolated species (Sánchez-Torres *et al*., 2008).

Basidiomycetes

Grapevines are susceptible to white rot fungi, which decompose lignin and polysaccharides (Fischer and Kassemeyer, 2003). Basidiomycetes are generally seen as less virulent pathogens and have therefore elicited little interest from researchers (Fischer, 2006). As a result, data pertaining to the diversity and distribution of basidiomycetes in different countries is limited (Fischer, 2002; Fischer *et al*., 2005; Fischer, 2006).

The fruiting bodies or basidiocarps on esca diseased vines were first identified in 1898 as *Phellinus igniarius* [known as *Fomes igniarius* (L. ex Fr.) Kickx.] (Ravaz, 1909). Another basidiomycete, *Stereum hirsutum*, was also found on grapevines early in the 20th century (Vinet, 1909; Viala, 1926). In a more recent study in France, *Stereum hirsutum* was found at low frequencies and *Phellinus punctatus* (P. Karst) Pilát was found more often (Larignon and Dubos, 1997). Upon taxonomic investigation, *Phellinus punctatus* was renamed *Fomitiporia punctata* (Fr.) Murrill (Fischer, 1996). Further characterization work showed that isolates of *Fomitiporia punctata* from grapevine are a new species, namely *Fomitiporia mediterranea* (Fischer, 2002). Even though the fruiting bodies of *F. punctata* and *F. mediterranea* are very similar, these fungi can be distinguished from each other with the ribosomal ITS region (Fischer, 2002). Previous identifications of

Fomitiporia punctata on grapevines proved to be incorrect and this species host range include *Salix* sp., *Sorbus* sp. and *Acer* sp. (Fischer and Binder, 2004).

The different basidiomycete species seem restricted to a specific area or continent. *Fomitiporia mediterranea* is the basidiomycete species associated with esca of grapevines in Europe and is found predominantly in the upper parts of grapevine trunks in white rotted wood (Fischer and Kassemeyer, 2003). Other basidiomycete fungi associated with esca include, *F. polymorpha* M. Fischer (North America), *F. australiensis* M. Fisch., J. Edwards, Cunnington & Pascoe, two unknown species from Australia, as well as three unknown species from South Africa (Fischer, 2006). 'Chlorotic leaf roll', which is similar to esca, is associated with *Fomitiporella vitis* Auger, Aguilera & Esterio (Chile) (Auger *et al*., 2005). The fungus associated with 'hoja de malvón', a disease with similar internal symptoms to esca, has been identified as *Inocutis jamaicensis* (Murrill) Gottlieb, J.E. Wright & Moncalvo and is suspected to be the main causal agent of this disease in Argentina (Lupo *et al*., 2006). Esca is known as Kav in Turkey, with similar symptoms to those observed in other European countries (Ari, 2000; Köklü, 2000). *Stereum hirsutum* and an unidentified *Phellinus* sp. are associated with these symptoms (Ari, 2000). Other lesser known and less common lignicolous fungi that can cause white rot on grapevines include *Armillaria mellea* (Vahl.: Fr.) Kumm., *Clitopilus hobsonii* (Berk. & Broome) P.D. Orton, *Flammulina velutipes* (M.A. Curt.: Fr.), *Pleurotus pulmonarius* (Fr.) Quél., *Inonotus hispidus* (Bull.: Fr.) P. Karst., *Trametes hirsuta* (Wulfen) Lloyd, *Trametes versicolor* (L.: Fr.) Quél., *Peniophora incarnate* (Pers.) P. Karst. and *Hirneola auricula-judae* Berk. (Fischer, 2000; Fischer and Kassemeyer, 2003).

Sánchez-Torres *et al*. (2008) stated that *S. hirsutum* could be a weak facultative parasite which also favours dry conditions and is found in the external layer of wood. This fungus can occasionally cause limited wood decay (Sánchez-Torres *et al*., 2008). However, in pathogenicity studies by Larignon and Dubos (1997), this fungus produced symptoms in young vines which were similar in intensity to those in esca-diseased vines i.e. soft-textured light-coloured necrosis. The low frequency of isolation and the limited distribution of *S. hirsutum* make the role of this fungus in esca unclear.

The number of basidiocarps found in a vineyard in relation to basidiomycetes isolated from internal symptoms is low. A ratio of approximately 100:1 of mycelium to

basidiocarps of *F. mediterranea* was predicted to occur in vineyards in Germany (Fischer, 2006). Basidiocarps of *F. australiensis* were rarely found and this could be due to the removal of dead vines on which they often occur (Fischer *et al*., 2005). Basidiocarps have also been found on other hardwood hosts outside the vineyard in the Mediterranean (Fischer, 2002; Fischer and Kassemeyer, 2003; Fischer, 2006). Basidiocarps of *S. hirsutum, Trametes hirsuta* and *Trametes versicolor* have been found in vineyards and some on wooden stakes, indicating these to be sources of inoculum (Reisenzein *et al*., 2000).

If fruiting bodies do develop, they are difficult to find and often in a poor state, making identification difficult (Fischer, 2006). Morphological features that can be used to distinguish among the different genera occurring on grapevines are summarized in Table 1. Using only morphological characteristics to identify the genera *Phellinus* and *Fomitiporia* can be difficult as they are very similar, but phylogenetic species recognition using the ITS region can be used to resolve the genus status of isolates (Fischer and Binder, 2004; Sánchez-Torres *et al*., 2008).

Molecular techniques that distinguish different species of *Fomitiporia* include sequencing of the ITS region with primers ITS4 and ITS5 (Fischer, 2002; Ciccarone *et al*., 2004; Pilotti *et al*., 2005) and restriction fragment length polymorphism (RFLP) of the ITS region (Cortesi *et al*., 2000; Pilotti *et al*., 2005). Species-specific primers have been developed for *F. mediterranea* (Fischer, 2006) as well as sequence-characterized amplified region (SCAR) primers (Pollastro *et al*., 2001).

The genetic variation among isolates of *F. punctata* has been investigated with random amplified polymorphic DNA (RAPD) and variations were found in a single vineyard and between vineyards (Pollastro *et al*., 2000a; Jamaux-Despréaux and Péros, 2003). Variation within species may be related to the geographic location of the isolates (Jamaux-Despréaux and Péros, 2003; Lupo *et al*., 2006). It has been suggested that *F. mediterranea* is spread via airborne basidiospores and that outcrossing occurs (Jamaux-Despréaux and Péros, 2003). Studies indicated that *F. mediterranea* reproduces sexually and therefore basidiospores are a source of inoculum (Cortesi *et al*., 2000). Heterothallism has been known to occur in certain *Phellinus* species (Fischer, 1996).

Of the different basidiomycete species occurring on grapevines, *F. mediterranea* has the widest host range (Table 2). *Fomitiporia mediterranea* is known to infect citrus trees (oranges, lemon and mandarin) in Greece causing decline and development of internal symptoms starting from the pruning wounds (Elena *et al*., 2006). Di Marco *et al*. (2004a) found that *F. mediterranea* on kiwifruit trees caused a similar disease to that found in grapevine with regards to the pathogens, the type of disease development and the symptom types within the wood. Species of *Inonotus* are also known to cause white rot in deciduous trees (Germain *et al*., 2002).

Phaeomoniella chlamydospora

Phaeomoniella chlamydospora is associated with black/ brown wood streaking (Pascoe and Cottral, 2000; Serra *et al*., 2000; Fischer and Kassemeyer, 2003). Together with *P. aleophilum*, it is involved in Petri disease and with *F. mediterranea*, this fungus is one of the three primary agents in esca (Fischer and Kassemeyer, 2003). It has been assumed that *Ph. chlamydospora* alone causes esca symptoms, as it was the only esca-associated fungus isolated from two to seven year old vines which showed symptoms in Australian vineyards (Edwards *et al*., 2001b).

This fungus enters the vine through wounds and spreads throughout the vine through the vessels followed by the accumulation of black deposits, but these are not continuous throughout the vine (Pascoe and Cottral, 2000). Potential sources of the fungus in newly planted vines include the rootstock and scion (Pascoe and Cottral, 2000; Edwards *et al*., 2001b; Halleen *et al*., 2003). Therefore infection can either occur in the nursery or after planting in the field (Pascoe and Cottral, 2000; Fourie and Halleen, 2004). A decline in the vine then occurs and productivity may be lost with a decrease in fruit production in preceding years (Pascoe and Cottral, 2000).

Phaeomoniella chlamydospora can be detected using molecular methods. Species-specific primers for *Ph. chlamydospora* have been developed from the ITS1 and ITS2 regions (Groenewald *et al*., 2000; Tegli *et al*., 2000). Retief *et al*. (2005) used species-specific primers to detect *Ph. chlamydospora*, but could not distinguish between live and dead fungal matter. Andolfi *et al*. (2009) developed antibodies that can detect exopolysaccharides secreted by *Ph. chlamydospora* and can be used to detect early

infections of this fungus. SCAR primers have also been developed for this fungus from a unique band from random amplified polymorphic DNA (RAPD) (Pollastro *et al*., 2001).

Colonies of *Ph. chlamydospora* appear green, and sometimes black pycnidia are formed on sporulating mycelium (Pascoe and Cottral, 2000). The conidiophores are thick-walled and pigmented and the phialides and hyphae are hyaline (Crous and Gams, 2000). The sexual stages of *Phaeomoniella* are unknown and low genetic variation found with RAPD and AFLP analyses confirms the absence of a teleomorph (Pollastro *et al*., 2001; Mostert *et al*., 2006a).

***Phaeoacremonium* spp.**

Twenty-five species of *Phaeoacremonium* have been isolated from either Petri or esca diseased grapevines (Crous *et al*., 1996; Mostert *et al*., 2005; Essakhi *et al*., 2008; Graham *et al*., 2009; Gramaje *et al*., 2009). Of these, the most predominant species found on grapevines is *P. aleophilum* (Larignon and Dubos 1997, Mugnai *et al*., 1999). *Phaeoacremonium parasiticum* (Ajello, Georg et C.J.K. Wang) W. Gams, Crous et M.J. Wingf. is commonly found on grapevines (Dupont *et al*. 2002; Mostert *et al*. 2006c).

Species-specific primers that amplify the ITS1 and ITS2 regions have been developed to detect *P. aleophilum* (Tegli *et al*., 2000). For 22 species of *Phaeoacremonium*, species-specific primers were developed from the β-tubulin and actin genes (Mostert *et al*., 2005). Aroca *et al*. (2007) developed a nested PCR that could detect seven *Phaeoacremonium* species in grapevine wood. Degenerate primers were developed to amplify a region of the β-tubulin gene of 11 *Phaeoacremonium* species (Aroca *et al*., 2008). They further developed species-specific probes to detect the four species occurring in Spain with real-time PCR using TaqMan®. This technique was used to detect naturally infected grapevine material and could therefore be used to test propagated nursery material (Aroca *et al*., 2008). The tool is being assessed to determine whether it can be used to detect *Phaeoacremonium* species in water and soil samples (Aroca *et al*., 2008).

The sexual stage of *Phaeoacremonium* was confirmed as *Togninia* (Mostert *et al*., 2003). The *Togninia* state has been found *in vitro* for several of the *Phaeoacremonium* species occurring on grapevines and include *Togninia austroafricana* L. Mostert, W. Gams & Crous (anamorph *P. austroafricanum* L. Mostert, W. Gams & Crous), *T.*

krajdenii L. Mostert, W. Gams & Crous (anamorph *P. kradjdenii* L. Mostert, Summerb. & Crous), *T. minima* (Tul. & Tul.) Berl. (anamorph *P. aleophilum*), *T. parasitica* L. Mostert, W. Gams & Crous (anamorph *P. parasiticum*), *T. viticola* L. Mostert, W. Gams & Crous (anamorph *P. viticola* J. Dupont) and *T. fraxinopennsylvanica* (Hinds) Hausner, Eyjolfsdorttir & J. Reid (anamorph *P. mortoniae* Crous & W. Gams) (Mostert *et al*., 2006b). The perithecia of *Togninia minima*, *T. fraxinopennsylvanica* and *T. viticola* have been found on grapevines in California (Eskalen *et al*., 2005a, b; Rooney-Latham *et al*., 2005b). These perithecia were found on dead vascular tissue on the surfaces of pruning wounds, as well as inside cracks on grapevine trunks and cordons. Apart from conidia, ascospores can be an additional source of inoculum (Rooney-Latham *et al*., 2005b).

Togninia minima is a heterothallic fungus (Mostert *et al*., 2003; Rooney-Latham *et al*., 2005a). Several genotypes of *P. aleophilum* can be found in a single vineyard (Borie *et al*., 2002). This indicates that the sexual stage of this fungus contributes to genetic diversity found in the field.

Phaeoacremonium species have been isolated from a large diversity of woody plants, but have also been found on larvae of bark beetles, as well as on humans (Crous *et al*., 1996; Mostert *et al*., 2006c, Aroca *et al*., 2008). Human infections are due to the opportunistic nature of *Phaeoacremonium*. Species that occur on grapevines, as well as humans, include *P. krajdenii*, *P. griseorubrum*, *P. parasiticum, P. rubrigenum,* and *P. venezuelense* (Mostert *et al*., 2005). *Phaeoacremonium* spp. can infect kiwifruit and cause similar symptoms as in grapevines (Di Marco *et al*., 2004a).

Phomopsis spp.

Fifteen species of *Phomopsis* have been reported to occur on grapevines (van Niekerk *et al*., 2005). Of these, *Phomopsis viticola*, was the most predominant species isolated. *Phomopsis viticola* is normally associated with *Phomopsis* cane and leaf spot, as well as black dead arm disease (Fischer and Kassemeyer, 2003). It has also been isolated from pruning wound stubs (Fourie and Halleen, 2004), shoots of esca diseased plants and from internal wood decay symptoms (Fischer and Kassemeyer, 2003; van Niekerk *et al*., 2005; Martin and Cobos, 2007; Sánchez-Torres *et al*., 2008). *Phomopsis viticola* caused the

most severe lesions in pathogenicity studies and is thought to be the most pathogenic *Phomopsis* species in South Africa (van Niekerk *et al*., 2005).

Identification according to morphological and cultural characteristics can be difficult (van Niekerk *et al*., 2005). Species identifications have been done with ITS phylogenies (Mostert *et al*., 2001; van Niekerk *et al*., 2005). SCAR primers have been developed for *Phomopsis viticola* and can be used to identify this species from infected wood (Pollastro *et al*., 2001).

Phomopsis species are generally not host specific and have wide host ranges (van Niekerk *et al*., 2005). *Phomopsis viticola*, however, has only been found on grapevines. *Phomopsis amygdali* (Del.) Tuset & Portilla is a severe pathogen of peaches and almonds (Farr *et al*., 1999). It has only twice been found on nursery grapevines in South Africa (Mostert *et al*., 2001; van Niekerk *et al*., 2005).

Eutypa lata

Eutypa lata is the causal agent of Eutypa dieback of grapevines (Carter and Price, 1973; Moller and Kasimatis, 1981; Munkvold *et al*., 1994). Eutypa dieback is an economically important disease and caused a loss in net income of over $260 million in 1999 in Californian wine grapes (Gubler *et al*., 2005). The disease caused a crop loss of 365 tons in Cabernet sauvignon blocks in Stellenbosch (South Africa) alone equating to a loss of R1.7 million in that season (Halleen *et al*., 2001; van Niekerk *et al*., 2003b). *Eutypa lata* enters the vine through pruning wounds (Moller and Kasimatis, 1978) and colonizes the wood with eventual necrosis and death of the area (Moller and Kasimatis, 1981). The disease will progress over a number of years and grape yields will start to decrease (Moller and Kasimatis, 1981; Munkvold *et al*., 1994).

The foliar symptoms are caused by the production of enzymes and toxins (such as eutypine) and are translocated from the wood to the foliar parts (Schmidt *et* al., 1999; Mauro *et al*., 1988; Tey-Rulh *et al*., 1991). Therefore, the point of infection can be a distance away from where the symptoms are seen (Moller and Kasimatis; 1981). Foliar symptoms include leaves which appear small, cupped, chlorotic or torn; stunting of new shoots; drying of inflorescences and poor fruit development. Characteristic V-shaped

necrosis can be seen within the trunk and arms of the infected grapevine (Mauro *et al*., 1988; Tey-Rulh *et al*., 1991).

In the southern vine growing regions in Europe, including Italy and Spain, *E. lata* is rarely found to be associated with esca diseased vines (Mugnai *et al*., 1999; Armengol *et al*., 2001). However, it has been isolated from esca diseased vines in France, Greece and Germany (Larignon and Dubos, 1997; Rumbos and Rumbou, 2001; Fischer and Kassemeyer, 2003; Péros *et al*, 2008). Larignon and Dubos (1997) regarded it as having a pioneer role in wood colonization since it was the main fungus isolated from sectorial brown necrosis and the zones adjoining decayed wood.

Eutypa lata spreads in vineyards via ascospores, which are released from perithecia and dispersed via rain and wind (Péros *et al*., 1997, Péros *et al*., 1999; Cortesi and Milgroom, 2001). *Eutypa lata* has a wide host range occurring on more than 80 woody host species (Bolay and Carter, 1985; Carter, 1986; Rolshausen *et al*., 2006).

Botryosphaeriaceae

Species within the family Botryosphaeriaceae are ubiquitous on grapevines and also cause black dead arm disease (Larignon and Dubos, 2001; Larignon *et al*., 2001; Surico *et al*., 2006). The awareness of trunk diseases caused by the Botryosphaeriaceae is increasing (van Niekerk *et al*., 2006). Symptoms include dieback, cankers, (Fischer and Kassemeyer, 2003; Úrbez-Torres *et al*., 2006; van Niekerk *et al*., 2006; Pitt *et al*., 2008), vascular streaking within the diseased vines (Úrbez-Torres *et al*., 2006; van Niekerk *et al*., 2006), bud mortality (resulting in reduced yields) (van Niekerk *et al*., 2006) and mild chlorosis in the leaves and these can be confused with esca (Fischer and Kassemeyer, 2003; van Niekerk *et al*., 2006). Information on the epidemiology of these pathogens is limited (Úrbez-Torres *et al*., 2006; van Niekerk *et al*., 2006).

Twelve Botryosphaeriaceae species have been isolated from grapevines in South Africa (van Niekerk *et al*., 2004; van Niekerk *et al*., 2006; van Niekerk *et al*., 2010). Of these, *Diplodia seriata* (previously *Botryosphaeria obtusa*), *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips [previously *Botryosphaeria parva* Pennycook & Samuels (Crous *et al*., 2006)] and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (previously *B. rhodina* (Berk. & M.A. Curtis) Arx) are the most

common (van Niekerk *et al*., 2003a; van Niekerk *et al*., 2004). *Neofussiccocum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips [previously *Botryosphaeria australis* Slippers, Crous & M.J. Wingf. (Crous *et al*., 2006)] is also commonly found and were shown to be the most pathogenic species on South African grapevines in pathogenicity studies using green shoots, mature canes and mature wood (van Niekerk *et al*., 2004). *Botryosphaeria dothidea* (Moug.) Ces. & De Not., *B. lutea* A.J.L. Phillips, *Diplodia corticola* A. J. L. Phillips, Alves & Luque, *Dothiorella iberica* A.J.L. Phillips, J. Luque & A. Alves (previously *B. iberica* A.J.L. Phillips, J. Luque & A. Alves), *Diplodia mutila* (Fr.) Mont. (previously *Botryosphaeria stevensii* Shoemaker), *Diplodia seriata, Dothiorella viticola* A.J.L. Phillips & J. Luque (previously *B. viticola* A.J.L. Phillips & J. Luque), *Fusicoccum aesculi* Sacc., *Lasiodiplodia theobromae, Lasiodiplodia crassispora* (Burgess & Barber), *N. australe, Neofusicoccum mediterraneum* (Crous, M.J. Wingf. & A.J.L. Phillips) and *N. parvum* are associated with grapevines in Australia, Portugal, Spain, South Africa and USA (California) (Phillips, 2002; Úrbez-Torres *et al*., 2006; Pitt *et al*., 2008; Sánchez-Torres *et al*., 2008; Úrbez-Torres *et al*., 2010a,b; van Niekerk *et al*., 2010). The variability in the virulence of the different species can be influenced by cultivar susceptibility, environmental conditions, the stages of host phenological development and the host tissue (van Niekerk *et al*., 2004).

In earlier research, the anamorphic characters were not clearly defined and so a number of names have been given to similar fungi (Phillips, 2002; van Niekerk *et al*., 2006). Therefore, the same species are known to cause many *Botryosphaeriaceae*-associated grapevine diseases, making their identification difficult (van Niekerk *et al*., 2006). Teleomorphs of *Botryosphaeriaceae* are not common in nature and there is also a low diversity of teleomorphs, which makes features unclear and difficult to identify to species level (Phillips, 2002). Anamorphic characters can be used to identify species of the Botryosphaeriaceae (Phillips, 2002). Pure cultures should therefore be used and the conditions and medium composition must be carefully controlled as the conidial characteristics vary in culture (Phillips, 2002). Mycelial pigment formation can vary as cultures age, for example, *B. dothidea* cultures on PDA appear white and eventually turn to grey then dark-grey and eventually black (Qui *et al*., 2008).

ITS, β-tubulin and the elongation translation factor-1α are used in phylogenetic analyses to identify species of the Botryosphaeriaceae (Úrbez-Torres *et al*., 2006; Qui *et al*., 2008; Úrbez-Torres *et al*., 2008). Restriction patterns of the ITS and 26S rRNA gene with the enzyme TaqI can also assist in identification (Alves *et al*., 2005). Identification of *D. seriata*, *D. mutila, B. dothidea* and *N. parvum* with molecular methods is more efficient than morphological features since the formation of pycnidia in these species take longer (Martin and Cobos, 2007). Intra-specific variation can also be found due to different hosts and geographical areas (Úrbez-Torres *et al*., 2006).

THE SYMPTOMS OF ESCA

External symptoms

External symptoms of esca such as general decline, leaf and berry symptoms, and apoplectic strokes can be confused with other grapevine diseases such as black dead arm (Surico, 2001; Surico *et al*., 2006; Sánchez-Torres *et al*., 2008) and *Phomopsis* cane and leaf spot (Sánchez-Torres *et al*., 2008). Esca proper, or chronic esca can, however, be distinguished by the presence of internal symptoms (including white rot) and external symptoms (Mugnai *et al*., 1999; Surico, 2001).

External symptoms can start in spring (Mugnai *et al*., 1999), generally when vines are between flowering and veraison (Edwards *et al*., 2001b) and can occur on the whole vine or on single branches (Mugnai *et al*., 1999). Weak or delayed growth can be seen if symptoms start in spring, but if symptoms start in late spring and summer, then shoots or branches may wilt (Mugnai *et al*., 1999). The bark on trunks and branches of infected vines can also crack and split (Bruno *et al*., 2007). In Spain, summer symptoms involve foliar symptoms, weak growth and short branches (Redondo *et al*., 2001). Typical winter symptoms include necrosis of the trunk and in pruning wounds, as well as basidiocarps of *F. punctata, S. hirsutum* and *Trametes versicolor* found on the main branches (Redondo *et al*., 2001).

Foliar symptoms do not occur early in the growing season, but become visible in summer and autumn (Mugnai *et al*., 1999). These symptoms include light green/ chlorotic rounded/ irregular spots, which occur between the veins and margins of the leaves

(Mugnai *et al*., 1999; Sparapano *et al*., 2001b; Bruno and Sparapano, 2006b), which eventually coalesce, leaving a green area along the veins or in uninfected areas (Surico *et al*., 2006). The coalesced areas can become necrotic (and can sometimes appear red in some cultivars and therefore the typical 'tiger stripe' symptom is formed (Mugnai *et al*., 1999; Sparapano *et al*., 2001b; Bruno and Sparapano, 2006b). Distortion of the lamina (Sparapano *et al*., 2001b) and wilt-like symptoms in the leaves can be due to xylem dysfunction within a diseased vine (Mugnai *et al*., 1999). Species of the Botryosphaeriaceae can also cause foliar symptoms on grapevines, which are similar to that of esca. However, the foliar symptoms are darker red in colour (Surico *et al*., 2006). Although not common, foliar symptoms can sometimes be seen on young vines (Mugnai *et al*., 1999; Surico, 2001). Increased infection in younger vines could be due to an increase of field inoculum, vines planted closer to each other and the use of machinery for harvesting and pruning (Surico *et al*., 2004).

Berry symptoms consist of diminutive spots which are dark brown, violet or purple and turn brown or violet-grey and begin to crack if the berry contains many spots, but this is rare (Mugnai *et al*., 1999; Ari, 2000; Bruno *et al*., 2007). The spots give the appearance which is known as 'black measles' which is common in California, and less frequently in southern Italy and France (Chiarappa, 1959b; Gubler *et al*., 2004). The spots are scattered, or in rows, on the berries towards the distal end (Mugnai *et al*., 1999; Bruno *et al*., 2007) and affected berries may be on a cluster which can be on a single branch or many branches and will eventually shrivel, wilt and rot (Bruno *et al*., 2007), or show reduced turgor (Reisenzein *et al*., 2000). Diseased red berries not showing spots appear violet in colour (Reisenzein *et al*., 2000). These symptoms may, or may not, be associated with foliar symptoms (Mugnai *et al*., 1999) and may vary from year to year (Bruno *et al*., 2007). Berry symptoms which are not accompanied by foliar symptoms are common in young vineyards (Pollastro *et al*., 2000b).

Apoplexy is the sudden wilt and collapse of the vine in summer where foliar symptoms are observed (Mugnai *et al*., 1999; Köklü, 2000; Bruno *et al*., 2007). This is another syndrome (the acute version of esca) which, in a European context, is favoured by hot summers especially when rainfall is followed by hot, dry weather (Viala, 1926; Mugnai *et al*., 1999; Bruno *et al*., 2007) and is mainly restricted to older vines (Surico *et

al., 2006). If it occurs in younger vines, then the reasons for this are not related to esca (Pollastro *et al*., 2000b; Surico *et al*., 2006). Here, the leaves wither and turn pale to grey green, eventually drying out entirely (Mugnai *et al*., 1999). Sudden wilting of the vine is possibly caused by the spongy decay caused by *Fomitiporia* (Serra *et al*., 2000) and can be related to improper xylem conductivity when toxin concentrations increase rapidly and if the transpiration rate is high (Bruno *et al.,* 2007). This may show that the cause of the foliar symptoms is different to those of apoplexy (which is probably due to water stress) (Surico *et al*., 2006).

Esca-like symptoms have also been observed in Argentina where it is called 'hoja de malvón' (Gatica *et al*., 2000; Gatica *et al*., 2004). In Chile, 'chlorotic leaf roll' is a disease similar to 'hoja de malvón' (Auger *et al*., 2005) and is different from esca, regarding the basidiomycete species involved, and the leaf symptoms (Fischer, 2006). Leaves appear chlorotic and curl downwards and also appear smaller than normal (Gatica *et al*., 2000; Gatica *et al*., 2004).

Internal wood symptoms

Symptom types in the wood occur due to physiological, as well as structural, changes within the plant, which include the host reactions to wounding (i.e. degradation and oxidation) and formation of tyloses due to growth-regulating substances and gums (Mugnai *et al*., 1999). Hyphae of tracheiphilous *Phaeoacremonium* species and *Ph. chlamydospora* are able to grow in the xylem and parenchyma cells and as a result of host-pathogen interactions, gummosis, wood discoloration and streaking occurs (Mugnai *et al*. 1999; Sparapano *et al*., 2000c; Sparapano *et al*., 2001b).

Up to seven different symptom types have been described (Serra *et al*., 2000). Even though symptom categories have been developed, the internal symptoms that are commonly found include brown wood streaking, black spotting, brown necrosis that can be sectorial, white rot (which may, or may not, be bordered by a black line) and brown margins of decayed wood (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Pollastro *et al*., 2000b; Serra *et al*., 2000; Sofia *et al*., 2006; Calzarano and Di Marco, 2007; Péros *et al*., 2008). Larignon and Dubos (1997) also identified a hard, pinkish brown margin/

necrosis. The white rot also appeared yellowish at times (Sánchez-Torres *et al*., 2008). The most common symptoms with the associated fungi isolated are listed in Table 3.

The symptom types of 'hoja de malvón' that have been observed are a central, soft, yellow necrosis which is bordered by a dark margin and a hard, light brown region (Gatica *et al*., 2000). *Phellinus* (later identified as *Inocutis jamaciensis*) was isolated from the soft white rot regions and, to a lesser extent, from sectorial brown areas and black lines bordering the decayed areas (Gatica *et al*., 2000; Gatica *et al*., 2004). Species of the Botryosphaeriaceae were frequently isolated from the margins of the hard brown zones (Gatica *et al*., 2000). Other fungi such as *Ph. chlamydospora* and *Phaeoacremonium* species were also associated with this fungus and isolated from the dark margins (Gatica *et al*., 2000; Gatica *et al*., 2004).

Some authors believe a succession of infections by fungi is necessary for wood decay to occur (Mugnai *et al*., 1996; Larignon and Dubos 1997). Calzarano and Di Marco (2007) and Serra *et al*. (2000) speculated that white wood rot development is favoured by a pre-existing necrosis (discolouration) caused by the colonization of other fungi found in the vine and, therefore, occurs in succession. However, Chiarappa (1997) and Sparapano *et al*. (2001b) found that *F. punctata* (*F. mediterranea*) does not require prior colonization of the other esca fungi to be able to infect the vine.

SYMPTOM EXPRESSION AND VARIABILITY

The diverse assortment and combination of fungi, the host and the environment can cause a variety of symptoms in the wood due to large number of interactions which are not yet understood. These different interactions can cause the discontinuity of symptom expression and influences the development of esca (Stefanini *et al*., 2000; Calzarano and Di Marco, 2007). Not only does the presence of the fungi contribute to external symptoms, but also vine age, propagation material, pruning, protection of wounds, climate, soil, irrigation, the state of the vine, soil type, slope of the land and the cultivar (Mugnai *et al*., 1999; Surico *et al*., 2000a; Surico *et al*., 2004). Vine training, which entails ongoing pruning, can also favour the development of esca (Mugnai *et al*., 1999; Surico *et al*., 2004). Factors such as rootstock characteristics, chemicals used for control,

topography, spacing, exposure and vigor have not yet shown to be significant (Surico *et al.*, 2004).

There is a discontinuity between foliar symptoms from year to year and the reasons why this occurs is still unclear (Mugnai *et al.*, 1999; Surico *et al.*, 2000a, b; Redondo *et al.*, 2001; Surico *et al.*, 2006). It is thought to be a result of the toxins that the white-rot basidiomycetes found in the wood produce (Surico, 2001). Sofia *et al.* (2006) found an increase in symptomatic vines over three years, possibly due to a higher contamination rate, or favorable environmental conditions.

The actual incidence of esca in a given vineyard is therefore difficult to quantify without continual observation over a number of years (Péros *et al.*, 2008; Quaglia *et al.*, 2009). Stefanini *et al.* (2000) proposed a statistical longitudinal model of symptom expression of esca which takes into consideration the probability of showing symptoms due to factors such as the presence of symptoms in the previous year and the proximity to diseased plants.

The correlation between foliar symptoms and causal fungi is difficult to determine. Foliar symptoms are not specific to the fungus internally and are probably determined by a combination of environmental parameters, the fungi found in the wood, the combination of metabolites present (and their method of action) and the physiology of the plant (Mugnai *et al.*, 1999; Surico, 2001; Péros *et al.*, 2008). *Phaeoacremonium* and *Phaeomoniella* caused black stripes in the wood and the vines showed foliar symptoms without the presence of white rot (Surico, 2001; Calzarano and Di Marco, 2007; Péros *et al.*, 2008). It is speculated that these species can then cause foliar symptoms without the basidiomycetes (Surico, 2001; Péros *et al.*, 2008).

Calzarano *et al.* (2009) found that the inception of esca symptoms was influenced by the concentration of mineral nutrients. Higher amounts in the growing season allowed for an increase of symptomatic vines as fungal virulence increased. However, levels of different minerals in the leaves and their composition in the berries did not differ significantly between asymptomatic and healthy vines.

Hyphae of *Ph. chlamydospora* infect cells intracellularly by spreading into the parenchyma and pith cells, where these cells start to produce tyloses and brown deposits (Pascoe and Cottral, 2000; Surico *et al.*, 2006). Vines which have blocked vessels could

obstruct water transport (Christen *et al*., 2007; Del Río *et al*., 2004) and change physiological processes and therefore show foliar symptoms (Péros *et al*., 2008). Other physiological occurrences can also assist in the obstruction of the vessels, such as defense reactions or enzyme/ toxin production (Christen *et al*., 2007).

Symptom expression and climate

Climate change can influence the spread of esca as rainfall in certain areas fluctuates (Surico *et al*., 2006). However, in Italy Surico *et al*. (2000a) found that a wet summer favoured the development of chronic esca and hot, dry summers favoured the acute form of esca. The acute esca can kill the vine in months whereas the chronic esca can take several years (Fischer and Kassemeyer, 2003). Hot, dry summers lead to drought stress and so changes in temperature could play a part in the development of apoplexy (Surico *et al*., 2000a).

Marchi *et al*. (2006) state that manifest (external symptoms present) and hidden (asymptomatic in some years) esca varies from year to year, depending on rainfall patterns in summer. Cool growing seasons favour visual symptoms and so reducing hidden esca. The drier seasons increase the level of hidden esca. The role of rainfall in the expression of esca symptoms is uncertain, but it is hypothesized that in wetter years there is a greater flow of phytotoxins to the leaves due to a constant water supply (Marchi *et al*., 2006). Mugnai *et al*. (1999), however, stated that disease development is not affected by the amount of water in the soil.

Symptom expression and metabolite production

Toxins and various metabolites produced by fungi act synergistically and cause symptom expression and contribute to disease development (Perrin-Cherioux *et al*., 2004; Strange 2007). It is further hypothesized that a long period of time is needed for fungi to colonize the wood and develop fungal metabolites, which accumulate and then cause the typical symptoms (Reisenzein *et al*., 2000).

Foliar and berry symptoms are due to fungal metabolites, which are produced in the wood, translocated via the xylem and accumulated in the leaves (Bruno and Sparapano, 2006b; Surico *et al*., 2006; Bruno *et al*., 2007; Péros *et al*., 2008). The host

defense compounds have also been found in the xylem sap and leaves of infected vines, indicating that these compounds are translocated from the infected tissues in the trunk to the leaves (Bruno and Sparapano, 2006b).

Phaeoacremonium aleophilum and *Ph. chlamydospora* secrete metabolites such as scytalone, isosclerone and pullulans and these are thought to be involved in the symptom expression of esca (and other trunk diseases) (Bruno and Sparapano, 2006a). These metabolites can be detected in berries, leaves and xylem sap in esca diseased vines during the seasonal growth of the vine and could possibly be used as an indicator of whether the plant is diseased or not (Bruno *et al*., 2007). Isosclerone causes large, coalescent necrotic and chlorotic spots on grapevine leaves, followed by lamina disfiguration and withering (Evidente *et al.,* 2000; Bruno and Sparapano, 2006a). With scytalone, leaves appear chlorotic and light green with irregular or rounded spots on either the margins or along the veins, which eventually spread throughout the whole leaf lamina (Evidente *et al*., 2000; Bruno and Sparapano, 2006a). Pullulan is a toxin that causes the development of thin films in the mesophyll tissue which makes it difficult for oxygen to permeate through the membranes and cause leaves to dry out and the margins and interveinal tissue to collapse (Sparapano *et al*., 2000a). Pullulan found in the woody tissue of the grapevines infected by *Ph. chlamydospora*, is potentially the cause of the brown wood streaking caused by this pathogen (Sparapano *et al*., 2000a).

In pathogenicity studies, leaves are able to take up these compounds and the symptoms produced are very similar to those produced in naturally infected plants (Bruno and Sparapano, 2006a, b). Leaves soaked in xylem sap from *Ph. chlamydospora*, *F. mediterranea* and *P. aleophilum* infected plants, showed symptoms, however, no symptoms were observed when leaves absorbed xylem sap from healthy plants (Bruno and Sparapano, 2006b).

EPIDEMIOLOGY OF ESCA

Esca is thought to spread via airborne spores, which may come from external and/ or internal sources (Surico *et al*., 2000b). According to Surico *et al*. (2000b), spatial analysis can provide information on how inoculum is spread through a vineyard. They state that if

esca spread via pruning tools, the disease would occur in rows. Furthermore, if the inoculum came from outside of the vineyard, then the disease would be spread randomly or in a uniform pattern; however, if the source of inoculum is found in the vineyard, then an aggregated or clustered pattern will occur. Several studies have shown that esca diseased vines were randomly scattered, did not occur on neighboring vines and were possibly spread by wind, insects or other agents (Reisenzein *et al*., 2000; Redondo *et al*., 2001; Sofia *et al*., 2006). Edwards *et al*. (2001b) found disease distribution to be random or clustered which could be due to micro-climate or soil. Pollastro *et al*. (2000b) found that diseased vines tend to aggregate in a field (especially in younger vineyards).

Basidiocarp formation of the fruiting bodies is generally correlated to the age of the plant (Fischer, 2006). These basidiocarps can be found on the trunks of infected vines or on dead vines after they have been pruned and left in or around the vineyard (Mugnai *et al*., 1999; Gatica *et al*., 2004). The spread of esca can be due to inoculum sources of *Fomitiporia* from outside the vineyards (Surico, 2001). Sofia *et al*. (2006) found that a 15-year-old symptomatic vineyard was in close proximity to a 60-year-old esca infected vineyard containing many basidiocarps, of which airborne basidiospores could have been the source of inoculum for the younger vineyard. Non-*Vitis* hosts can also harbor basidiocarps (Fischer, 2006). *Stereum hirsutum* produces basidiocarps willingly on trellising poles, and although they are not found commonly in the vineyard, the basidiospores can be spread via wind (Mugnai *et al*., 1999).

Fruiting bodies produced by the basidiomycetes are able to release spores when the relative humidity is more than 80 % and the temperature is above 10°C (Fischer, 2009). The release of the spores is not influenced by rainfall. However, the activity of the fruiting bodies can be influenced by drought in summer (Fischer, 2009). When basidiospores are released, they are able to colonize the host by infecting pruning wounds (Mugnai *et al*., 1999; Fischer, 2009).

Phaeoacremonium spp. and *Ph*. *chlamydospora* may be found in grapevine propagation material, which, in many cases, originates from infected mother plants (Mugnai *et al*., 1999; Pascoe and Cottral, 2000; Zanzotto *et al*., 2001; Halleen *et al*., 2003). Aerial inoculums of *Phaeoacremonium* spp. and *Ph*. *chlamydospora* have also been found. Spores of *Ph*. *chlamydospora* and *Phaeoacremonium* spp. were captured on

petroleum-jelly covered slides in the field and found to be present on the vines throughout the year and could either be dispersed by air or by water-splash (Larignon and Dubos, 1997; Eskalen and Gubler, 2001; Surico *et al*., 2006). Quaglia *et al*. (2009) found that *Ph*. *chlamydospora* spores were released during or after rainfall in the coldest months of the year in Italy. Spore release of *Ph*. *chlamydospora* and *P*. *aleophilum* were more profound in late spring when temperatures rose to between 15 and 18°C (Surico *et al*., 2006). Berries in Californian vineyards were infected with *P*. *aleophilum*, indicating the presence of aerial conidia or ascospores in the vineyard during summer (Eskalen and Gubler, 2001; Rooney *et al*., 2004). Sporulating pycnidia of *Ph*. *chlamydospora* have also been found in cracks of trunks (Edwards *et al*., 2001a).

Phaeoacremonium aleophilum and *Ph*. *chlamydospora* can infect pruning wounds (Edwards *et al*., 2001a; Halleen *et al*., 2007). *Phaeomoniella chlamydospora* was shown to be a more aggressive wound invader than *Phaeoacremonium* spp. in pathogenicity trials (Halleen *et al*., 2007). Pruning wounds can stay susceptible to infection by these pathogens for up to four months after pruning (Gubler *et al*., 2001).

PATHOGENICITY STUDIES

The fungi involved in esca and the foliar symptoms are a combination of many abiotic and biotic factors, which are difficult to replicate (Surico *et al*., 2006). Factors such as the cultivar used and the method of inoculation can influence the outcome of a pathogenicity study (Halleen *et al*., 2007).

In 1912, Petri used undetermined species of *Celphalosporium* and *Acremonium* to reproduce early internal esca symptoms. Chiarappa (1959b) found a relationship between the black measles and wood decay and that *P*. *igniarius* was able to cause the wood decay *in vitro*. In 1987, Larignon and Dubos found that mitosporic fungi were able to act with the basidiomycetes to produce esca.

The interactions among *F*. *mediterranea*, *Ph*. *chlamydospora* and *P*. *aleophilum* have been studied *in vitro* and *in planta*. An antagonistic affect of *P*. *aleophilum* towards *F*. *mediterranea* was observed on solid media (inhibiting the growth of *F*. *mediterranea*) and in vines (limiting the effect of *F*. *mediterranea* on the wood) (Sparapano *et al*.,

2000c; 2001a). *Fomitiporia mediterranea* was not inhibited by *Ph. chlamydospora* and their growth was often intertwined with no competitive interactions observed (Sparapano *et al*., 2000c; 2001b). *Phaeoacremonium aleophilum* and *Ph. chlamydospora* both competed for substrate, but did not directly challenge each other (Sparapano *et al*., 2000c; 2001a, b). The relative position of mycelial plugs of *F. mediterranea*, in combination with *Ph. chlamydospora* and *P. aleophilum* on grapevine calli, influenced the percentage reduction in the callus growth (Sparapano *et al*., 2001b). When *F. mediterranea* was placed outside the inoculation site of *Ph. chlamydospora* and *P. aleophilum*, a higher reduction in callus growth was observed than when *F. mediterranea* was placed in between the two fungi or close to *P. aleophilum*. This confirms the competitive effect between *F. mediterranea* and *P. aleophilum*.

In vitro pathogenicity studies using grapevine shoots can show symptoms after two months when inoculated with Petri disease fungi and this provides a quick method in determining the pathogenicity of these fungi (Zanzotto *et al*., 2008). Susceptibility and resistance studies, as well as sensitivity and tolerance relationships with plants and pathogens, can also be conveniently studied using bioassays and tissue culture (Sparapano *et al*., 2001c).

Fomitiporia is able to infect vines without the presence of other fungi when inoculated onto current pruning wounds on young vines, or deep inside older vines (Sparapano *et al*., 2000b). This shows that *F. mediterranea* can operate as a primary pathogen (Sparapano *et al*., 2000b). Inoculation of *F. mediterranea*, *Ph. chlamydospora* and *P. aleophilum* in wood tissue showed dark-brown wood streaking and decay downwards and upward from the inoculation point in the trunk and branches (Mugnai *et al*., 1999; Sparapano *et al*., 2000c). *Fomitiporia mediterranea* grew slowly in wood tissues causing a gradual decay, but when inoculated in the spurs, it was not able to spread to the rest of the plant (Sparapano *et al*., 2001b). Wood discoloration followed by the spongy decay has been observed with *F. mediterranea* inoculations (Sparapano *et al*., 2000c). No differences in virulence were observed among different strains due to their slow growth (Sparapano *et al*., 2000b).

Pathogenicity trials with the different pathogens will produce symptoms over various time intervals. *Fomitiporia punctata*, for instance, will produce wood decay and

possible white rot within two years (in rootstocks, spurs and branches), but external symptoms are difficult to reproduce and are inconsistent (Sparapano *et al*., 2000b; 2000c). Gatica *et al*. (2000) performed pathogenicity studies *in vitro* by placing 5 mm inoculum discs of *I. jamaicensis* (associated with 'hoja de malvón'), at the base of tissue culture plants, and found that, after 30 days, the growth was reduced and external symptoms were observed. These symptoms included, chlorosis and reddish edges on leaves with some leaves rolled downwards and darkened stems. After 40 days of inoculation with *Ph. chlamydospora*, similar symptoms were observed, but plants inoculated with *Botryodiplodia* sp. caused them to desiccate and die after six days. Wood deterioration was found after eight months of inoculation with basidiomycete strains into trunks and branches of grapevines and external symptoms within three years of inoculation (Sparapano *et al*., 2000c; 2001b). Tissue culture plants may allow one to produce faster results, but the response of woody plants may differ. Further studies are required to confirm the relevance of tissue culture pathogenicity reactions.

In a pathogenicity study conducted by Laveau *et al*. (2009), grapevines were inoculated in a glasshouse and internal discolouration of *E. lata*, *Ph. chlamydospora*, *P. aleophilum*, *D. seriata*, *N. parvum* and *F. mediterranea* was measured after 5 and 15 months. *Eutypa lata*, *N. parvum* and *Ph. chlamydospora* appeared to be the most virulent strains. *Neofusicoccum parvum* caused cankers five months after inoculation that were larger than the cankers caused by the other species. *Fomitiporia mediterranea* caused the smallest lesions and had black necrotic spots in the xylem and eventual white rot.

Pathogenicity studies which include the wood rot fungi require one to several years to obtain wood rot symptoms. A more rapid method would aid in understanding the relative role of the basidiomycete fungi associated with esca. By examining the known toxins and enzymes that are produced by the basidiomycetes, one can determine if these metabolites could produce internal and external symptoms.

TOXINS AND ENZYMES PRODUCED BY THE ESCA FUNGI

Cell walls, cuticles, and microbial compounds produced by the plant act as barriers against invasion of pathogens (Bruno and Sparapano, 2006b). A host recognizes a

pathogen with the help of plant-derived compounds, which elicit a response, or by compounds which the pathogen produces (Bruno and Sparapano, 2006c). Toxins may have three modes of action which include enzyme inhibition, interfering with defense responses of the host plant or interfering with the properties of the plant's membranes (Tabacchi *et al*., 2000; Strange, 2007). Enzymes produced by fungi can detoxify plant-derived compounds or the break down of physical barriers (Bruno and Sparapano, 2006c).

Toxins secreted by esca fungi

Little is known about the toxins secreted by the basidiomycete fungi associated with esca. Tabacchi *et al*. (2000) found that culture filtrates of *F. mediterranea* contained p-hydroxy-benzaldehyde and a new chromanone, which is biogenetically similar to eutypine called 6-formyl-2,2,-dimethyl-4-chromanone. *Stereum hirsutum* is able to produce sterehirsutinal and compounds which are structurally close to those of eutypine (Dubin *et al*., 2000). Perrin-Cherioux *et al*. (2004) discussed that the aldehyde substituent on sterehirsutinal is thought to be accountable for its biological activity. Sterehirsutinal is difficult to synthesize; however, low amounts inhibited growth of Gamay callus (Perrin-Cherioux *et al*., 2004). Tabacchi *et al*., (2000) did not find sterehirsutinal in sap or leaves, and concluded that this toxin is not transported into the vessels (Tabacchi *et al*., 2000).

Scytalone, isosclerone and pullulan are produced both *in vivo* and *in planta* and have been linked to leaf and berry symptoms (Evidente *et al*., 2000; Sparapano *et al*., 2000a; Bruno and Sparapano, 2006a; Bruno *et al*., 2007). *Phaeomoniella chlamydospora* has been found to produce phytotoxic pullulans, whereas *P. aleophilum* produces less pullulans, scytalone and isosclerone (Surico, 2001). *Phaeoacremonium aleophilum* also produced seven napthalenones, similar to napthoquinone pigments that can be produced by phytopathogenic fungi, which inhibit the plants natural defense system and therefore increase the virulence of the fungus (Abou-Mansour *et al*., 2004).

Enzymes secreted by esca fungi

The production of tannase, laccase and peroxidase assist the esca pathogens to degrade the wood and break down antimicrobial compounds produced by the plant (Bruno and

Sparapano, 2006c). White rot fungi secrete extracellular enzymes having a diverse composition, which degrade lignin, but there are few comparative studies looking at the production of these enzymes which are produced (Kachlishvili *et al*., 2006). With regard to this, one would like to determine if the white rot fungi found on grapevines produce the same enzymes.

Fomes igniarius and *F. punctata* are able to produce laccase (Chiarappa, 1959a; Alessandro *et al*., 2000). Laccase and peroxidase are produced by *F. mediterranea* and used to degrade lignin (Chiarappa, 1959a; Mugnai *et al*., 1999; Bruno and Sparapano, 2006c). *Stereum hirsutum* did not produce lignin peroxidase, laccase and manganese peroxidase (Chiarappa, 1959a; Del Río *et al*., 2004).

Phaeoacremonium has a larger variety of enzymes, which helps it to be a pioneer fungus and adaptable to different hosts (Santos *et al*., 2006b). Pioneer organisms need to grow in the presence of growth inhibitors, which the host produces, in order to survive (Mugnai *et al*., 1999). Laccase is produced by *Phaeoacremonium* species (Del Río *et al*., 2004; Santos *et al*., 2006b). Some *Ph. chlamydospora* and *P. aleophilum* isolates are also able to produce peroxidase (Mugnai *et al*., 1999). *Phaeoacremonium aleophilum* has a high specific activity for lignin peroxidase and not for manganese peroxidase (Del Río *et al*., 2004). These fungi thereby also play a role in the degradation of lignin (Santos *et al*., 2006b). *Phaeomoniella chlamydospora*, however, does not produce these lignin degrading enzymes (Del Río *et al*., 2004). *Phaeoacremonium* isolates produce more xylanase than *Phaeomoniella* isolates and therefore possibly having a greater ability to degrade xylan (Santos *et al*., 2006b). Hemicellulose and cellulose are also more easily degraded by *Phaeoacremonium* isolates and are thought to be more virulent than *Phaeomoniella* isolates as they have a more damaging effect on grapevine cells (Santos *et al*., 2006b). *Phaeomoniella chlamydospora* and *P. aleophilum* can both produce pectic enzymes (Surico, 2001). *Phaeoacremonium* and *Phaeomoniella* isolates are not able to degrade chitin (Santos *et al*., 2006b).

Other trunk disease pathogens are able to secrete a large range of enzymes which degrade other components of the plant cell wall (Schmidt *et al*., 1999). *Eutypa lata* is known to target glucose rich polymers (Rolshausen *et al*., 2008) and produces extra-cellular enzymes such as cellulase, xylanase, 1,3-β-glucanase, chitinase, protease

(Schmidt *et al*., 1999) and lignin peroxidase (Del Río *et al*., 2004). A range of toxins and enzymes are therefore secreted by the esca fungi and this assists in the break down of plant components and so assists in the pathogenicity of the fungi.

MANAGEMENT OF ESCA

Knowledge regarding the fungi involved in esca, their epidemiology, the mode of action and the vineyard age will aid in the development of a management strategy. However, it is difficult to determine these management strategies due to the inconsistent nature of the symptom expression of the disease (Di Marco *et al*., 2000).

Methods to control the disease in the past included very traditional, yet primitive, methods, still used in the Mediterranean region today. For example, by inserting an acorn or stone into a crack on the vine (Rui and Battel, 1962; Rumbos and Rumbou, 2001) the wood would be exposed to air and would slow down the development of foliar symptoms (Rui and Battel, 1962).

Traditionally, sodium arsenite was the only chemical available for the control of esca and considered to be successful (Mugnai *et al*., 1999). However, it was found to be carcinogenic and toxic and therefore banned in various countries in Europe prior to 2001 (Mugnai *et al*., 1999; Di Marco *et al*., 2000; Darrieutort and Lecomte, 2007). Sodium arsenite was then banned in all European countries in 2001 (Darrieutort and Lecomte, 2007). To date, no chemical control has been successful in the control of the disease.

The first line of management is ensuring clean grafting material to nurseries. Both *Ph*. *chlamydospora* and *Phaeoacremonium* species are present in mother block vines and subsequently in young vines (Mugnai *et al*., 1999; Pascoe and Cottral, 2000; Fourie and Halleen, 2002). Vines can become infected with *Ph*. *chlamydospora* in nurseries through contaminated grafting tools and water (Whiteman *et al*., 2003; Pollastro *et al*., 2009). Hot water treatment should be applied in all nurseries, as it ensures that conidial germination and colony growth rate of the pathogens and pests are reduced before planting (Fourie and Halleen, 2004; Gramaje *et al*., 2008; Waite and Morton, 2007).

Biological control products based on *Trichoderma harzianum* can increase root growth and prevent root and pruning wound infection (Fourie *et al*., 2001; Di Marco *et*

al., 2004b; Di Marco and Osti, 2007). Fungicides such as cyproconazole, flusilazole, penconazole and tetraconazole can be painted on pruning wounds to seal them (Di Marco *et al*., 2000). Halleen and Fourie (2005) found that spray-treating pruning wounds with flusilazole can reduce *Ph*. *chlamydospora* infections by 82 % and *Phomopsis* infections by 53 %. They also found that benomyl was able to reduce *Ph*. *chlamydospora* by 77 %.

Another potential method of control against esca could be to use pathogenically active secondary fungal metabolites (Perrin-Cherioux *et al*., 2004). However, no conclusive results have been found. A control measure which inhibits/ inactivates lignin degrading enzymes could be effective (Chiarappa, 1959a). More research should also be conducted on plant stimulants or defense responses and a more cost effective method of application would have to be developed (Darrieutort and Lecomte, 2007).

Most *Vitis* cultivars are susceptible to esca with no resistant cultivars found yet (Mugnai *et al*., 1999; Christen *et al*., 2005; Sánchez-Torres *et al*., 2008). By using the least susceptible cultivars such as Roussane (Marchi, 2001), Matilde (Sparapano *et al*., 2000b), Aglianico (Zanzotto *et al*., 2008), Montepulciano and Merlot (Quaglia *et al*., 2009), one could possibly decrease the incidence of disease.

Prevention of the disease is possibly the best manner in which the disease can be controlled. Healthy farming practices, such as pruning wound protection, appropriate sanitation and using disease free propagation material, should be implemented (Mugnai *et al*., 1999; Ari, 2000; Di Marco *et al*., 2000; Köklü, 2000; Gatica *et al*., 2000). Early detection methods will aid the timing of management practices, but pruning wound protection and using clean planting material are probably the best measures to use in prevention as there are no sufficient chemical control measures available.

CONCLUSIONS

Esca is a complex disease that has many factors still to be determined. These include the role of the different fungi in the disease complex, the identity of the basidiomycete fungi, the reasons for the variability of the external and internal symptoms and the epidemiology of the basidiomycetes. Knowledge regarding these aspects will aid in the development of effective management strategies. In South Africa, research has been done on many of the

grapevine trunk diseases including Petri disease, Botryosphaeria dieback and cankers, Eutypa dieback and Phomopsis cane and leaf spot; however, not on esca. As the incidence of esca is increasing in the world, it is important that studies be conducted in order to better understand the disease.

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Table 1. Morphological characters that can be used to identify basidiomycete genera found on grapevines.[1]

Genus	Substrate	Basidiocarp shape	Fruiting body mitism	Consistency	Setae	Basidiospore shape	Basidiospore colour
Fomitiporella	Deciduous wood	Resupinate/ effused - reflexed	Dimitic	Perennial	Lacking	Ellipsoid/ Globose	Brownish
Fomitiporia	Deciduous/ coniferous wood	Resupinate/ effused - reflexed/ pileate	Monomitic/ dimitic	Perennial	Lacking or rare; hymenial	Globose	Hyaline
Inocutis	Deciduous wood	Pileate	Monomitic	Annual	Lacking	Ellipsoid	Yellowish/ brownish
***Inonotus* s.s.**	Deciduous wood	Resupinate/ effused - reflexed/ pileate	Monomitic/ dimitic	Annual/ perennial	Mostly present; hymenial/ hyphoid	Ellipsoid/ Globose	Yellowish/ brownish
***Phellinus* s.s.**	Deciduous wood	Resupinate/ effused - reflexed/ pileate	Dimitic	Perennial	Hymenial	Ellipsoid/ subglobose	Hyaline

[1] (Wagner and Fischer, 2002).

Table 2. A list of known basidiomycete species associated with esca on grapevine, their geographical location and host ranges.

Basidiomycete species	Geographical Location	Host range[1]
Fomitiporia australiensis	Australia	*Vitis vinifera*
Fomitiporia polymorpha	North America	*Vitis vinifera*
Fomitiporia punctata	Europe	*Vitis vinifera*
Fomitiporia mediterranea	Europe	*Acer negundo, Actinidia chinensis*, *Actinidia deliciosa, Corylus avellana*, *Lagerstroemia indica*, *Laurus nobilis*, *Ligustrum vulgare*, *Olea europaea*, *Quercus ilex*, *Platanus x acerifolia* (plane trees), *Robinia pseudoacacia*, *Vitis vinifera*
Fomitiporella vitis	Chile	*Vitis vinifera*
Inocutis jamaciensis	Argentina	*Vitis vinifera*, *Eucalyptus* spp.
Stereum hirsutum[2]	Europe, North America	*Vitis vinifera*
Trametes hirsuta[2]	Europe	*Vitis vinifera*

[1] (Elena and Paplomatas, 2002; Pilotti *et al*., 2005; Fischer, 2006; Pérez *et al*., 2008).

[2] These fungi play a minor role in esca.

Table 3. Fungi generally isolated from the different symptom types found in esca diseased vines.[1]

Symptom type found in the wood	Organism most frequently isolated from this symptom type	Other organisms also found in this symptom type
Healthy wood	*Botryosphaeria obtusa*	*Phaeomoniella chlamydospora*
Brown wood streaking	*Phaeomoniella chlamydospora*	*Phaeoacremonium aleophilum*
Brown necrosis	*Phaeomoniella chlamydospora*	*Phaeoacremonium aleophilum, Phomopsis* sp., *Botryosphaeria* sp., *Eutypa lata*
White rot/ Spongy decay	*Fomitiporia mediterranea*	*Stereum hirsutum*
White rot bordered by black margin	*Fomitiporia mediterranea*	*Fomitiporia* sp., sterile mycelium

[1] (Larignon and Dubos, 1997 ; Mugnai *et al.*, 1999; Serra *et al.*, 2000; Sofia *et al.*, 2006; Calzarano and Di Marco, 2007).

CHAPTER 2

THE SYMPTOMS AND FUNGI ASSOCIATED WITH ESCA IN SOUTH AFRICA

ABSTRACT

In the past, only a few incidences of esca diseased grapevines were reported from the Slanghoek and Rawsonville areas (Western Cape). However, it was believed to be of little importance and, therefore, the disease has not been studied in South Africa. In this study, vines with internal or external symptoms of esca were sampled from table, raisin and wine grape cultivars from 37 production areas in the Western Cape, Northern Cape and Limpopo provinces. Most vines were over the age of ten years old. However, younger vines (age two - three) were also found to be infected. External symptoms, including dieback, tiger striped leaves, berry symptoms (shrivelling and insufficient colouring) and apoplexy, resembled those found on grapevines in Europe and the USA, although the typical tiger stripe symptom was observed less frequently. The internal symptoms and the fungi associated with them were similar to European symptoms and included white rot, black and brown wood streaking, brown necrosis within white rot, sectorial brown necrosis and central brown/ red/ black margin. The fungi isolated mostly from the white rot were the basidiomycetes (30.4 %). Black and brown wood streaking was primarily caused by *Phaeomoniella chlamydospora* (45.4 %). Brown necrosis within the white rot was caused by basidiomycetes (20.4 %), *Phaeoacremonium aleophilum* (15.9 %) and *Ph. chlamydospora* (13.6 %). *Phaeomoniella chlamydospora* (20.8 %) and the Botryosphaeriaceae (10.7 %) were the fungi isolated the most from the sectorial brown necrosis and *Ph. chlamydospora* (29.1 %) from the central brown/ red/ black margin. Basidiocarps were found on only a few grapevines. Given the wide distribution of esca in the grape growing regions investigated, this disease should be considered as an important limiting factor in the productive lifespan of vineyards and the quality of produce in South Africa.

INTRODUCTION

Esca is one of the most destructive diseases of grapevine (Mugnai *et al*., 1999). The disease affects the trunks and arms of grapevines and is widespread in older vineyards in Europe (Mugnai *et al*., 1999; Surico *et al*., 2006). It is believed to be caused by a complex of fungi that, according to some theories, infect the plant in succession and affect different parts of the woody tissue (Larignon and Dubos, 1987; Mugnai *et al*., 1996; Mugnai *et al*., 1999, Tabacchi *et al*., 2000, Surico, 2001; Surico *et al*., 2006).

The combination of fungi associated with esca diseased vines varies between countries. These mostly include *Fomitiporia mediterranea* M. Fischer (which is the most common basidiomycete species in Europe), *F. polymorpha* M. Fischer (North America), *F. australiensis* M. Fisch., J. Edwards, Cunnington & Pascoe (Australia), *Phellinus* sp. (Europe and North America), *Stereum hirsutum* (Willd.) Pers., *Phaeomoniella chlamydospora* (W. Gams, Crous & M.J. Wingf. & L. Mugnai) Crous & W. Gams, *Phaeoacremonium* spp., *Eutypa lata* Tul. & C. Tul, Botryosphaeriaceae and *Phomopsis* species (Mugnai *et al*., 1996; Larignon and Dubos, 1997; Armengol *et al*., 2001; Rumbos and Rumbou, 2001; Fischer, 2002; Fischer and Kassemeyer, 2003; Fischer and Binder, 2004; Fischer, 2006; Martin and Cobos, 2007). However, it is generally accepted that the primary organisms responsible for esca are basidiomycetes, *Ph. chlamydospora* and *P. aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai. The latter two cause Petri disease, the forerunner for young esca, and when combined with the basidiomycete fungi, the complex will form esca proper. Species such as *Eutypa lata* (Larignon and Dubos, 1997; Mugnai *et al*., 1999), members of the Botryosphaeriaceae (Mugnai *et al*., 1999) such as *Diplodia seriata* De Not. (Armengol *et al*., 2001; Crous *et al*., 2006; Calzarano and Di Marco, 2007) and *Phomopsis* species, particularly *Phomopsis viticola* (Sacc.) Sacc. (van Niekerk *et al*., 2005), are all involved in their own symptomology. However, the role of these fungi in wood degradation, as well as the interactions with the primary fungi in the esca complex, still needs to be determined (Péros *et al*., 2008).

In recent years the incidence of esca in Europe has increased markedly. There are great economic losses associated with replacing esca-infected vineyards (Rumbos and Rumbou, 2001). In Italy the disease is found in all the grape growing regions and in some

instances affects 90 – 100% of vineyards between the ages of 15 to 25 years (average of 1 – 50 %). The average annual increase is 4 – 5% (Mugnai *et al*., 1999). Higher incidences of the disease are found in vineyards of 10-years and older (Mugnai *et al*., 1999; Reisenzein *et al*., 2000; Surico, 2001; Romanazzi *et al*., 2009). Younger vines (two to six years old) in California have shown susceptibility to esca infection (Gubler *et al*., 2004) but foliar symptoms may, or may not, be present (Mugnai *et al*., 1999; Surico, 2001).

Most cultivars are susceptible to esca (Mugnai *et al*., 1999) with no *Vitis* cultivars found to be resistant to trunk diseases (Christen *et al*., 2005). The combination of fungi, environmental conditions and the host itself all play a role in the severity of the disease (Stefanini *et al*., 2000; Surico, 2001; Calzarano and Di Marco, 2007). The process of wood degradation and the way in which the organisms interact with each other is still unclear (Calzarano and Di Marco, 2007). In turn, this expresses uncertainty as to how the internal symptoms affect symptom expression and the development of esca (Surico *et al*., 2004; Calzarano and Di Marco, 2007).

Foliar symptoms include the typical ‘tiger stripe’ symptom which can appear red in some cultivars (Viala, 1926; Mugnai *et al*., 1999; Bruno and Sparapano, 2006). These tiger stripes appear as light green/ chlorotic rounded/ irregular spots which occur between the veins and margins of the leaves (Mugnai *et al*., 1999; Sparapano *et al*., 2001; Bruno and Sparapano, 2006). The tiger stripes eventually coalesce and become necrotic, leaving a green area along the veins of unaffected areas (Surico *et al*., 2006). Foliar symptoms do not occur early in the growing season (spring), but become visible in summer and autumn (Mugnai *et al*., 1999). It is very difficult to correlate foliar and internal symptoms as the various fungi in the disease complex produce various metabolites which may influence symptom expression (Serra *et al*., 2000). Additionally, blocked vessels within the plant could change physiological processes and contribute to foliar symptoms (Péros *et al*., 2008).

Berry symptoms consist of diminutive spots which are dark brown, violet or purple and are known as ‘black measles’. Berry symptoms are common in California (Chiarappa, 1959; Gubler *et al*., 2004) and southern Italy (Mugnai *et al*., 1999) and have also been reported in France (Mugnai *et al*., 1999) and Austria (Reisenzein *et al*., 2000). Infected berries not showing spots tend to have a violet colour and show a reduced turgor,

causing them to shrivel (Reisenzein *et al*., 2000). These symptoms may, or may not, be associated with foliar symptoms (Mugnai *et al*., 1999).

Apoplexy, the sudden wilt and collapse of the vine, is the acute syndrome of esca (Viala, 1926). It is usually observed during hot summers, especially when rainfall is followed by hot, dry weather (Mugnai *et al*., 1999; Bruno *et al*., 2007). Apoplexy is mainly restricted to older vines (Surico *et al*., 2006).

The internal symptoms associated with esca in Europe have been described by various authors as a combination of different symptom types. Up to seven different symptom types have been described (Serra *et al*., 2000). Commonly found internal symptoms include brown wood streaking, black spotting, brown necrosis that can be sectorial, white rot which can be bordered by a black line and brown margins of decayed wood (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Pollastro *et al*., 2000; Serra *et al*., 2000; Sofia *et al*., 2006; Calzarano and Di Marco, 2007; Péros *et al*., 2008).

Some authors believe a succession of infections by fungi is necessary for wood decay to occur (Mugnai *et al*., 1996; Larignon and Dubos 1997). Calzarano and Di Marco (2007) and Serra *et al*. (2000) speculated that white wood rot development is favoured by a pre-existing necrosis (discolouration) caused by the colonization of other fungi found in the vine and, therefore, occurs in succession. However, Chiarappa (1997) and Sparapano *et al*. (2001b) found that *F. punctata* (*F. mediterranea*) does not require prior colonization of the other esca fungi to be able to infect the vine.

Esca has been studied in various grapevine growing countries such as Australia, France, Germany, Greece, Italy, Portugal, Spain and the United States of America (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Pascoe and Cottral, 2000; Armengol *et al*., 2001; Edwards *et al*., 2001b; Redondo *et al*., 2001; Rumbos and Rumbou, 2001; Fischer and Kassemeyer, 2003; Feliciano *et al*., 2004; Gubler *et al*., 2004; Sofia *et al*., 2006; Martin and Cobos, 2007). There is little information available on the occurrence of esca in South Africa. Few incidences have been reported prior to 1981 in the Rawsonville and Slanghoek areas of the Western Cape Province and it was thought to be of no economic concern. *Stereum hirsutum* and *Phellinus igniarius* (L.) Quél. were believed to be the causal organisms, although no study was ever conducted to confirm this (Marais, 1981).

The aims of this study were to determine the extent of esca diseased vines in the different grapevine production areas of South Africa. The external and internal symptoms associated with diseased vines were also investigated. As well as the identification of the fungi isolated from specific symptom types found in these diseased vines, with specific reference to the basidiomycete fungi.

MATERIALS AND METHODS

Sampling of esca diseased vines

Vineyards showing symptoms of esca or general decline were identified in all the major wine, raisin and table grape production areas of South Africa in the Western Cape, Northern Cape and Limpopo provinces between 2001 and 2008. Esca diseased vines were identified as having internal discoloration and white rot, which may, or may not, be accompanied with external symptoms. External symptoms were not always visible, since several vines were collected during winter when farmers traditionally remove old or unproductive vineyards. The diseased vines were removed and immediately taken to the laboratory where fungal isolations were made.

Fungal isolations from diseased vines

Cross and longitudinal sections were made at various places in the cordons and trunk of each plant to investigate internal necrosis. For fungal isolations, wood sections with internal necrosis were selected and cut into two smaller sections adjacent to each other, in order to obtain two mirror images of the same symptom type. This was also done to facilitate the use of two sterilization techniques to ensure fungal isolation from soft, spongy material. A photograph of each wood section showing the various symptom types was taken. Photographs of the wood section were used to identify and compare the different internal symptom types. Fungi that were isolated were linked to the specific symptom type and recorded on the different photographs. From this, the total number of fungi isolated from each symptom type from each section of wood was calculated. The one section was flame sterilized by holding the wood with sterile forceps, lightly spraying it with 70 % ethanol and passing it through a flame. The other piece was triple sterilized

as follows: 30 seconds in 70 % ethanol, 2 minutes in 3.5 % NaOCl and 30 seconds in 70 % ethanol. Twelve small sections of wood (1 x 1 x 2 mm) from each of the different symptom types were then aseptically removed with a scalpel and placed onto Potato Dextrose Agar (PDA, Biolab, South Africa) plates containing 250 mg chloramphenicol (four pieces per plate). Plates were incubated at 23 - 25°C for approximately four weeks and the growth was monitored daily.

Selection and storage of cultures

Fungi isolated from the various symptom types and suspected of being involved with esca were recorded, identified (where possible) and hyphal tipped or single spored for later identification. Isolates were also selected to represent the various geographical regions. Pure cultures were stored in sterile distilled water in 14 ml McCartney bottles kept at 4°C. Representative isolates are stored in a fungal culture collection at ARC Infruitec-Nietvoorbij, Stellenbosch and the STE-U culture collection of the Department of Plant Pathology, Stellenbosch University.

Identification of fungi

Fungal isolates obtained from the various symptom types were identified to genus level with phenotypic characteristics including cultural growth and micromorphology. For a selected number of isolates, species identification was done with PCR and sequence comparisons (Chapter 3).

RESULTS

Distribution of esca

Diseased vines representative of the search criteria were found in 31, 5 and 1 production areas in the Western Cape, Northern Cape and Limpopo Provinces, respectively (Table 1). This represented all the sampled areas and from which diseased vines were found. In total, 212 vines were collected from vineyards covering an area of 207 hectares. Of these vines, fungi were isolated from 181 vines, representing 18 cultivars.

The majority of diseased vines were found in older vineyards. A total of 3 % of the diseased vines sampled were between 1 and 10 years of age, 39 % were between 11 and 20 years, 56 % were older than 21 years and the ages of the remaining 3 % of diseased vines sampled were unknown.

Esca symptoms observed in the field

Although extensive surveys of the same number of vineyards of the same age in each of the regions could not be conducted, symptoms typical to those described for esca were observed in several vineyards. External symptoms included foliar and berry symptoms, as well as apoplexy.

The first symptom observed in the beginning of the growing season was typical dieback symptoms. These consisted of vine arms not producing any green shoots (Figure 1). Apoplexy, the sudden die-back and wilt in the hot summer months (Figure 2) was also observed, however, this was not a frequent occurrence.

Foliar symptoms (Figure 3 and 4), when present, appeared in a short window period between the end of January and the end of March. Foliar symptoms observed in some vineyards included typical tiger stripe symptoms, as found in European vineyards and general scorching on the leaves where eventual necrosis occurred together with general vine decline symptoms (which included smaller leaves, shoots with shortened internodes and stunted shoots). The tiger stripe symptoms included scorching on the edge of the leaf lamina and chlorosis along the veins with red cultivars, showing a reddish scorching along the edges of the leaves. White cultivars did not have this red tinge and appeared whitish in colour.

Examples of berry symptoms were also observed. Symptoms on red berries included a paler discoloration and shriveled berries (Figure 5 A-D). Symptoms on white berries appeared as black spots on the berries. This was only observed on one occasion on berries of a Hanepoot vine located in Stellenbosch (Figure 5 E, F).

Although specific surveys were not conducted to determine the incidence of basidiocarps in this study, attempts were made to find them. They were observed in some vineyards but were, however, difficult to find. An investigation of all the plants in a 26-year-old Chenin blanc vineyard (1 ha) located at Nietvoorbji in Stellenbosch revealed

seven basidiocarps. They appeared as ‘putty’-like substances which were brown, hard and porous and were generally found on the underside of grapevine arms and cordons, in many cases where the arm met the trunk (Figure 6). The basidiocarps were not identified or linked to the basidiomycete mycelium found inside the vines and further identification using PCR will be performed in future studies.

Internal wood symptoms

Five predominant symptom types were identified. These included white rot, black and brown wood streaking, brown necrosis within the white rot, sectorial brown necrosis, and a central brown/ red/ black margin (Figure 7).

The white rot areas appeared white, yellow or yellow-orange in colour and ranged from spongy to hard in texture (Figure 8). Black and brown wood streaking took the form of brown to black dots in transverse sections that were either separate or clustered. These were assumed to be the typical Petri disease symptoms. A brown necrosis, which appeared as a dark brown/ brown margin within the white rot, was identified as another symptom type. This symptom type had to be surrounded by white rot on all sides. The sectorial brown necrosis started from the edge of the vine and then progressed inwards sometimes taking on a V-shape. This sectorial necrosis appeared brown to dark brown. The central brown, red or black margin surrounded the other symptom types and appeared darker in colour than neighboring symptoms (especially when surrounding the sectorial necrosis).

Fungi associated with specific symptom types

In total, 24 715 isolations were made and 13 669 fungal isolates were obtained from the five symptom types associated with esca diseased vines in South Africa (Table 2). Basidiomycetes (4695), *Ph. chlamydospora* (4595) and *P. aleophilum* (2386) were by far the most frequently isolated pathogens associated with these symptoms and represent 34.3, 33.6 and 17.5 % of the total number of isolates obtained, respectively. Other pathogens included Botryosphaeriaceae, other *Phaeoacremonium* spp., *Eutypa lata*, *Phomopsis viticola*, other *Phomopsis* spp. as well as *Pleurostomophora richardsiae* (Nannf.) L. Mostert, W. Gams & Crous. Most of the isolates were retrieved from the

central brown/ red/ black margin (5257), white rot (4554) and sectorial brown necrosis (2405), followed by black and brown wood streaking (914) and brown necrosis within white rot (539). The number of isolates obtained for each taxon per symptom type is presented in Table 2.

The basidiomycetes were the primary cause of the white rot (30.4 %; Table 2). *Phaeoacremonium aleophilum*, *Ph. chlamydospora* and *Phaeoacremonium* spp. were also found in the white rot, but at much lower incidences (7.4, 4.8 and 2.1 %, respectively). It appeared that the first stage of the white rot was often orange in colour and still hard in texture, which then progressed to become whiter and semi-spongy to spongy and soft (Figure 8). The development of symptom types could be seen in the differences between trunks of younger vines versus older vines and sometimes within the same vine. In younger vines, the sizes of the brown discoloured wood (Figure 8 C) were often larger in size than the area of white rot, indicating that there could be a succession of infection and symptom development in the wood. The white rot dominated over the other symptom types as wood degradation advanced (Figures 8 E, F).

Black and brown wood streaking was primarily caused by *Ph. chlamydospora* (45.4 %). No *Phomopsis* species, *Pleurostomophora richardsiae* or *Eutypa lata* were found in this symptom. The Botryosphaeriaceae, *Phaeoacremonium* spp., *Phaeoacremonium aleophilum* and the basidiomycetes occurred at a relatively low frequency (1.1, 1.1, 1.4, 1.8 % respectively). From the brown necrosis within the white rot, six fungi were isolated in the following frequencies: basidiomycetes (20.4 %), *P. aleophilum* (15.9 %), *Ph. chlamydospora* (13.6 %), Botryosphaeriaceae (1.5 %), *Pleurostomophora richardsiae* (1.1 %) and *E. lata* (0.4 %). *Phomopsis* was the only fungus not isolated from this symptom type. The most predominant species isolated from the sectorial brown necrosis were *Ph. chlamydospora* (20.8 %). Botryosphaeriaceae (10.7 %), *P. aleophilum* (9.4 %), *E. lata* (7.5 %), the basidiomycetes (5.5 %), *Phomopsis viticola* (6.3 %), *Phaeoacremonium* spp. (1.2 %), *Phomopsis* spp. (0.1 %) and *Pleurostomophora richardsiae* (0.026 %) comprised the rest of the species isolated from this area. *Phaeomoniella chlamydospora* (29.1 %) was the primary fungus that was isolated from the central brown/ red/ black margin. The basidiomycetes (15.1 %), *P. aleophilum* (13.6 %), *Phaeoacremonium* spp. (2.5 %), Botryosphaeriaceae (2.1 %),

Phomopsis viticola (1.5 %), *E. lata* (1.1 %) and *Pleurostomophora richardsiae* (0.01 %) comprised the rest of the species isolated from this area. *Phomopsis* spp. were not isolated from this region.

DISCUSSION

Esca occurs over a wide range of grapevine producing areas in South Africa. Esca was found in 31 production areas in the Western Cape, five production areas in the Northern Cape and in one production area in the Limpopo province. Wine, table and raisin grapes on a range of cultivars were affected. This illustrates the prominence of the disease in the country.

In the present study, 95 % of the esca diseased vines were from vineyards older than ten years. This correlates with other studies which showed that higher incidences of disease were common in vines aged ten and older (Mugnai *et al*., 1999; Reisenzein *et al*., 2000; Péros *et al*., 2008; Romanazzi *et al*., 2009). Petri disease, or young esca, can appear in nursery vines. However, esca proper with white rot symptoms take much longer to develop and is hence generally found in older grapevines (Mugnai *et al*., 1999; Graniti *et al*., 2000; Surico, 2001; Surico *et al*., 2006).

External symptoms observed in a few South African vineyards were very similar to those found in Europe and other countries, including foliar, berry and apoplexy symptoms. The recent observations made in South African vineyards were also consistent with external symptoms reported by Marais (1981) resembling tiger-stripes, apoplexy, dieback and decline of the vines. Apoplexy was rarely found in this study, but general decline symptoms were much more prevalent. Measle-like berry symptoms were only found on one occasion and the typical tiger stripe symptoms were rarely found and only in a short window period in summer. This observation differs with results found in Europe where tiger stripe symptoms are common (Mugnai *et al*., 1999), but it is similar to the situation in Australian vineyards, where foliar symptoms are also not common (Pascoe and Cottral, 2000). However, given the nature of this study where all the vineyards were not visited during the same time period, conclusions regarding the occurrence of various symptoms can not be made.

Basidiocarps were found for the first time on South African vines, but were not frequently seen. In European countries, basidiocarps can develop on dead *Vitis* trunks (Mugnai *et al*., 1999; Fischer and Kassemeyer, 2003). They can also develop on other hardwood species (Fischer and Kassemeyer, 2003; Fischer, 2006) or on wooden stakes or trellising, which could be a source of inoculum for nearby vineyards (Mugnai *et al*., 1999; Reisenzein *et al*., 2000). In the present study, other hosts around the vineyard were not investigated for basidiocarps.

In the current study, not all symptom types and not all of the fungal species were found within one vine, and so the occurrence of external symptoms was likely to vary as well. The combination of fungal species in the wood, their contribution to wood degradation, their interactions amongst each other and the toxins produced by the different species are all thought to play a role in symptom expression. This interaction, however, it is not fully understood (Mugnai *et al*., 1999; Stefanini *et al*., 2000; Surico *et al*., 2000a, b; Surico *et al*., 2006; Calzarano and Di Marco, 2007). External symptoms can also be influenced by vine age, farming practices, soil type, the slope of the land and the cultivar type (Surico *et al*., 2000a), as well as environmental and seasonal factors (Mugnai *et al*., 1999). The expression of external symptoms have been found to be inconsistent in other studies (Mugnai *et al*., 1999; Stefanini *et al*., 2000; Surico *et al*., 2000a, b; Surico *et al*., 2006; Calzarano and Di Marco, 2007).

Species of the basidiomycetes, Botryosphaeriaceae, *E. lata*, *P. aleophilum,* other *Phaeoacremonium* spp., *Ph. chlamydospora*, *Phomopsis viticola*, other *Phomopsis* spp. as well as *Pleurostomophora richardsiae* were found in this study. Five symptom types including white rot; black and brown wood streaking; brown necrosis within the white rot; sectorial brown necrosis; and a central brown/ red/ black margin were found. Internal symptoms observed and the fungi isolated from each type were similar to those observed in Europe (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Pollastro *et al*., 2000; Sofia *et al*., 2006; Calzarano and Di Marco, 2007; Péros *et al*., 2008; Sánchez-Torres *et al*., 2008). The fungi associated with esca and their frequencies were also similar in Italy, France and Spain (Armengol *et al*., 2001). Marais (1981) reported internal symptoms of yellow rot surrounded by a black zone.

From the white rot, basidiomycete species were the most predominant species with low number of *P. aleophilum* and *Ph. chlamydospora* isolated in this study. Many other studies showed that *F. mediterranea* was the main casual agent in the white rot, but *P. aleophilum* and *Ph. chlamydospora* were also found in low numbers in some cases (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Pollastro *et al*., 2000; Sofia *et al*., 2006; Calzarano and Di Marco, 2007; Péros *et al*., 2008; Sánchez-Torres *et al*., 2008).

The black and brown wood streaking was primarily caused by *Ph. chlamydospora* in South Africa. This is consistent with other studies (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Pollastro *et al*., 2000; Sofia *et al*., 2006; Calzarano and Di Marco, 2007; Péros *et al*., 2008; Sánchez-Torres *et al*., 2008). In this study, basidiomycetes, *P. aleophilum* and Botryosphaeria species were also isolated from black and brown wood streaking, but below 2 % for each species. *Dendrophoma pleurospora* f. *vitigena* and an unidentified sterile mycelium have also been isolated from this region (Serra *et al*. 2000) but were not found in this study.

From the brown necrosis within the white rot, basidiomycetes, *P. aleophilum* and *Ph. chlamydospora* were isolated. The highest numbers of isolates from this symptom were the basidiomycetes (20 %). Serra *et al*. (2000) isolated mostly sterile mycelium and *Fomitiporia* sp. from this symptom type. Even though basidiomycetes are more commonly associated with white rot, pathogenicity studies showed that basidiomycetes can also cause a brown discolouration (Sparapano *et al*., 2001).

Phaeomoniella chlamydospora was the primary fungus isolated from the central brown/ red/ black margin. Other studies have also found *Phaeoacremonium* spp., *Ph. chlamydospora* and the Botryosphaeriaceae in the black line and within the brown discoloured wood (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Pollastro *et al*., 2000; Serra *et al*., 2000; Sofia *et al*., 2006; Calzarano and Di Marco, 2007; Péros *et al*., 2008; Sánchez-Torres *et al*., 2008; Sofia *et al*., 2006).

Phaeomoniella chlamydospora and species from the Botryosphaeriaceae were the most numerous fungi isolated from the sectorial brown necrosis. From observations, the brown sectorial necrosis could be assumed to be the first to develop and with progression, these fungi may overgrow the other adjacent symptom types. In another study, it was found that sterile mycelium, *Sphaeropsis* sp. (Botryosphaeriaceae), *Phomopsis* sp. and *E.*

lata were isolated from the brown necrosis (Serra *et al*., 2000). In the present study, *Eutypa lata* was rarely isolated (only 1.7 % of the total isolates) and predominantly from the sectorial brown necrosis. *Eutypa lata* is thought to be a pioneer fungus (Larignon and Dubos, 1997) and has been isolated from esca diseased vines. However, it causes Eutypa dieback, which has its own symptomology (Mugnai *et al*., 1999).

Members of the Botryosphaeriaceae have also been known to be associated with esca diseased vines and are generally isolated from the dark brown margins, which border the decayed wood (Mugnai *et al*., 1999). *Phomopsis* species, particularly *Phomopsis viticola*, have also been associated with grapevine trunk diseases (van Niekerk *et al*., 2005). The majority of the *Phomopsis* isolates were isolated from the sectorial brown necrosis, but they appeared to play a minor role due to low frequencies found in comparison with the other fungi. The *Phomopsis* spp. were only found in the sectorial brown necrosis. The *Phomopsis* spp. and *Phomopsis viticola* were not isolated in the black and brown wood streaking or in the brown wood streaking within the white rot. The role of the *Phomopsis* spp. (Péros *et al*., 2008) and the role of the Botryosphaeriaceae are unclear (Calzarano and Di Marco, 2007).

Pleurostomophora richardsiae was rarely found in the present study. This is a lesser known grapevine trunk fungus that can cause vascular streaking lesions in pruning wound and trunk inoculations (Halleen *et al*., 2007) and its involvement in esca is not certain. It has been frequently isolated from young diseased vines, but often from graft unions specifically around the pith region (Halleen and Groenewald, 2005).

Stereum hirsutum was not isolated in this study, indicating that it is not associated with esca in South Africa. This fungus was believed to be the casual agent in South African esca diseased vines (Marais, 1981). The role of *Stereum hirsutum* in esca is still unclear (Reisenzein *et al*., 2000), since pathogenicity studies conducted in Austria could not reproduce any typical esca symptoms. However, Larignon and Dubos (1997) considered it a pioneer fungus as it caused inner necrosis, as well as wood degradation, despite the fact that it was isolated from only 20% of the esca-affected vines investigated in France.

CONCLUSIONS

Esca is more prominent and widespread in South Africa than previously thought and occurs in the three provinces where grapevine production dominates. External symptoms were observed in the field and five internal symptom types were identified. The five internal symptoms included a white rot; black and brown wood streaking; brown necrosis within the white rot; sectorial brown necrosis; and a central brown/ red/ black margin. The external symptoms and the predominant fungi isolated were similar to those observed in Europe and the USA. The isolated fungi included basidiomycetes, *Ph. chlamydospora* and *Phaeoacremonium* species (mostly *P. aleophilum*), confirming these taxa as the main causal organisms of esca diseased vines. Additionally, *Phomopsis* species, Botryosphaeriaceae and *E. lata* were also found, but to a lesser extent.

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Table 1. The geographic distribution of esca diseased vines in South Africa, the cultivars affected by the disease and their age.

Province	District[1]	Town	Affected Cultivars[2]
Western Cape	Botriver	Botrivier	Chenin blanc (20, 23)
	Calitzdorp	Calitzdorp, De Rust, Ladismith, Montagu, Oudtshoorn, Prins Albert	Chenin blanc (28, 38), Colombar (27), Fransdruif (33), Hanepoot (37), Merlot (3), Pinotage (29), Red Muscadel (31)
	Cape Point	Constantia	Sauvignon blanc (18, 25)
	Darling	Darling	Chenin blanc (21, 23)
	Lutzville Valley	Klawer, Lutzville	Chenin blanc (41), Colombar (20), Fransdruif (35)
	Overberg	Grabouw	Chardonnay (15), Sauvignon blanc (15)
	Paarl	Franschoek, Paarl, Wellington	Cabernet sauvignon (13, 14), Chenin blanc (18, 20, 25, 40) Hanepoot (22)
	Robertson	Ashton, Bonnievale, Klaas Voogds	Red Globe (10)[#], Sauvignon blanc (20), Shiraz (30)
	Stellenbosch	Somerset West, Stellenbosch	Cabernet sauvignon (15, 19, 31, 32), Chenin blanc (26), Hanepoot (12), Malbec (12), Pinotage (28), Ruby cabernet (22), Tinta barocca (28), Sauvignon blanc (16, 23, 25)
	Swartland	Piketberg, Porterville, Malmesbury, Riebeek Kasteel, Riebeeck Wes	Dan Ben Hannah (19), Chenin blanc (19, 20, 36), Colombar (15), Pinotage (36)
	Tulbagh	Tulbagh	Chenin blanc (24, 28)
	Tygerberg	Durbanville	Chenin blanc (26, 34), Sauvignon blanc (23), Shiraz (21)
	Walker Bay	Hermanus	Chardonnay (21)
	Worcester	De Doorns, Rawsonville, Slanghoek	Chenin blanc (11, 20), Hanepoot (40), Sultana (18)[#], Red Globe (9)
Northern Cape		Keboes, Keimoes, Kanon Eiland, Marchand, Prieska	Chenin blanc (18), Colomino (18), Sultana (26, 40)[* #]
Limpopo		Marken	Prime seedless (5)[#]

[1] Districts as described by the South African Wine Industry Information & Systems (SAWIS).
[2] Cultivars affected by esca with the age of the sampled vines indicated in brackets.
[#] Table grape cultivars; [*] Raisin cultivars; those which are not indicated are wine grapes

Table 2. The total number of isolations and fungi isolated from each of the five symptom types associated with esca diseased vines collected in South Africa.

Symptom Type	Isolated fungi [a]									Total number of isolates[b]	Total number of isolations
	Basidiomycetes	***Phaeoacremonium aleophilum***	***Phaeoacremonium* spp.**	***Phaeomoniella chlamydospora***	***Phomopsis viticola***	***Phomopsis* spp.**	***Botryosphaeriaceae***	***Eutypa lata***	***Pleurostomophora richardsiae***		
White rot	3026 (30.4)	738 (7.4)	208 (2.1)	478 (4.8)	9 (0.1)	0 (0)	38 (0.4)	43 (0.4)	14 (0.1)	**4554 (33.3)**	**9944**
Black and brown wood streaking	32 (1.8)	26 (1.4)	19 (1.1)	818 (45.4)	0 (0)	0 (0)	19 (1.1)	0 (0)	0 (0)	**914 (6.7)**	**1803**
Brown necrosis within white rot	202 (20.4)	158 (15.9)	14 (1.4)	135 (13.6)	0 (0)	0 (0)	15 (1.5)	4 (0.4)	11 (1.1)	**539 (3.9)**	**992**
Sectorial brown necrosis	215 (5.5)	368 (9.4)	45 (1.2)	813 (20.8)	247 (6.3)	3 (0.1)	419 (10.7)	294 (7.5)	1 (0.03)	**2405 (17.6)**	**3904**
Central brown/ red/ black margin	1220 (15.1)	1096 (13.6)	205 (2.5)	2351(29.1)	124 (1.5)	0 (0)	171 (2.1)	89 (1.1)	1 (0.01)	**5257 (38.5)**	**8072**
Number of isolates[b]	**4695 (19)**	**2386 (9.7)**	**491 (2)**	**4595 (18.6)**	**380 (1.5)**	**3 (0.1)**	**662 (2.7)**	**430 (1.7)**	**27 (0.1)**	**13669**	**24715**

[a] The number in brackets represents the percentage of isolates of a specific fungus from the total number of isolations that was made from that symptom type.
[b] The number in brackets represents the percentage of isolates retrieved from that symptom type.

Fig. 1. Vines which showed signs of dieback in the vineyard. A. Missing vines in the field; B – C. Dieback as seen in a few of the vines; D – E. Vine showing loss of leaves; F. Foliar symptoms may be visible.

Fig. 2. A wilted and dead vine due to apoplexy: berries appear shriveled and berry size as well as bunch size was uneven. Upon sectioning of the vine, white rot was present.

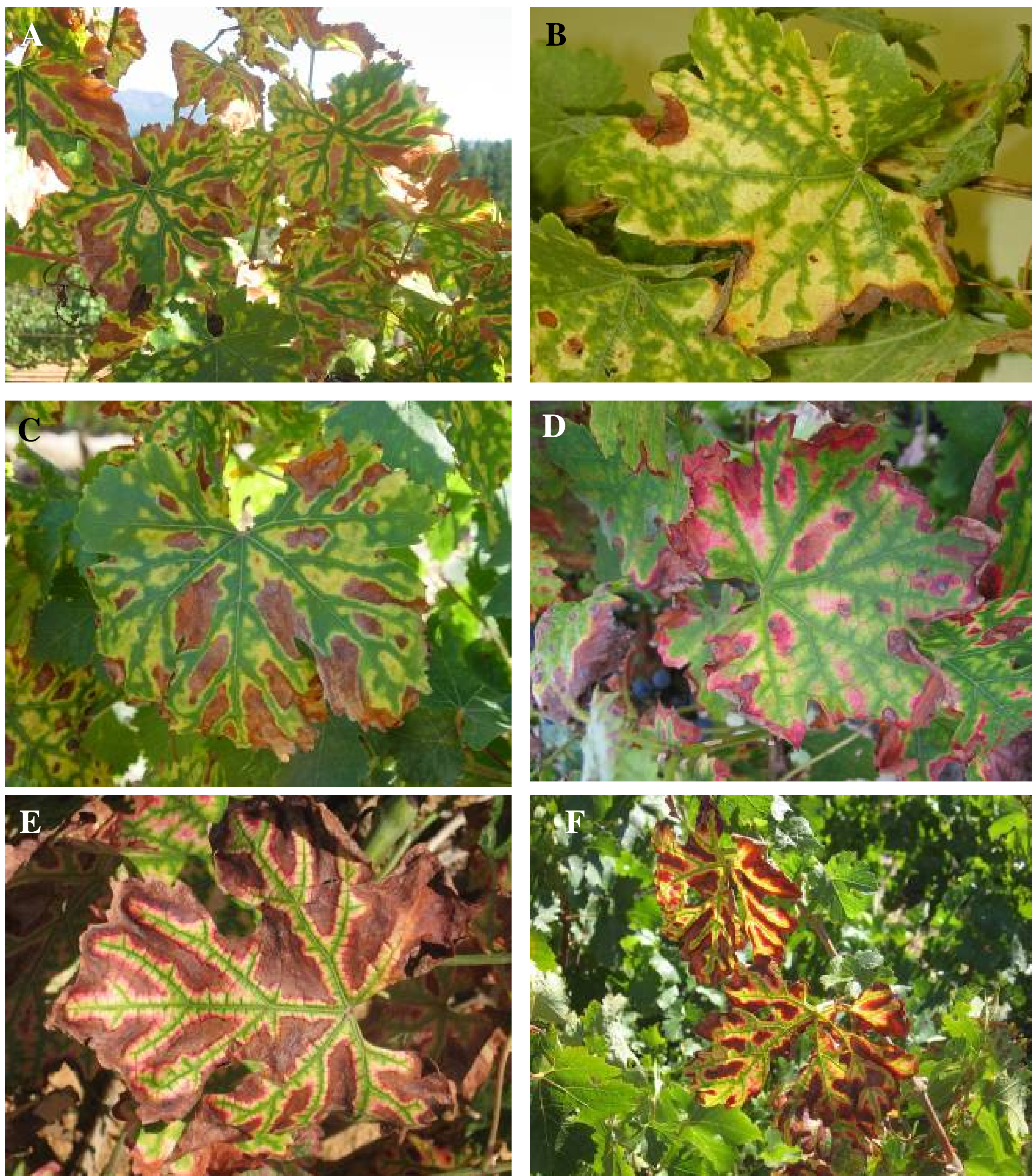

Fig. 3. Variation in foliar symptoms found on diseased vines. A – C: Tiger stripes on white cultivars; D – F: Tiger stripes found on red cultivars showing the reddish scorching on the perimeter of the leaves. Not all esca diseased red cultivars had leaves with red scorching.

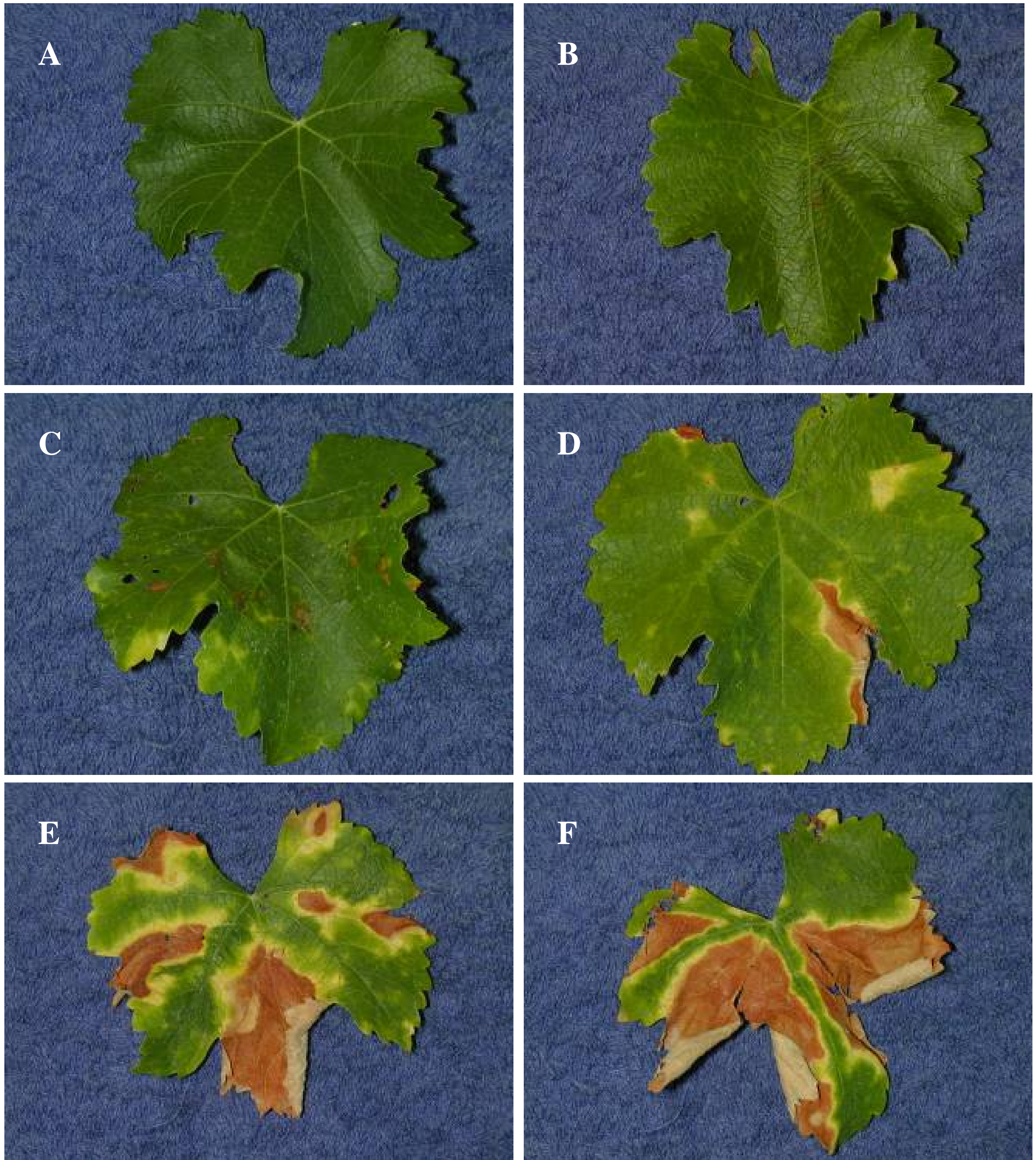

Fig. 4. The progression of foliar symptoms on a white cultivar. A. Healthy leaf; B. Very small chlorotic spots; C. Chlorotic areas increase in size and necrosis started; D. Necrosis started on the perimeter with chlorosis preceding; E. Tiger stripe formation with chlorosis along the veins and necrosis on the tips of the leaves moving inwards; F. Diseased leaf before total necrosis and death.

Fig. 5. External symptoms found on diseased berries. A. Healthy bunch compared to a diseased bunch showing shriveling and discoloration; B. Diseased bunch amongst healthy bunches which have started to discolour; C – D. Shriveling of berries and uneven berry size; E - F: Black measles observed in a Hanepoot vineyard in Stellenbosch. Here berries were cracked and split.

Fig. 6. Basidiocarps found on grapevines sampled from various areas. A. Wellington; B. Upington; C. Wellington; D. Stellenbosch; E. Paarl; F. Stellenbosch.

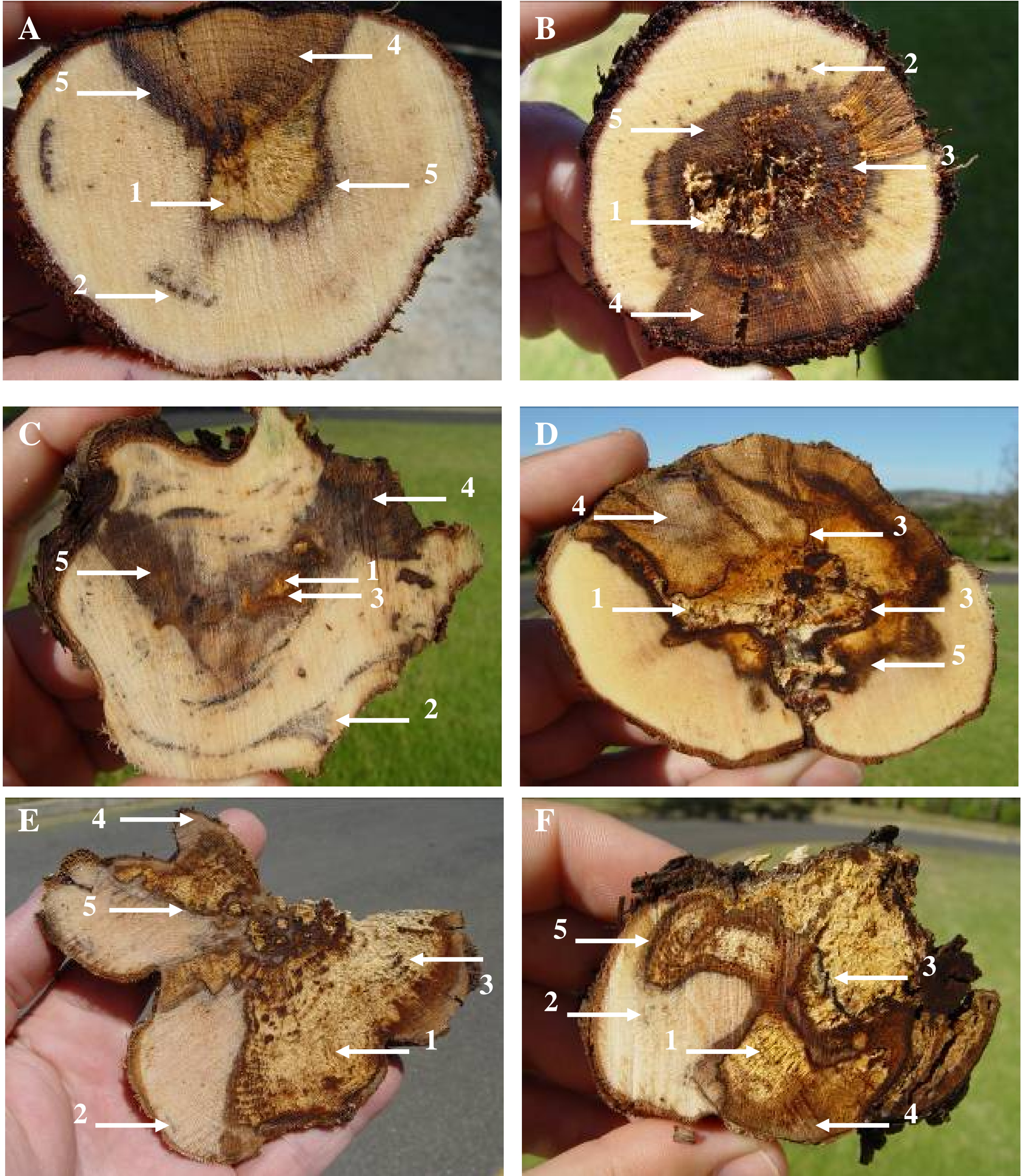

Fig. 7 A - F. Cross sections showing the internal symptoms and symptom types found in diseased vines. 1. White rot; 2. Black and brown wood streaking; 3. Brown necrosis within white rot; 4. Sectorial brown necrosis; 5. Central brown/ red/ black margin. Not all vines exhibit all the symptoms at the same time but the white rot was always present.

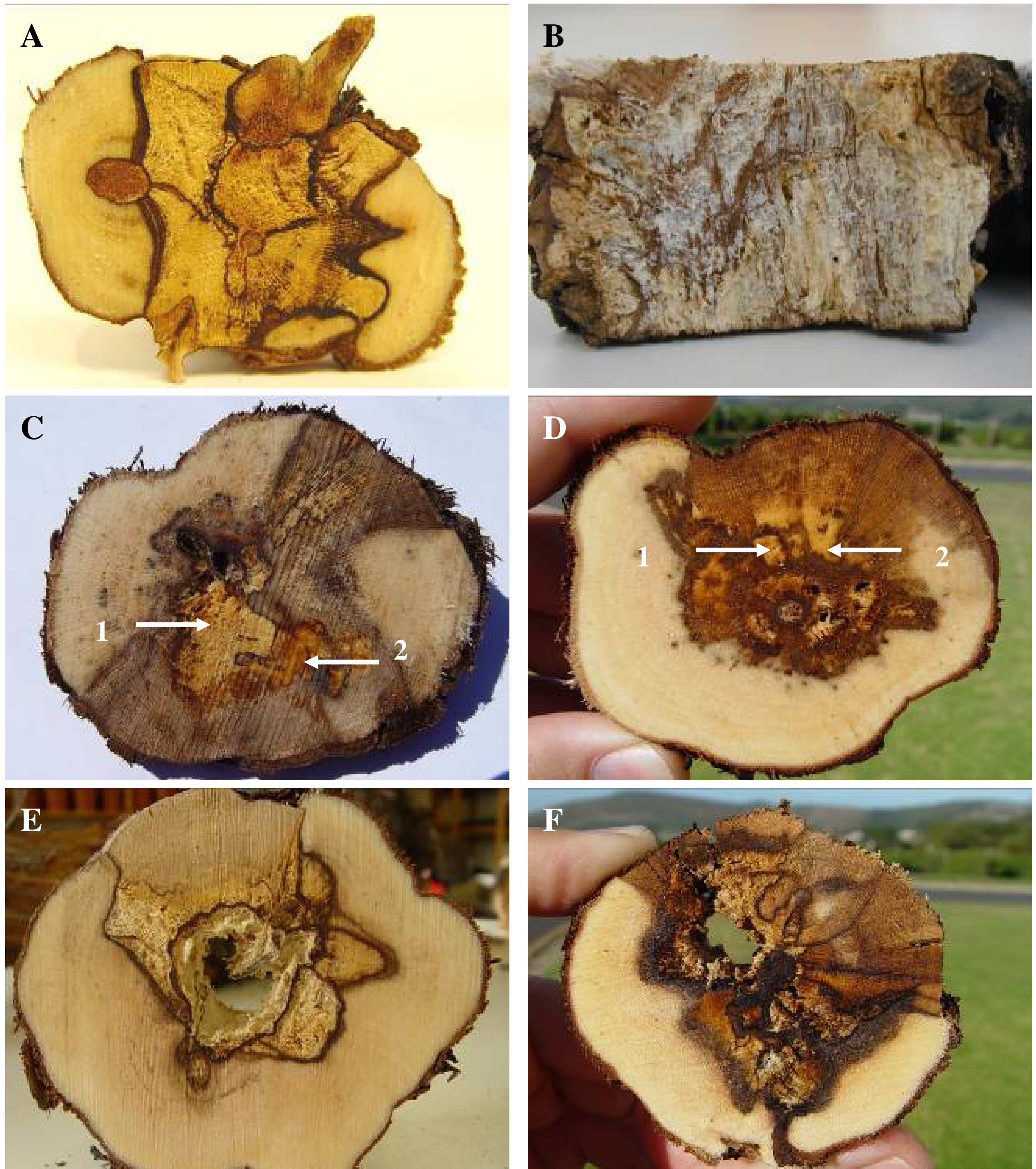

Fig. 8. Sections that illustrate the variation in white rot. A. White rot surrounded by dark lines, no other symptom types visible; B. Cross section showing soft rot; C and D. Arrow 1 shows the typical white rot and arrow 2 illustrates the orange colored rot; E. Soft rot which has been completed deteriorated wood, leaving a hollow center; F. Deterioration in the trunk due to the white rot.

CHAPTER 3

CHARACTERIZATION OF THE FUNGI ASSOCIATED WITH ESCA DISEASED GRAPEVINES IN SOUTH AFRICA

ABSTRACT

The etiology of esca, a disease caused by a complex of fungi, has not yet been investigated in grapevines in South Africa. Grapevines showing the presence of foliar and/ or internal symptoms of esca were collected from various grape growing regions in South Africa. Isolations were made from typical internal wood symptoms associated with esca. Fungal isolates were characterized by cultural growth patterns, morphology on media and phylogenetic inference with sequences. The gene regions sequenced included the internal transcribed spacers and the 5.8S rRNA gene for the basidiomycetes and *Phomopsis* isolates, the partial β-tubulin gene for *Phaeoacremonium* isolates and the partial translation elongation 1-α gene for the Botryosphaeriaceae isolates. *Phaeomoniella chlamydospora* and six species of *Phaeoacremonium* (including *P. aleophilum*, *P. alvesii*, *P. parasiticum*, *P. iranianum*, *P. mortoniae* and *P. sicilianum*) were also isolated, of which the latter three are reported for the first time in South Africa. The other taxa that were found include *Eutypa lata*, *Phomopsis viticola*, *Phomopsis* sp. 1, *Diaporthe ambigua*, *Diplodia seriata*, *Neofusicoccum australe* and *N. parvum*. The basidiomycete isolates were distributed over 10 well supported monophyletic clades among genera of the Hymenochaetales, of which two clades could be identified as species of *Fomitiporia* and *Phellinus*. This diverse array of basidiomycete species shows that the disease is more complex than what was previously thought.

INTRODUCTION

Esca has been studied in various grapevine producing countries, including Argentina, Australia, France, Germany, Greece, Italy, Portugal, Spain and the USA (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Gatica *et al*., 2000; Pascoe and Cottral, 2000; Armengol *et al*., 2001; Edwards *et al*., 2001; Redondo *et al*., 2001; Rumbos and Rumbou, 2001; Fischer and Kassemeyer, 2003; Feliciano *et al*., 2004; Gubler *et al*., 2004; Sofia *et al*., 2006; Martin and Cobos, 2007). Esca of grapevines is a relatively unknown disease in South Africa, with only a few incidences reported in the past (Marais, 1981). There is little information available on the causal fungi of esca in South Africa, especially for the basidiomycete fungi.

Esca is a complex disease which is caused by different fungi. Petri disease is seen as the precursor of esca (Fischer 2006), of which *Phaeomoniella* (*Ph.*) *chlamydospora* (W. Gams, Crous & M.J. Wingf. & L. Mugnai) Crous & W. Gams and various species of *Phaeoacremonium* are involved (Pascoe and Cottral, 2000; Fischer and Kassemeyer, 2003). The white rot symptoms associated with esca are caused by basidiomycete fungi such as *Fomitiporia* (*F.*) *mediterranea* M. Fischer, *F. polymorpha* M. Fischer and *F. australiensis* M. Fisch., J. Edwards, Cunnington & Pascoe (Fischer, 2002; Fischer and Kassemeyer, 2003; Fischer and Binder, 2004; Fischer, 2006). The Petri disease fungi, together with the basidiomycetes, have traditionally been seen as the causal agents of esca (Mugnai *et al*., 1999).

Other trunk disease fungi have been isolated from esca diseased vines, making the etiology of the disease difficult. Trunk disease fungi regularly isolated from esca diseased vines include species of the Botryosphaeriaceae, *Eutypa lata* Tul. & C. Tul and *Phomopsis viticola* (Sacc.) Sacc. (Fischer and Kassemeyer, 2003; Calzarano and Di Marco, 2007; Péros *et al*., 2008). Their role in esca is not clear, since they cause diseases such as Botryosphaeria canker, Eutypa dieback and Phomopsis cane and shoot blight respectively (Mauro *et al*., 1988; Tey-Rulh *et al*., 1991; Mugnai *et al*., 1999; Molyneux *et al*., 2002; Mahoney *et al*., 2003; van Niekerk *et al*., 2005).

The fungi associated with esca in Italy, France and Spain include *F. mediterranea*, *Ph. chlamydospora*, *P. aleophilum*, *Phomopsis viticola* and species

within the Botryosphaeriaceae (Larignon and Dubos, 1997; Mugnai *et al*., 1999; Sánchez-Torres *et al*., 2008). In Germany and Greece, the same species, as well as *Cylindrocarpon* species, were found (Rumbos and Rumbou, 2001; Fischer and Kassemeyer, 2003). A study in Castilla y León, Spain, found *F. mediterranea*, *Stereum hirsutum* (Willd.: Fr.) Pers. and *Phomopsis viticola* only on a few occasions (Martin and Cobos, 2007). However, another study found that *F. mediterranea* and *S. hirsutum* were the most frequently isolated species in the Comunidad Valenciana in Spain (Sánchez-Torres *et al*., 2008). *Stereum hirsutum* has been isolated in low frequencies from esca diseased vines in Europe and is seen as a secondary invader that does not attack living wood (Reisenzein *et al*., 2000; Fischer, 2006).

The perception that basidiomycetes are not a threat to grapevines has limited the research in this field (Fischer, 2006). Therefore, data pertaining to the distribution and identity of basidiomycetes from the different countries is poorly represented (Fischer, 2002; Fischer *et al*., 2005). Basidiomycete taxa seem to be specific to a continent or geographic region. *Fomitiporia mediterranea* is the most common species in Europe. *Fomitiporia australiensis* is restricted to Australia and *F. polymorpha* to North America (Fischer, 2006). *Inocutis jamaicensis* and *Fomitiporella vitis*, associated with 'hoja de malvón' and chlorotic leaf roll, respectively, occur on grapevines in South America (Fischer, 2006).

Various studies on South African grapevines have investigated Petri disease fungi (Crous *et al*., 2000; Groenewald *et al*., 2001; Mostert *et al*., 2006b), Botryosphaeriaceae (van Niekerk *et al*., 2004), *Phomopsis* species (Mostert *et al*., 2001; van Niekerk *et al*., 2005) and Diatrypaceae (Mostert *et al*., 2004; Safodien, 2007). There has been no extensive characterization on the basidiomycetes in South Africa. In a review of basidiomycete taxa from grapevines worldwide, ten basidiomycete isolates from esca diseased grapevines in South Africa were included (Fischer, 2006). The ITS phylogeny revealed that these South African isolates formed three unrelated and new clades. One was closely related to *F. mediterranea* and two were related to *Inocutis s.l.*

The extent of fungal taxa found in esca-related symptoms of grapevines in South Africa is not known. Also, limited information is available on the basidiomycete taxa associated with the white rot of esca in South Africa. Therefore the aim of this study was

to characterize the different fungi associated with esca diseased vines collected from geographically different grape growing regions in South Africa.

MATERIALS AND METHODS

Sampling of esca diseased vines

Vineyards showing leaf symptoms of esca, general decline or dieback were identified and sampled from all the major grapevine producing areas in South Africa from 2001 until 2008. Vines showing typical symptoms (external and/ or internal) were removed and taken to the laboratory, where transverse sections of the wood were made. Esca diseased vines were identified as having brown-black internal discoloration accompanied by white rot.

Fungal isolations and storage

Cross and longitudinal sections were made at various places in the cordons and trunk of each plant to investigate internal necrosis. For fungal isolations, wood sections with internal necrosis were selected and cut into two smaller sections adjacent to each other, in order to obtain two mirror images of the same symptom type. This was also done to facilitate the use of two sterilization techniques to ensure fungal isolation from soft, spongy material. A photograph of each wood section showing the various symptom types was taken. The one section was flame sterilized by holding the wood with sterile forceps, lightly spraying it with 70 % ethanol and passing it through a flame. The other piece was triple sterilized as follows: 30 seconds in 70 % ethanol, 2 minutes in 3.5 % NaOCl and 30 seconds in 70 % ethanol. Twelve small sections of wood (1 x 1 x 2 mm) from each of the different symptom types were then aseptically removed with a scalpel and placed onto Potato Dextrose Agar (PDA, Biolab, South Africa) plates containing 250 mg chloramphenicol (four pieces per plate). Plates were incubated at 23 - 25°C for approximately four weeks. The growth was monitored daily.

Isolates were identified according to morphological and cultural characteristics as species of basidiomycetes (Fischer 2002), Botryosphaeriaceae (van Niekerk *et al*., 2004; Crous *et al*., 2006; Damm *et al*., 2007; Phillips *et al*., 2008), *Eutypa* (Glawe and Rodgers,

1982), *Phaeoacremonium* (Essakhi *et al*., 2008; Mostert *et al*., 2006b), *Phomopsis* (Mostert *et al*., 2001; van Niekerk *et al*. 2005) and *Phaeomoniella chlamydospora* (Crous and Gams, 2000). The cultures were purified through hyphal tipping or single sporing, if possible, and pure cultures were grown on PDA. Cultures were stored in sterile water in 14 ml McCartney bottles kept at 4°C. All of the basidiomycete isolates and a selection of isolates of the other genera were deposited in the fungal culture collection at the ARC Infruitec-Nietvoorbij in Stellenbosch. The selected isolates used in this study were also placed in the STE-U culture collection at the Department of Plant Pathology, Stellenbosch University.

Phenotypic characterization

Seventeen *Phomopsis* isolates that showed growth patterns different from *Phomopsis viticola* were selected from the primary isolations for further characterization. The basidiomycetes and Botryosphaeriaceae isolates were plated onto PDA and incubated at 25°C. After two weeks 18 out of 137 isolates of Botryosphaeriaceae were selected on the basis of different cultural growth patterns. Similarly, after four weeks, a selection of 134 of the 350 basidiomycete isolates was made. The isolates represented all of the sampled geographical regions and included all of the different cultural growth patterns.

A further selection of 31 basidiomycete isolates, representative of the different phylogenetic clades, was made for a growth study. These isolates were grown on PDA and mycelial plugs of 2 mm diameter were re-plated out onto PDA and incubated at 25°C. After 14 days, the diameters of the colonies were measured. These same basidiomycete isolates were further used in the toxin and enzyme analyses (Chapter 4).

Cultural growth patterns were determined for 38 isolates of *Phaeoacremonium*. Mycelial plugs of 2 mm diameter of the *Phaeoacremonium* isolates were grown on PDA, Malt extract agar (MEA; 2 % malt extract, Oxoid Ltd, England; 1.5 % agar, Difco, Le Pont de Claix, France) and on Oatmeal Agar (OA; Difco, Le Pont de Claix, France). Plates were incubated at 25°C for eight days and the radial growth of the colonies was measured. After 16 days, the colour of the colonies was determined (Rayner, 1970). From these results, 17 *Phaeoacremonium* isolates were selected for further identification.

Botryosphaeriaceae isolates were grown on sterile pine needles on water agar (WA, Biolab, South Africa) to induce the formation of pycnidia (Crous *et al*., 2006). Microscopic slides of the selected Botryosphaeriaceae, *Phomopsis* and *Phaeoacremonium* isolates were made in lactic acid. Conidial dimensions were measured under a light microscope (Axioskop, Zeiss, Germany). Twenty-four spores were measured from each isolate and 95 % confidence intervals were calculated.

The selected isolates of the different taxa were stored in 14 ml McCartney glass bottles filled with sterile distilled water in the culture collection of the Department of Plant Pathology, University of Stellenbosch at $8^{o}C$ (Table 1).

DNA isolation and amplification

DNA was extracted from the selected 134 basidiomycetes, 18 Botryosphaeriaceae and 17 *Phomopsis* and 17 *Phaeoacremonium* isolates. The CTAB based method was used as described by Damm *et al*. (2008). Mycelia were scraped from agar plates and placed in 2 ml Eppendorf tubes. Approximately 0.5 g of glass beads and 600 µl of CTAB extraction buffer were added to each tube. The Eppendorf tubes were then shaken in a Retsch® MM301 (Retsch® GmbH and Co.) at a frequency of 30 shakes/ s. Tubes were then incubated in a water bath set at $65^{o}C$ for approximately 30 - 45 minutes. To each tube, 400 µl of chloroform:isoamylalcohol (24:1) was added and inverted 5 - 10 times. Samples were centrifuged for 10 minutes at 14 000 rpm. The supernatant was collected and to this, cold 7.5 M ammonium acetate solution was added until a concentration of 2.5 M was reached. Six hundred µl of cold isopropanol was added and the tubes inverted. Tubes were then placed in the freezer for 15 minutes and centrifuged at 14 000 rpm for 15 minutes. The supernatant was discarded and one ml of cold 70 % ethanol was added. The samples were centrifuged again at 14 000 rpm for seven minutes, the supernatant discarded and the pellet was dried at room temperature overnight. The pellet was then resuspended in 100 µl of dH_2O.

The partial β-tubulin region was amplified for the *Phaeoacremonium* isolates with the primers T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995) which amplified a region of approximately 600 bp. The reaction mixture for PCR contained 1 µl of undiluted DNA, 1×PCR buffer (Bioline, London, United Kingdom), 2.5

pmol of each primer, 200 μM of each dNTP, 0.5 U of Taq Polymerase (Bioline), 1.5 mM $MgCl_2$ (Bioline) and the reaction was made up to a total volume of 25 μl with sterile water. The PCR amplification cycles included a denaturing step at 96°C for five minutes followed by 36 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 90 s followed by an extension step at 72°C for seven minutes.

For the Botryosphaeriaceae isolates, a region of 350 bp of the elongation factor-1α was amplified with the primers EF1-728F and EF1-986R (Carbone and Kohn, 1999). The PCR reaction contained 1 μl of undiluted DNA, 1× PCR buffer, 4 pmol of each primer, 0.2 mM of each dNTP, 1.5 U of Taq Polymerase, 2.5 mM $MgCl_2$ and the reaction was made up to a total volume of 25 μl with sterile water. The PCR amplification cycles included a denaturing step at 95°C for eight minutes followed by 35 cycles of 95°C for 15 s, 55°C for 20 s and 72°C for 60 s followed by an extension step at 72°C for five minutes.

The internal transcribed spacers 1 and 2 and the 5.8S rRNA gene were amplified for the *Phomopsis* species. The ITS1 and ITS4 primers (White *et al*., 1990) were used, which amplified a region of approximately 510 - 530 bp. The PCR reaction contained 1 μl of undiluted DNA, 1× PCR buffer, 4 pmol of each primer, 0.2 mM of each dNTP, 1.5 U of Taq Polymerase, 2.5 mM $MgCl_2$ and the reaction was made up to a total volume of 25 μl with sterile water. The parameters used were a denaturing step at 96°C for five minutes followed by 30 cycles of 96°C for 30 s, 55°C for 30 s and 72°C for 90 s followed by an extension step at 72°C for seven minutes.

The ITS region was also amplified for the basidiomycetes. The ITS1 and ITS4 primers were used and amplified a region of approximately 500 - 600 bp. Due to the presence of dikaryotic mycelium of basidiomycete fungi (Clark and Anderson, 2004) the PCR products were cloned to ensure sequencing of a single copy. A PCR reaction for each isolate was performed to amplify the ITS region using two μl undiluted DNA, 0.5 mM of each primer, 0.2 mM of each dNTP, 1× PCR buffer without $MgSO_4$ (Fermentas Life Sciences, St. Leon-Rot, Germany), 0.5 U of Pfu Taq Polymerase (Fermentas Life Sciences) four mM $MgSO_4$ (Fermentas Life Sciences), and the reaction was made up to a total volume of 25 μl with sterile water. The parameters used were a denaturing step at

95°C for three minutes, followed by 40 cycles of 95°C for one minute, 45°C for one minute and 72°C for two minutes, followed by an extension step at 72°C for five minutes.

The PCR reactions were run on a GeneAmp PCR System 9700. All PCR products were visualized under UV light on a one % agarose gel stained with ethidium bromide. The PCR products were cleaned using the MSB Spin PCRapase kit (Invitek, Berlin, Germany). The ITS products of the basidiomycetes were cloned using the CloneJET™ PCR cloning kit (Fermentas Life Sciences) according to manufacturer's instructions. Colonies were selected and a PCR reaction performed to obtain a product which was then cleaned using the MSB Spin PCRapase kit (Invitek, Germany).

The cleaned products were sequenced in both directions using an ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA) with the primers used in the initial PCR reactions. The products were then analyzed on an ABI Prism 3130XL DNA sequencer (Perkin-Elmer, Norwalk, CN).

Phylogenetic analyses

Sequences were edited and a consensus sequence made from the forward and reverse sequences using Geneious Pro v3.6.2. Reference sequences representing the relevant species for Botryosphaeriaceae (van Niekerk *et al*., 2004), *Phaeoacremonium* (Essakhi *et al*., 2008; Mostert *et al*., 2006b), *Phomopsis* (van Niekerk *et al*., 2005) and the basidiomycetes (Fischer, 2006) were obtained from GenBank and included in the different alignments. The sequences were automatically aligned using MAFFT v6 (Katoh *et al*., 2002) and further manual alignment was performed using Sequence alignment editor v2.0a11 (Rambaut, 2002).

Maximum parsimony analyses were performed using PAUP (Phylogenetic Analysis Using Parsimony) v4.0b10 (Swofford, 2003), using the heuristic search option, with 10 random taxon additions for all the datasets. Tree bisection and reconstruction was used as the branch swapping algorithm. All characters were unordered and of equal weight. Gaps were treated as missing data. Bootstrap support values were calculated from 1000 heuristic search replicates. Measures calculated for parsimony included tree length (TL), consistency index (CI), retention index (RI) and the rescaled consistency index (RC) values.

RESULTS

Phenotypic characterization

One hundred and thirty-four basidiomycetes, 18 Botryosphaeriaceae and 17 *Phomopsis* and 17 *Phaeoacremonium* isolates were characterized in this study. The origin, cultivar and age of the isolates are listed in Table 1. The STE-U number as well as the collection date is also stated.

The 38 *Phaeoacremonium* isolates had 12 cultural growth patterns which are summarized in Table 2. The colony textures were in most cases felty on MEA and PDA, and woolly on OA. Only two isolates, one of *P. parasiticum* (Ajello, Georg & C.J.K. Wang) W. Gams, Crous & M.J. Wingf. (STE-U 6990) and one of *P. iranianum* L. Mostert, Gräfenhan, W. Gams & Crous (STE-U 6999) were felty on OA. Variable colony characters were observed among the isolates of *P. aleophilum*, *P. alvesii* L. Mostert, Summerb. & Crous , *P. iranianum*, *P. parasiticum* and *P. sicilianum* Essakhi, Mugnai, Surico & Crous. Conidial dimensions were measured for 17 isolates representing the different cultural growth patterns and are shown in Table 3.

Of the 17 isolates of *Phomopsis,* seven isolates formed pycnidia and were identified as *Phomopsis viticola*. The alpha conidia dimensions are given in Table 4. The production of beta conidia was variable and in one case (STE-U 7008) only beta conidia were produced.

The colony growth of the 18 Botryosphaeriaceae isolates varied from pale grey to dark grey or olivaceous coloured and the colony textures were mostly woolly. The colony colour of *Diplodia seriata* De Not isolates varied. *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips had pale olivaceous grey colonies. Four isolates of *D. seriata* and one of *N. parvum* formed pycnidia. The conidial dimensions of these isolates are reported in Table 5. Conidia of *N. parvum* were hyaline and aseptate, while those of *D. seriata* were brown and aseptate with the inner walls appearing rough in texture.

The basidiomycete isolates were grouped into 15 cultural growth patterns reported in Table 6. The different taxa, as determined by the phylogenetic analysis, comprised of various cultural growth patterns. However, Taxa 7 and 8 were distinctly

only orange-brown in colour with fluffy mycelial growth. Since these fungi do not form fruiting structures in culture, only the colony diameters were measured for selected isolates of each taxon (Table 7). Taxon 4 was the slowest growing taxon with a colony diameter ranging from 29 to 41 mm. Taxon 7 was also slow growing (45 - 57 mm in diameter), but overlapped with other taxa. The colony diameter of *Fomitiporia*, Taxon 1 and Taxon 5, had more variation among their isolates than the other taxa.

Phaeomoniella chlamydospora isolates were identified on the basis of their distinct olive green to white yeast-like growth on PDA, pigmented conidiophores and small oblong-ellipsoidal conidia (Crous and Gams, 2000). The cultures of *Ph. chlamydospora* were not characterized further since previous studies have shown that only one species, which has very little variation, occurs on grapevine (Mostert *et al*., 2006a; Pottinger *et al*., 2002).

Phylogenetic analyses

The β-tubulin alignment of 17 *Phaeoacremonium* isolates included 603 nucleotides (including gaps) of which 335 nucleotides were parsimony-informative. A maximum parsimony analysis was conducted and isolates were grouped in well supported clades with known *Phaeoacremonium* species (Fig. 1). Identities were conclusive in most cases with bootstrap supports of 100%. These isolates were identified as *P. aleophilum* (STE-U 6986, 6991, 6996, 6997 and 7002), *P. iranianum* (STE-U 6998 and 6999), *P. mortoniae* (STE-U 6987), *P. parasiticum* (STE-U 6990 and 6993) and *P. sicilianum* (STE-U 6992, 6994 and 6994). Only four isolates (STE-U 6988, 6989, 7000 and 7001) grouped with other isolates of *P. alvesii* with a bootstrap support of 96 %.

The parsimony analysis for the *Phomopsis* isolates included 494 nucleotides (including gaps) of which 111 were parsimony-informative. The phylogram resulted in six major clades and several sub-groups (Fig. 2). Identities were conclusive for two species with bootstrap supports of 100%. Fourteen of these isolates were identified as *Phomopsis viticola*, and one isolate grouped with *Diaporthe ambigua* Nitschke. Two isolates grouped with *Phomopsis* sp. 1 (STE-U 7010 and 7016) with a bootstrap support of 71 %.

The EF alignment of the Botryosphaeriaceae included 341 nucleotides (including gaps) of which 215 were parsimony-informative. Isolates STE-U 7020, 7026, 7031, 7032, 7033, 7034 and 7035 grouped with *Diplodia seriata* with a bootstrap support of 63 %. *Diplodia seriata* was the most predominant species found (Fig. 3). *Diplodia seriata* grouped basal to *D. pinea* (Desm.) J. Kickx f. (bootstrap support of 64 %) and *D. scrobiculata* J. de Wet, Slippers & M.J. Wingf. (bootstrap support of 95 %). Isolates STE-U 7024, 7025, 7028, 7029 and 7030 grouped with reference sequences of *Neofusicoccum australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips with a bootstrap support of 77 %. The remainder of the isolates (STE-U 7021, 7023, 7026, 7027, 7036 and 7037) grouped with isolates of *N. parvum* with a bootstrap support of 100 %.

The identities of the *Eutypa* isolates were determined by Safodien (2007). The phylogenetic tree of the ITS region revealed that the isolates STE-U 5694, 5695, 5696, 5697, 5698, 5699 and 5700 clustered with *E. lata* reference sequences with a bootstrap support of 83 %. The phylogenetic tree of the β-tubulin region, which included the additional isolates STE-U 5692 and 5693, also grouped with the *E. lata* sequences with a bootstrap support of 100 %.

The ITS alignment of the 134 basidiomycete isolates included 931 nucleotides (including gaps) of which 548 were parsimony-informative. The sequences grouped into eight well supported monophyletic clades (Taxa 1 to 8) of which the genus identity is uncertain. A comparison was only made with known sequences on GenBank. Two clades clustered with the genera *Phellinus* and *Fomitiporia* (Fig. 4). Taxa 1, 2, 3, 4, 5, 7, 8, *Phellinus* sp. and *Fomitiporia* sp. each had a bootstrap support of 100 %. Taxon 6 had a bootstrap support of 82 %. Taxa 1 to 4 grouped with *Fomitiporella* cf. and *Inocutis* cf. and Taxa 5 to 8 grouped with *Inonotus*. Taxon 1 represented 42 % of the basidiomycete isolates, followed by *Fomitiporia,* which comprised 25 % of the isolates. *Phellinus* was the next largest Taxon (8 %) followed by Taxon 5 (7 %), Taxon 7 (4 %), Taxon 3 (3 %) and Taxon 2 (2 %). Taxa 4, 6 and 8 occurred the least and each comprised 1 % of the isolates. The majority of basidiomycete taxa included isolates from different regions and was not restricted to a specific location. Four taxa were restricted to a specific locality. Taxon 4 came only from Stellenbosch, Taxon 6 from Malmesbury and Taxon 8 from

Botrivier, however, only two isolates of each were found. *Phellinus* sp. isolates were all obtained from Keimoes, Kanon Eiland, Prieska and Upington (Northern Cape) and Marchand in Limpopo. It was also found that in a few cases, more than one (but not more than three) basidiomycete taxa were found within one plant. It could be possible that more species exist as only a certain section of the wood was examined and isolated from.

DISCUSSION

A variety of fungi are associated with esca symptoms in diseased vines in South Africa. These fungi include species of basidiomycetes and Botryosphaeriaceae, *Eutypa lata*, *Phaeoacremonium* spp.*, Phaeomoniella chlamydospora* and *Phomopsis* spp. There are also new species reported for the first time in the country.

Six species of *Phaeoacremonium* were isolated and include *P. aleophilum, P. alvesii, P. iranianum, P. mortoniae*, *P. parasiticum* and *P. sicilianum*. There are 25 species of *Phaeoacremonium* world-wide that have been isolated from either Petri diseased or esca grapevines (Crous *et al*., 1996; Mostert *et al*., 2006b; Essakhi *et al*., 2008; Graham *et al*., 2009; Gramaje *et al*., 2009). *Phaeoacremonium aleophilum* is the most common species on grapevines (Crous *et al*., 1996; Mostert *et al*., 2006b), followed by *P. parasiticum* (Mostert *et al*., 2006b), which has been confirmed in this study. In South Africa, *P. aleophilum*, *P. austroafricanum*, *P. krajdenii*, *P. parasiticum*, *P. scolyti*, *P. subulatum*, *P. viticola* and *P. venezuelense* have been previously isolated from grapevines (Mostert *et al*., 2006b). This is the first report of *P. mortoniae*, *P. iranianum* and *P. sicilianum* in South Africa.

Colony colours of *Phaeoacremonium* on the different media were useful for identification purposes, but variation within a species, as well as overlap among species, did occur. The colony radius for one isolate of *P. aleophilum* and two isolates of *P. sicilianum* were 2 mm longer and, for one isolate of *P. iranianum*, 4 mm longer than that observed by Mostert *et al*. (2006b) and Essakhi *et al*. (2008). Conidial sizes overlapped among the different species and were not useful for identification. However, more variation was observed in the conidial shapes than found by Mostert *et al*. (2005; 2006b)

and Essakhi *et al*. (2008). Phylogenetic analysis with β-tubulin sequences were the most accurate option for identification of *Phaeoacremonium* species.

In the current study, *Phomopsis viticola*, *Phomopsis* sp. 1, and *Diaporthe ambigua* were found to be associated with esca. The identification of species was mostly based on the ITS phylogeny, since not all the isolates sporulated. The conidial shape and sizes of the *Phomopsis viticola* isolates corresponded with those obtained by Mostert *et al*. (2001).

Van Niekerk *et al*. (2005) showed that a variety of 15 *Phomopsis* species occur on grapevines in South Africa. Of these, *Phomopsis viticola* and *Phomopsis amygdali* are the most pathogenic and cause the most severe lesions on pruning wounds and nursery plants in pathogenicity studies. *Phomopsis viticola* is commonly found on grapevines and is especially associated with Phomopsis cane and leaf blight (Mostert *et al*., 2001; van Niekerk *et al*., 2005). *Phomopsis* sp. 1 has a wider host range, including *Protea* sp., *Pyrus* sp. and *Prunus* sp. (Mostert *et al*., 2001; van Niekerk *et al*., 2005). *Diaporthe ambigua* rarely occurs on grapevine and is more commonly associated with cankers on *Malus* sp., *Prunus* sp. and *Pyrus* sp. (Smit *et al*., 1996; Crous *et al*., 2000; van Niekerk *et al*. 2005). In the current study, the *D. ambigua* isolate was obtained from a vineyard in Porterville.

Three species of the Botryosphaeriaceae, namely *D. seriata*, *N. parvum* and *N. australe*, were found associated with esca symptoms. However, eleven species of the Botryosphaeriaceae have been previously isolated from grapevines in South Africa (van Niekerk *et al*., 2004; van Niekerk *et al*., 2006). Of these, *D. seriata* (previously *Botryosphaeria obtusa*), *Neofusicoccum parvum* [previously *Botryosphaeria parva* (Crous *et al*., 2006)] and *Lasiodiplodia theobromae* (previously *B. rhodina*) were the most common (van Niekerk *et al*., 2003; van Niekerk *et al*., 2004). *Neofussiccocum australe* [previously *Botryosphaeria australis* (Crous *et al*., 2006)] was also commonly found and was the most pathogenic species on South African grapevines (van Niekerk *et al*., 2004). *Diplodia seriata*, *N. parvum*, *N. australe* and other Botryosphaeria species are associated with grapevines in other countries, such as Australia, USA (California), Portugal and Spain (Phillips, 2002; Pitt *et al*., 2008; Sánchez-Torres *et al*., 2008; Úrbez-Torrez *et al*., 2006).

Cultural growth patterns of the Botryosphaeriaceae overlap among species and can not be used as distinguishing characteristics, although the conidial morphology could be used to distinguish *D. seriata* and *N. parvum*. The gene regions that have been used to delineate species within the Botryosphaeriaceae include the EF, ITS and β-tubulin regions (van Niekerk *et al*., 2004; Úrbez-Torres *et al*., 2006; Phillips *et al*., 2008). The EF region used in this study gave low bootstrap support for two of the *Diplodia* species, namely *D. pinea* (64 %) and *D. seriata* (63 %). Other analyses have also shown similar bootstrap support for these species. In a study using only the ITS region, no support was found for the *D. pinea* clade, which grouped basal to *D. seriata* and *D. scrobiculata* (Phillips *et al*., 2007). The combined analysis of EF and ITS gave low bootstrap support for the *D. seriata* (67 %) and *D. pinea* clades (62 %) (Damm *et al*., 2007). *Diplodia pinea*, *D. scrobiculata* and *D. seriata* are phylogenetically closely related and also share morphological features, namely aseptate conidia that become pigmented within the pycnidium (Phillips *et al*., 2007).

In the current study, *Ph. chlamydospora* was commonly associated with esca diseased vines. *Phaeomoniella chlamydospora* is possibly the most ubiquitous species associated with esca-diseased vines or declining grapevines worldwide (Mostert *et al*., 2006a). Six additional species of *Phaeomoniella* have been found on other hosts. *Phaeomoniella zymoides* and *Ph. pinifoliorum* have been found on pine needles (Lee *et al*., 2006). *Phaeomoniella dura*, *P. effusa*, *P. prunicola*, *P. tardicola* and *Ph. zymoides* have been found on *Prunus* spp. trees in South Africa (Damm *et al*., 2010).

Genera of the Diatrypaceae that occur on grapevines include *Cryptosphaeria*, *Cryptovalsa*, *Diatrype*, *Diatrypella*, *Eutypa* and *Eutypella* (Trouillas *et al*., 2010). In South Africa *Cryptovalsa ampelina*, *Eutypa lata*, *Eutypa leptoplaca* and *Eutypella vitis* has been found on grapevines (Mostert *et al*., 2004; Safodien, 2007). In the present study, only *Eutypa lata* was found to be associated with esca symptoms (Safodien, 2007). *Eutypa lata* has also been found on esca diseased vines in Italy, Germany, Spain and France (Mugnai *et al.,* 1999; Fischer and Kassemeyer, 2003; Martin and Cobos, 2007; Péros *et al*., 2008).

Ten different basidiomycete taxa, not corresponding with known species on GenBank, were found in the current study. Two taxa could be linked to the genera of

Fomitiporia and *Phellinus*. According to the Index Fungorum online database, there are 51 *Fomitiporia* spp. and 466 *Phellinus* spp. The other taxa could possibly be *Fomitiporella*, *Inonotus* or *Inocutis* species. According to the Index Fungorum, there are 19 *Fomitiporella* spp., 231 *Inonotus* spp. and nine *Inocutis* spp. Two of the taxa, *Fomitiporia* sp. and Taxon 1, contained the majority of the basidiomycete isolates. For naming these phylogenetic taxa, the basidiocarps need to be linked to the sequence identity. Sequences were compared with known sequences on GenBank and not with morphology of the other described species and so these could be related to Basidiomycetes already described. However, basidiocarps are difficult to find and are often in a poor state, which makes field identifications difficult (Fischer, 2006). Isolated cultures do not form identifiable structures. Variable colony characters can occur within a taxon and may change after subculturing. Therefore, cultural characteristics cannot be used for identification purposes. *Phellinus* and *Fomitiporia* are also highly similar in basidiocarp morphology; therefore, phylogenetic species recognition using the ITS region is rather used for identification purposes (Fischer and Binder, 2004; Sánchez-Torres *et al*., 2008).

The diversity of basidiomycete taxa found in other countries was far less than that found in this study. *Fomitiporia mediterranea* is the most common basidiomycete species associated with esca in Europe (Fischer, 2006), *F. australiensis* in Australia (Fischer *et al*., 2005) and *F. polymorpha* and *F. robusta* in North America (Fischer and Binder, 2004; Fischer, 2006). *Fomitiporella vitis* is associated with chlorotic leafroll in Chile (Auger *et al*., 2005) and *Inocutis jamaciensis* with 'hoja de malvón' in Argentina (Bettuci *et al*., 2005). This illustrates that there are not more than four predominant species that occur in each of these countries. In the present study, ten taxa were found in South Africa, possibly due to the wide area of investigation which consisted of different climatic regions. Most of the taxa were found in the Western Cape Province. Stellenbosch had the highest diversity, with four taxa present (Taxon 1, 4, 7 and *Fomitiporia* sp.). However, this could be due to the bias in number of samples analyzed from this location. Some of the taxa were restricted to a specific area. In the Northern Cape or Limpopo provinces, which are known for their warmer climate, only *Phellinus* sp. (11 isolates) were found. These areas were also geographically isolated from the other

grapevine production areas in the Western Cape. Taxon 2 (three isolates) was only found in Oudtshoorn and Calitzdorp, which is about 400 km from the Cape Peninsula.

White rot symptoms varied in colour and texture, ranging from yellow to white and hard to soft (Chapter 2). This could be due to either the stage at which the white rot occurs, or the specific basidiomycete taxon present. Such different types of white rot have not been observed in Europe and this could be due to the presence of only one primary basidiomycete, *F. mediterranea*. The different basidiomycete taxa found in South African grapevines could contribute to the inconsistency in external symptoms observed due to their interactions with each other within the plant.

CONCLUSIONS

Basidiomycetes associated with esca diseased vines in South Africa belonged to ten new taxa not yet reported on grapevines. The large diversity of taxa is in contrast with the one to four species usually associated with esca in other countries. This is possibly due to the diverse areas of South Africa which were sampled. Knowing which basidiomycetes are present on grapevines in South Africa and having an ITS barcode for each species will aid in developing early detection tools. *Phaeomoniella chlamydospora, E. lata, Phaeoacremonium aleophilum, P. alvesii, P. parasiticum*, *P. iranianum*, *P. mortoniae*, *P. sicilianum, Phomopsis viticola*, *Phomopsis* sp. 1, *Diaporthe ambigua*, *Diplodia seriata*, *Neofusicoccum parvum* and *Neofusicoccum australe* were also isolated from grapevines showing esca symptoms, together with the basidiomycetes. Most of the species are mostly known from vines in the country. This is the first report of *P. iranianum, P. mortoniae*, and *P. sicilianum* in South Africa. Further work should include finding the basidiocarps of the basidiomycete taxa and linking them to the phylogenetic taxa. The basidiomycetes also need to be linked to the external symptoms on grapevines. External symptoms can be caused by toxins secreted by the fungi; therefore, the toxins that the basidiomycete taxa produce should also be researched. The epidemiology of the new basidiomycete taxa is not known and determining the spore dispersal patterns of these fungi would aid in developing effective control strategies.

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Table 1. Isolation details of *Phaeoacremonium, Phomopsis,* Botryosphaeriaceae, *Eutypa* and basidiomycete isolates obtained from esca diseased grapevines (*Vitis vinifera*).

STE-U Number	Species	Origin	Cultivar	Age of vine (years)	Collection date	Collector
	Phaeoacremonium					
6986	*P. aleophilum*	Hermanus	Chardonnay	21	2008/02/13	C. White, F. Halleen
6987	*P. mortoniae*	Hermanus	Chardonnay	21	2008/02/13	C. White, F. Halleen
6988	*P. alvesii*	Klawer	Chenin blanc	41	2008/01/31	F. Halleen
6989	*P. alvesii*	Klawer	Chenin blanc	41	2008/01/31	F. Halleen
6990	*P. parasiticum*	Klawer	Chenin blanc	41	2008/01/31	F. Halleen
6991	*P. aleophilum*	Vredendal	Colombar	± 35	2008/01/30	F. Halleen
6992	*P. sicilianum*	Oudtshoorn	Colombar	31	2008/02/07	F. Halleen
6993	*P. parasiticum*	De Rust	Fransdruif	33	2008/02/07	F. Halleen
6994	*P. sicilianum*	Calitzdorp	Hanepoot	37	2008/02	F. Halleen
6995	*P. sicilianum*	Calitzdorp	Hannepoot	37	2008/02	F. Halleen
6996	*P. aleophilum*	Wellington	Cabernet Sauvignon	13	2008/02/18	C. White, F. Halleen
6997	*P. aleophilum*	Calitzdorp	Hanepoot	37	2008/02	F. Halleen
6998	*P. iranianum*	Calitzdorp	Chenin blanc	44	2008/02/06	F. Halleen
6999	*P. iranianum*	Calitzdorp	Chenin blanc	44	2008/02/06	F. Halleen
7000	*P. alvesii*	De Rust	Chenin blanc	38	2008/02/06	F. Halleen
7001	*P. alvesii*	De Rust	Chenin blanc	38	2008/02/06	F. Halleen

7002	*P. aleophilum*	Calitzdorp	Hanepoot	37	2008/02	F. Halleen
	Phomopsis/ Diaporthe					
7003	*Diaporthe ambigua*	Porterville	Colombar	15	2005/03/01	F. Halleen
7004	*P. viticola*	Stellenbosch	Cabernet Sauvignon	Unknown	2005/05/05	F. Halleen
7005	*P. viticola*	Stellenbosch	Cabernet Sauvignon	Unknown	2005/05/05	F. Halleen
7006	*P. viticola*	Stellenbosch	Cabernet Sauvignon	Unknown	2005/02/17	F. Halleen
7007	*P. viticola*	Stellenbosch	Cabernet Sauvignon	Unknown	2005/05/05	F. Halleen
7008	*P. viticola*	Stellenbosch	Cabernet Sauvignon	15	2005/05/17	F. Halleen
7009	*P. viticola*	Stellenbosch	Cabernet Sauvignon	15	2005/05/17	F. Halleen
7010	*Phomopsis* sp. 1	Stellenbosch	Cabernet Sauvignon	15	2005/05/17	F. Halleen
7011	*P. viticola*	Stellenbosch	Cabernet Sauvignon	19	2005/05/24	F. Halleen
7012	*P. viticola*	Stellenbosch	Cabernet Sauvignon	19	2005/05/24	F. Halleen
7013	*P. viticola*	Stellenbosch	Cabernet Sauvignon	19	2005/05/24	F. Halleen
7014	*P. viticola*	Stellenbosch	Cabernet Sauvignon	19	2005/05/24	F. Halleen
7015	*P. viticola*	Stellenbosch	Sauvignon blanc	25	2005/06/02	F. Halleen
7016	*Phomopsis* sp. 1	Stellenbosch	Sauvignon blanc	25	2005/06/02	F. Halleen
7017	*P. viticola*	Lutzville	Colombar	22	2008/01/30	F. Halleen
7018	*P. viticola*	Somerset West	Cabernet Sauvignon	31	2008/02/20	C. White, F. Halleen
7019	*P. viticola*	Ashton	Sauvignon blanc	20	2008/02/29	C. White, F. Halleen
	Botryosphaericeae					
7020	*Diplodia seriata*	Paarl	Chenin blanc	18	2005/02/03	F. Halleen
7021	*Neofusicoccum parvum*	Paarl	Chenin blanc	18	2005/02/14	F. Halleen
7022	*Neofusicoccum parvum*	Paarl	Chenin blanc	18	2005/02/14	F. Halleen

7023	*Neofusicoccum parvum*	Paarl	Chenin blanc	18	2005/02/14	F. Halleen
7024	*Neofusicoccum australe*	Paarl	Hanepoot	22	2005/02/14	F. Halleen
7025	*Neofusicoccum australe*	Porterville	Colombar	15	2005/03/01	F. Halleen
7026	*Diplodia seriata*	Porterville	Colombar	15	2005/03/01	F. Halleen
7027	*Neofusicoccum parvum*	Porterville	Colombar	15	2005/03/02	F. Halleen
7028	*Neofusicoccum australe*	Stellenbosch	Cabernet Sauvignon	15	2005/05/17	F. Halleen
7029	*Neofusicoccum australe*	Stellenbosch	Cabernet Sauvignon	19	2005/05/24	F. Halleen
7030	*Neofusicoccum australe*	Stellenbosch	Pinotage	28	2005/05/25	F. Halleen
7031	*Diplodia seriata*	Stellenbosch	Sauvignon blanc	25	2005/06/02	F. Halleen
7032	*Diplodia seriata*	Stellenbosch	Sauvignon blanc	25	2005/06/21	F. Halleen
7033	*Diplodia seriata*	Klawer	Fransdruif	35	2008/01/30	F. Halleen
7034	*Diplodia seriata*	Tulbagh	Chenin blanc	24	2007/11/06	F. Halleen
7035	*Diplodia seriata*	Rawsonville	Chenin blanc	20	2007/11/28	F. Halleen
7036	*Neofusicoccum parvum*	Darling	Chenin blanc	21	2007/10/22	F. Halleen
7037	*Neofusicoccum parvum*	Constantia	Sauvignon blanc	25	2007/10/16	F. Halleen
	Eutypa lata					
5699	*Eutypa lata*	Stellenbosch	Sauvignon blanc	23	2003/03/13	F. Halleen
5700	*Eutypa lata*	Stellenbosch	Sauvignon blanc	23	2003/03/13	F. Halleen
5692	*Eutypa lata*	Stellenbosch	Chenin blanc	26	2002/11/25	F. Halleen
5693	*Eutypa lata*	Stellenbosch	Chenin blanc	26	2002/11/25	F. Halleen
5694	*Eutypa lata*	Stellenbosch	Chenin blanc	26	2002/11/25	F. Halleen
5695	*Eutypa lata*	Stellenbosch	Chenin blanc	26	2002/11/25	F. Halleen
5696	*Eutypa lata*	Stellenbosch	Chenin blanc	26	2002/11/25	F. Halleen

5697	*Eutypa lata*	Stellenbosch	Chenin blanc	26	2002/11/25	F. Halleen
5698	*Eutypa lata*	Stellenbosch	Chenin blanc	26	2002/11/25	F. Halleen
	Basidiomycetes					
7038	Taxon 1	Stellenbosch	Sauvignon blanc	23	2003/01/29	F. Halleen
7039	Taxon 1	Stellenbosch	Sauvignon blanc	23	2003/01/29	F. Halleen
7040	*Fomitiporia* sp.	Stellenbosch	Sauvignon blanc	23	2003/01/29	F. Halleen
7041	*Fomitiporia* sp.	Stellenbosch	Sauvignon blanc	23	2003/01/29	F. Halleen
7042	Taxon 4	Stellenbosch	Chenin blanc	26	2002/11/25	F. Halleen
7043	Taxon 4	Stellenbosch	Chenin blanc	26	2002/11/25	F. Halleen
7045	Taxon 1	Stellenbosch	Sauvignon blanc	23	2003/03/13	F. Halleen
7046	Taxon 1	Porterville	Colombar	15	2004/11/15	F. Halleen
7047	Taxon 1	Porterville	Colombar	15	2004/11/15	F. Halleen
7048	Taxon 1	Paarl	Chenin blanc	18	2005/02/03	F. Halleen
7049	*Fomitiporia* sp.	Paarl	Hanepoot	22	2005/02/04	F. Halleen
7050	*Fomitiporia* sp.	Paarl	Hanepoot	22	2005/02/14	F. Halleen
7051	Taxon 1	Paarl	Chenin blanc	18	2005/02/14	F. Halleen
7052	*Fomitiporia* sp.	Paarl	Chenin blanc	18	2005/02/14	F. Halleen
7053	*Fomitiporia* sp.	Paarl	Chenin blanc	18	2005/02/14	F. Halleen
7054	Taxon 1	Porterville	Dan Ben Hanna	19	2003/11/27	F. Halleen
7055	*Phellinus* sp.	Marken	Prime seedless	5	2003/11/27	F. Halleen
7056	*Fomitiporia* sp.	Stellenbosch	Hanepoot	12	2005/02/25	F. Halleen
7057	*Fomitiporia* sp.	Stellenbosch	Malbec	12	2005/02/25	F. Halleen
7058	Taxon 1	Porterville	Colombar	15	2005/03/02	F. Halleen

7059	Taxon 1	Porterville	Colombar	15	2005/03/02	F. Halleen
7060	Taxon 1	Porterville	Colombar	15	2005/03/02	F. Halleen
7061	Taxon 1	Porterville	Colombar	15	2005/03/02	F. Halleen
7062	Taxon 1	Porterville	Colombar	15	2005/03/02	F. Halleen
7063	Taxon 1	Porterville	Colombar	15	2005/03/02	F. Halleen
7064	Taxon 1	Porterville	Colombar	15	2005/03/02	F. Halleen
7065	Taxon 1	Porterville	Colombar	15	2005/03/01	F. Halleen
7066	Taxon 1	Porterville	Colombar	15	2005/03/01	F. Halleen
7067	Taxon 1	Slanghoek	Hanepoot	40	2005/03/02	F. Halleen
7069	*Fomitiporia* sp.	Stellenbosch	Cabernet Sauvignon	15	2005/05/17	F. Halleen
7070	Taxon 1	Stellenbosch	Cabernet Sauvignon	15	2005/05/17	F. Halleen
7071	Taxon 1	Stellenbosch	Cabernet Sauvignon	15	2005/05/17	F. Halleen
7072	*Fomitiporia* sp.	Stellenbosch	Cabernet Sauvignon	15	2005/05/17	F. Halleen
7073	Taxon 1	Stellenbosch	Cabernet Sauvignon	15	2005/05/17	F. Halleen
7074	Taxon 1	Stellenbosch	Cabernet Sauvignon	19	2005/05/24	F. Halleen
7075	Taxon 1	Stellenbosch	Cabernet Sauvignon	19	2005/05/24	F. Halleen
7076	Taxon 7	Stellenbosch	Pinotage	28	2005/05/25	F. Halleen
7077	*Fomitiporia* sp.	Stellenbosch	Sauvignon blanc	25	2005/06/02	F. Halleen
7078	Taxon 1	Stellenbosch	Sauvignon blanc	25	2005/06/02	F. Halleen
7079	Taxon 1	Stellenbosch	Sauvignon blanc	25	2005/06/07	F. Halleen
7080	Taxon 1	Stellenbosch	Sauvignon blanc	25	2005/06/07	F. Halleen
7081	*Fomitiporia* sp.	Stellenbosch	Sauvignon blanc	25	2005/06/20	F. Halleen
7082	*Fomitiporia* sp.	Stellenbosch	Sauvignon blanc	25	2005/06/21	F. Halleen

7083	Taxon 1	Stellenbosch	Cabernet Sauvignon	19	2005/06/23	F. Halleen
7084	Taxon 1	Slanghoek	Hanepoot	40	2005/07/08	F. Halleen
7086	*Fomitiporia* sp.	Klaas voogds	Red Globe	10	2005/09/12	F. Halleen
7088	Taxon 1	Rawsonville	Chenin blanc	11	2005/11/03	F. Halleen
7090	Taxon 7	Stellenbosch	Ruby Cabernet	22	2007/08/02	F. Halleen
7092	Taxon 1	De Doorns	Sultana	18	2007/07/07	F. Halleen
7093	*Fomitiporia* sp.	Wellington	Chenin blanc	20	2007/09/07	F. Halleen
7094	*Fomitiporia* sp.	Wellington	Chenin blanc	20	2007/09/07	F. Halleen
7095	*Fomitiporia* sp.	Wellington	Chenin blanc	20	2007/09/07	F. Halleen
7096	*Fomitiporia* sp.	Franschhoek	Chenin blanc	40	2007/09/19	F. Halleen
7097	*Fomitiporia* sp.	Somerset West	Sauvignon blanc	16	2007/09/26	F. Halleen
7098	*Phellinus* sp.	Kanon Eiland	Sultana	Unknown	2007/09/15	F. Halleen
7099	*Phellinus* sp.	Kanon Eiland	Sultana	Unknown	2007/09/15	F. Halleen
7100	*Phellinus* sp.	Keimoes	Chenin blanc	18	2007/09/15	F. Halleen
7101	*Phellinus* sp.	Keimoes	Colomino	18	2007/09/15	F. Halleen
7102	*Phellinus* sp.	Keimoes	Colomino	18	2007/09/15	F. Halleen
7103	*Phellinus* sp.	Marchand	Sultana	40	2007/09/15	F. Halleen
7104	*Phellinus* sp.	Marchand	Sultana	40	2007/09/15	F. Halleen
7105	*Phellinus* sp.	Marchand	Sultana	40	2007/09/15	F. Halleen
7106	Taxon 7	Constantia	Sauvignon blanc	25	2007/10/16	F. Halleen
7107	Taxon 1	Constantia	Sauvignon blanc	25	2007/10/16	F. Halleen
7108	*Fomitiporia* sp.	Constantia	Sauvignon blanc	18	2007/10/16	F. Halleen
7109	Taxon 3	Constantia	Sauvignon blanc	18	2007/10/16	F. Halleen

7110	Taxon 1	Constantia	Sauvignon blanc	18	2007/10/16	F. Halleen
7112	Taxon 1	Bonnievale	Sauvignon blanc	20	2007/10/07	F. Halleen
7113	Taxon 1	Bonnievale	Sauvignon blanc	20	2007/10/07	F. Halleen
7115	*Fomitiporia* sp.	Durbanville	Chenin blanc	26	2007/09/27	F. Halleen
7117	Taxon 1	Durbanville	Shiraz	21	2007/09/27	F. Halleen
7118	Taxon 1	Durbanville	Shiraz	21	2007/09/27	F. Halleen
7119	*Fomitiporia* sp.	Durbanville	Sauvignon blanc	23	2007/09/27	F. Halleen
7120	Taxon 1	Durbanville	Sauvignon blanc	23	2007/09/27	F. Halleen
7121	*Fomitiporia* sp.	Durbanville	Sauvignon blanc	23	2007/09/27	F. Halleen
7122	*Fomitiporia* sp.	Durbanville	Sauvignon blanc	23	2007/09/27	F. Halleen
7123	Taxon 1	Durbanville	Sauvignon blanc	23	2007/09/27	F. Halleen
7124	*Fomitiporia* sp.	Darling	Chenin blanc	23	2007/10/22	F. Halleen
7125	Taxon 5	Darling	Chenin blanc	23	2007/10/22	F. Halleen
7126	Taxon 5	Darling	Chenin blanc	23	2007/10/22	F. Halleen
7127	Taxon 5	Darling	Chenin blanc	21	2007/10/22	F. Halleen
7128	Taxon 5	Darling	Chenin blanc	21	2007/10/22	F. Halleen
7129	Taxon 5	Darling	Chenin blanc	21	2007/10/22	F. Halleen
7130	Taxon 1	Malmesbury	Chenin blanc	36	2007/10/22	F. Halleen
7131	Taxon 5	Malmesbury	Pinotage	36	2007/10/22	F. Halleen
7132	Taxon 5	Malmesbury	Pinotage	36	2007/10/22	F. Halleen
7133	Taxon 6	Malmesbury	Pinotage	36	2007/10/22	F. Halleen
7134	Taxon 6	Malmesbury	Pinotage	36	2007/10/22	F. Halleen
7135	*Fomitiporia* sp.	Grabouw	Chardonnay	15	2007/11/08	F. Halleen

7136	Taxon 3	Grabouw	Sauvignon blanc	15	2007/11/08	F. Halleen
7137	*Fomitiporia* sp.	Botrivier	Chenin blanc	20	2007/11/08	F. Halleen
7138	Taxon 8	Botrivier	Chenin blanc	20	2007/11/08	F. Halleen
7139	Taxon 8	Botrivier	Chenin blanc	20	2007/11/08	F. Halleen
7140	*Fomitiporia* sp.	Riebeeck Wes	Chenin blanc	19	2007/11/06	F. Halleen
7141	Taxon 1	Riebeeck Kasteel	Chenin blanc	20	2007/11/06	F. Halleen
7142	Taxon 1	Tulbagh	Chenin blanc	24	2007/11/06	F. Halleen
7143	Taxon 5	Tulbagh	Chenin blanc	24	2007/11/06	F. Halleen
7144	Taxon 1	Tulbagh	Chenin blanc	28	2007/11/06	F. Halleen
7145	Taxon 1	Rawsonville	Chenin blanc	20	2007/11/28	F. Halleen
7146	Taxon 1	Rawsonville	Chenin blanc	20	2007/11/28	F. Halleen
7147	Taxon 2	Oudtshoorn	Pinotage	29	2008/02/06	F. Halleen
7148	Taxon 1	De Rust	Chenin blanc	38	2008/02/06	F. Halleen
7149	Taxon 1	De Rust	Chenin blanc	38	2008/02/06	F. Halleen
7150	Taxon 1	De Rust	Red muskadel	31	2008/02/06	F. Halleen
7151	Taxon 1	De Rust	Red muskadel	31	2008/02/06	F. Halleen
7152	Taxon 1	De Rust	Fransdruif	33	2008/02/07	F. Halleen
7153	Taxon 5	Ladismith	Chenin blanc	28	2008/02/06	F. Halleen
7154	Taxon 2	Calitzdorp	Hannepoot	37	Feb-08	F. Halleen
7155	Taxon 2	Calitzdorp	Hannepoot	37	Feb-08	F. Halleen
7156	Taxon 1	Lutzville	Colombar	22	2008/01/30	F. Halleen
7157	Taxon 1	Klawer	Fransdruif	35	2008/01/30	F. Halleen
7158	Taxon 1	Klawer	Chenin blanc	41	2008/01/31	F. Halleen

7159	Taxon 1	Klawer	Chenin blanc	41	2008/01/31	F. Halleen
7160	Taxon 1	Klawer	Chenin blanc	41	2008/01/31	F. Halleen
7161	Taxon 1	Klawer	Chenin blanc	41	2008/01/31	F. Halleen
7162	Taxon 1	Klawer	Chenin blanc	41	2008/01/31	F. Halleen
7163	*Fomitiporia* sp.	Franschhoek	Cabernet Sauvignon	14	2008/02/12	C. White, F. Halleen
7164	*Fomitiporia* sp.	Franschhoek	Cabernet Sauvignon	14	2008/02/12	C. White, F. Halleen
7165	Taxon 7	Franschhoek	Chenin blanc	25	2008/02/13	C. White, F. Halleen
7166	*Fomitiporia* sp.	Hermanus	Chardonnay	21	2008/02/13	C. White, F. Halleen
7167	*Fomitiporia* sp.	Hermanus	Chardonnay	21	2008/02/13	C. White, F. Halleen
7168	*Fomitiporia* sp.	Hermanus	Chardonnay	21	2008/02/13	C. White, F. Halleen
7169	*Fomitiporia* sp.	Wellington	Cabernet Sauvignon	13	2008/02/18	C. White, F. Halleen
7170	*Fomitiporia* sp.	Wellington	Cabernet Sauvignon	13	2008/02/18	C. White, F. Halleen
7171	*Fomitiporia* sp.	Somerset West	Tinta Barroca	28	2008/02/19	C. White, F. Halleen
7172	Taxon 1	Somerset West	Cabernet Sauvignon	32	2008/02/19	C. White, F. Halleen
7173	Taxon 7	Somerset West	Cabernet Sauvignon	31	2008/02/20	C. White, F. Halleen
7174	Taxon 3	Ashton	Sauvignon blanc	20	2008/02/29	C. White, F. Halleen
7175	Taxon 1	Ashton	Shiraz	30	2008/02/29	C. White, F. Halleen
7176	Taxon 1	Montagu	Colombar	27	2008/02/29	C. White, F. Halleen
7177	Taxon 5	Montagu	Colombar	27	2008/02/29	C. White, F. Halleen
7178	Taxon 3	Montagu	Colombar	27	2008/02/29	C. White, F. Halleen
7179	*Phellinus* sp.	Keboes	Sultana	Unknown	2008/02/27	F. Halleen
7180	*Phellinus* sp.	Prieska	Sultana	Unknown	2008/04/17	F. Halleen

Table 2. Cultural growth characteristics of *Phaeoacremonium* isolates grown on malt extract agar (MEA), potato dextrose agar (PDA) and oatmeal agar (OA).

Species	STE-U isolates used in growth study	Total number of isolates investigated	Growth pattern on PDA	Colony colour[1]			Radial growth after 8 days (mm)
				MEA	PDA	OA	MEA
P. aleophilum	6991, 6996, 7002	14	Yellow pigment in agar	Greyish Sepia (15'''i)	Smoke grey (21''''f)	Vinaceous buff (17'''d)	5 - 10
P. aleophilum	6986	1	Slow growing brown	Isabelline (17''i)	Pale mouse grey (15'''''d)	Greyish sepia	5
P. aleophilum	6997	1	Dark Brown	Dark mouse grey (13'''''K) with yellow pigment	Smoke grey	Isabelline (17''i)	13
P. alvesii	6988, 6989, 7001	4	Pink colour	Venacious purple (65'''b); Pale venacious (5'''f)	Pale venacious; buff (19''f)	Fawn (13'''); Venacious purple (65'''b)	10 - 14.5
P. alvesii	7000	1	Single pale brown	Buff	Buff	Greyish sepia	10
P. iranianum	6999	1	Pale Yellow	Buff	Buff	Buff	8.5

P. iranianum	6998	1	Circular Brown	Venacious buff	Greyish sepia	Smoke grey	13
P. mortoniae	6987	1	Very pale	Buff	Buff yeast like growth	Buff	12
P. parasiticum	6993	3	Pale coloured	Pale purplish grey (1""d)	Greyish sepia	Pale mouse grey	12
P. parasiticum	6990	3	Brown coloured sectorial	Venacious buff mixed with buff	Greyish sepia	Mouse grey (13""")	12
P. sicilianum	6992, 6995	6	Brown coloured	Venacious buff mixed with Pale mouse grey	Greyish sepia	Pale mouse grey	12 - 14
P. sicilianum	6994	2	Circular brown coloured colony	Pale mouse grey	Greyish sepia	Pale mouse grey	13.5

[1] Colony colour descriptions and codes according to Rayner (1970).

Table 3. Conidial dimensions of *Phaeoacremonium* isolates measured from isolates grown on MEA.

Species	STE-U Number	Length (μm)	Width (μm)	Conidial Shape
P. aleophilum	6986	(4–)5–7(–8)	2-4	Mostly oblong-ellipsoidal or cylindrical, occasionally reniform
	6991	(4.5–)5–6	2(–3)	
	6996	(4–)4–5(–7)	2(–2.5)	
	6997	(4–)5–6(–7)	(1–)2	
	7002	(4–)4–5(–6)	(1.5–)2(–3)	
P. alvesii	6988	(4.5–)5–6(–7)	2	Ovoid or oblong-ellipsoidal and sometimes reniform to allantoid
	6989	(4–)5(–6)	(1.5–)2(–2)	
	7000	(4–)5.5–8(–10)	(1–)2 –3	
	7001	(4–)4–5.5(–6)	(1.5–)2(–2.5)	
P. iranianum	6998	(3–)4–5(–6)	(1–)2(–2.5)	Oblong-ellipsoidal
	6999	(4–)4–6(–7)	(1.5–)2(–2.5)	
P. mortoniae	6987	(4–)5– 7	1–2.5(–5)	Oblong-ellipsoidal and sometimes reniform
P. parasiticum	6990	(5–)7(–8)	(1.5–)2 –3(–3)	Oblong-ellipsoidal and sometimes allantoid to broadly oblong
	6993	(4–)5–6(–8)	(2–)2(–3)	
P. sicilianum	6992	5–6(–7)	(1.5–)2	Mainly allantoid, with some being subcylindrical.
	6994	(4–)5–7(–8)	2	
	6995	5–6(–7)	2(–2.5)	

Table 4. Conidial dimensions of the alpha conidia of the *Phomopsis viticola* isolates that formed pycnidia on PDA.

STE-U Number	Length (μm)	Width (μm)	Presence of Beta conidia
7004	(7–)8.5–10(–11)	2–3	Present
7007	(6–)9–11.5(–14)	(2.5–)3(–3.5)	Absent
7008	–[1]	–[1]	Present
7011	(9–)9.5–11	(2.5–)3(–3.5)	Absent
7013	(8–)9–11(–14)	2–3	Absent
7015	(8–)10–12(–13.5)	2– 3	Present
7017	(9–)10–13(–14)	2–3	Absent
7018	(8–)10–13(–15)	(2–)3(–4)	Absent

[1] No alpha conidia were formed.

Table 5. Conidial dimensions of Botryosphaeriaceae isolates grown on WA containing autoclaved pine needles to allow for sporulation.

STE-U Number	Species	Length	Width
7031	*D. seriata*	(21–)22– 25	10–11
7033	*D. seriata*	21–23(–24)	(8–)9–10
7034	*D. seriata*	(21–)22–25	(10–)10.5–12
7035	*D. seriata*	(20–)22–25	9–10
7021	*N. parvum*	(13–)15–18(–19)	6–8

Table 6. Description of the cultural growth patterns of the sequenced basidiomycete isolates after 4 weeks on PDA.

Taxon	STE-U Number	Origin	Cultural growth patterns
1	**7038; 7039; 7045; 7046; 7047; 7048; 7051; 7058; 7059; 7060; 7061; 7062; 7063; 7064; 7065; 7066; 7067; 7070; 7071; 7073; 7074; 7075; 7078; 7079; 7080; 7083; 7084; 7088; 7092; 7107; 7110; 7112; 7113; 7117; 7118; 7120; 7120; 7123; 7130; 7141; 7142; 7144; 7145; 7146; 7148; 7149; 7150; 7151; 7152; 7156; 7157; 7158; 7159; 7160; 7161; 7162; 7172; 7175; 7176**	Ashton; Bonnievale; Constantia; De Doorns; De Rust; Durbanville; Klawer; Lutzville; Malmesbury; Montagu; Paarl; Piketburg; Porterville; Rawsonville; Riebeeck Kasteel; Slanghoek; Somerset West; Stellenbosch; Tulbagh	Cotton white/ pale yellow; Flat brown; Flat orange-yellow; Flat white/ pale yellow (cream); Fluffy cream with dark mycelial strands; Slow growing clear shades of brown with sparse mycelium; Sparse white; Speckled white/ yellow/ brown; Tufty orange/ brown; Tufty white; White brown/ radiating growth streaky growth; Woolly light brown; Woolly sparse white
2	**7147; 7154; 7155**	Oudtshoorn; Calitzdorp	Flat orange-yellow; Speckled white/yellow/ brown
3	**7109; 7136; 7174; 7178**	Ashton; Constantia; Grabouw; Montagu	Flat white/ pale yellow (cream); Slow growing shades of brown with sparse mycelium; Wooly light brown; Woolly sparse white
4	**7042; 7043**	Stellenbosch	Flat various yellow/ brown/ white tones; Slow growing clear shades of brown with sparse mycelium
5	**7125; 7126; 7127; 7128; 7129; 7131; 7132; 7143; 7153; 7177**	Darling; Ladismith; Malmesbury; Montagu; Tulbagh	Cotton white/ pale yellow; Flat brown; Flat orange-yellow; Flat white/ pale yellow (cream); Speckled white/ yellow/ brown
6	**7133; 7134**	Malmesbury	Flat brown; Speckled white/ yellow/ brown
7	**7076; 7090; 7106; 7165; 7173**	Constantia; Franschhoek; Somerset West; Stellenbosch	Fluffy orange-brown
8	**7138; 7139**	Botrivier	Fluffy orange-brown
Fomitiporia	**7040; 7041; 7049; 7050; 7052; 7053; 7056; 7057; 7069; 7072; 7077; 7081; 7082; 7086; 7093; 7094; 7095; 7096; 7097; 7108; 7115; 7119; 7121; 7122; 7124; 7135; 7137; 7140; 7163; 7164; 7166; 7167; 7168; 7169; 7170; 7171**	Botrivier; Constantia; Darling; Durbanville; Franschhoek; Grabouw; Hermanus; Klaas voogds; Paarl; Riebeek Wes; Somerset West; Stellenbosch; Wellington	Flat brown; Flat various yellow/ brown white tones; Flat white/ pale yellow (cream); Speckled white/ yellow/ brown; Tufty orange/ brown; Woolly light brown
Phellinus	**7055; 7098; 7099; 7100; 7101; 7102; 7103; 7104; 7105; 7179; 7180**	Kanon Eiland; Keboes; Keimoes; Marchand; Marken; Prieska	Flat brown; Speckled white/ yellow/ brown; Tufty orange/ brown

Table 7. Colony diameter of the basidiomycete isolates grown on PDA at 25°C for 14 days.

STE-U Number	Taxon	Average diameter (mm)
7038	1	75 ± 2
7058	1	52 ± 7
7084	1	60 ± 15
7141	1	63 ± 17
7148	1	70 ± 15
7147	2	85 ± 0
7154	2	83 ± 3
7155	2	85 ± 0
7109	3	80 ± 1
7136	3	72 ± 10
7174	3	63 ± 1
7042	4	41 ± 2
7043	4	29 ± 22
7126	5	43 ± 6
7143	5	51 ± 4
7153	5	70 ± 6
7133	6	85 ± 0
7134	6	78 ± 2
7090	7	45 ± 2
7106	7	51 ± 4
7165	7	57 ± 3
7138	8	85 ± 0
7139	8	71 ± 7
7069	*Fomitiporia* sp.	46 ± 14
7096	*Fomitiporia* sp.	65 ± 23
7122	*Fomitiporia* sp.	85 ± 0
7135	*Fomitiporia* sp.	77 ± 2
7168	*Fomitiporia* sp.	83 ± 2
7055	*Phellinus* sp.	79 ± 2
7098	*Phellinus* sp.	85 ± 0
7105	*Phellinus* sp.	85 ± 0

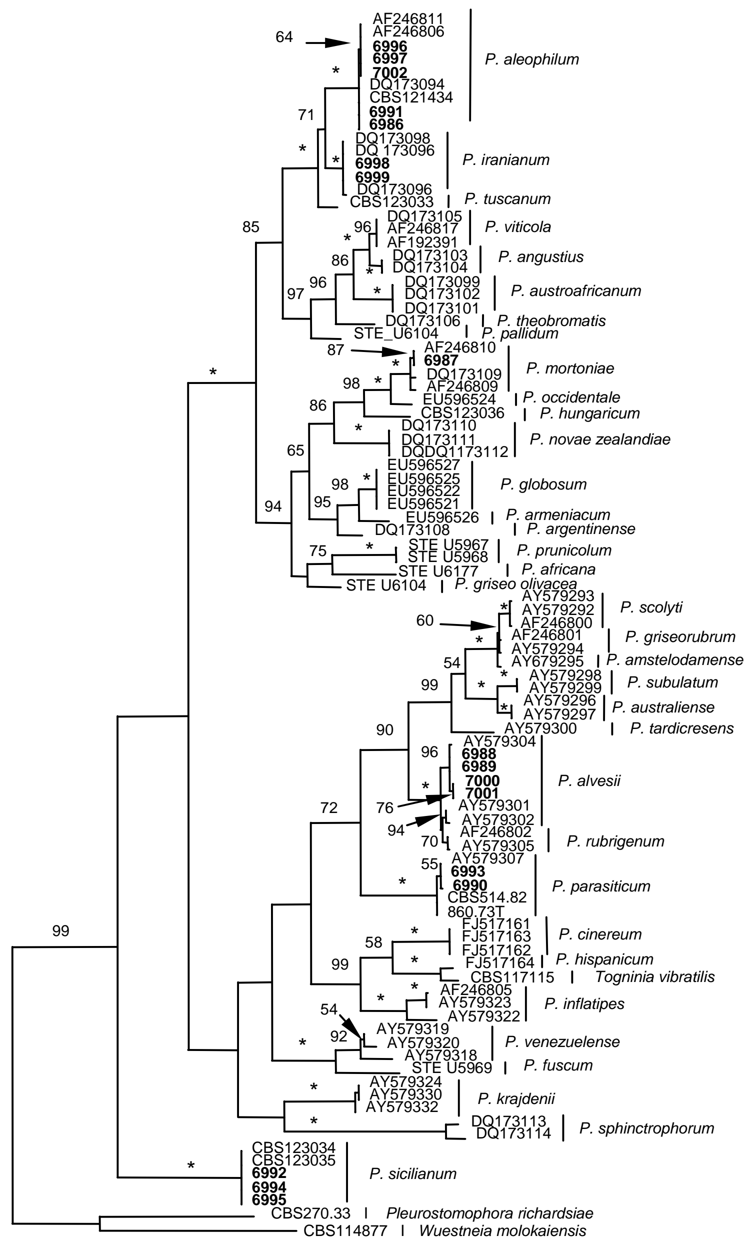


Fig. 1. One of 42 most parsimonious trees obtained from heuristic searches of the β-tubulin sequences (length: 1691 steps; CI: 0.458; RI: 0.862; RC: 0.394) of the *Phaeoacremonium* isolates. Bootstrap support values (1000 replicates) are shown above the nodes and bootstrap values of 100 % are indicated by an asterisk (*). The outgroups were *Pleurostomophora richardsiae* and *Wuestneia molokaiensis*. Isolates in bold print are isolates from this study.

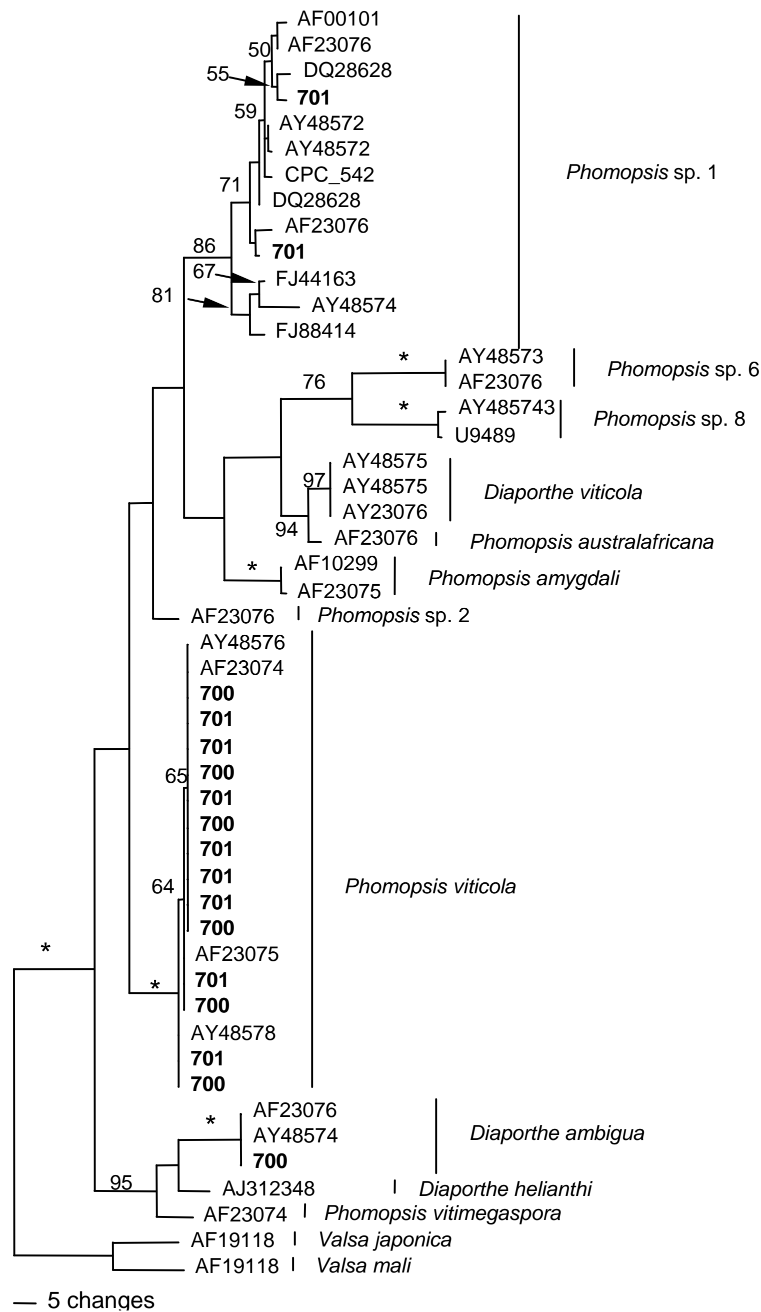


Fig. 2. One of 22 most parsimonious trees obtained from heuristic searches of the ITS sequences (length: 325 steps; CI: 0.578; RI: 0.847; RC: 0.490) of the *Phomopsis* isolates. Bootstrap support values (1000 replicates) are shown above the nodes and bootstrap values of 100 % are indicated by an asterisk (*). The outgroups used were *Valsa japonica* and *Valsa mali*. Isolates in bold print are from this study.

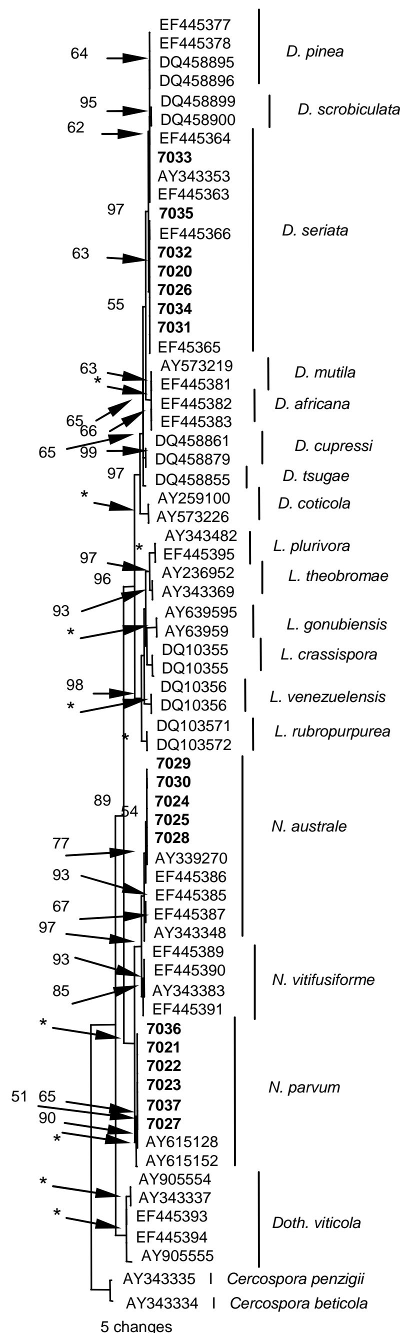
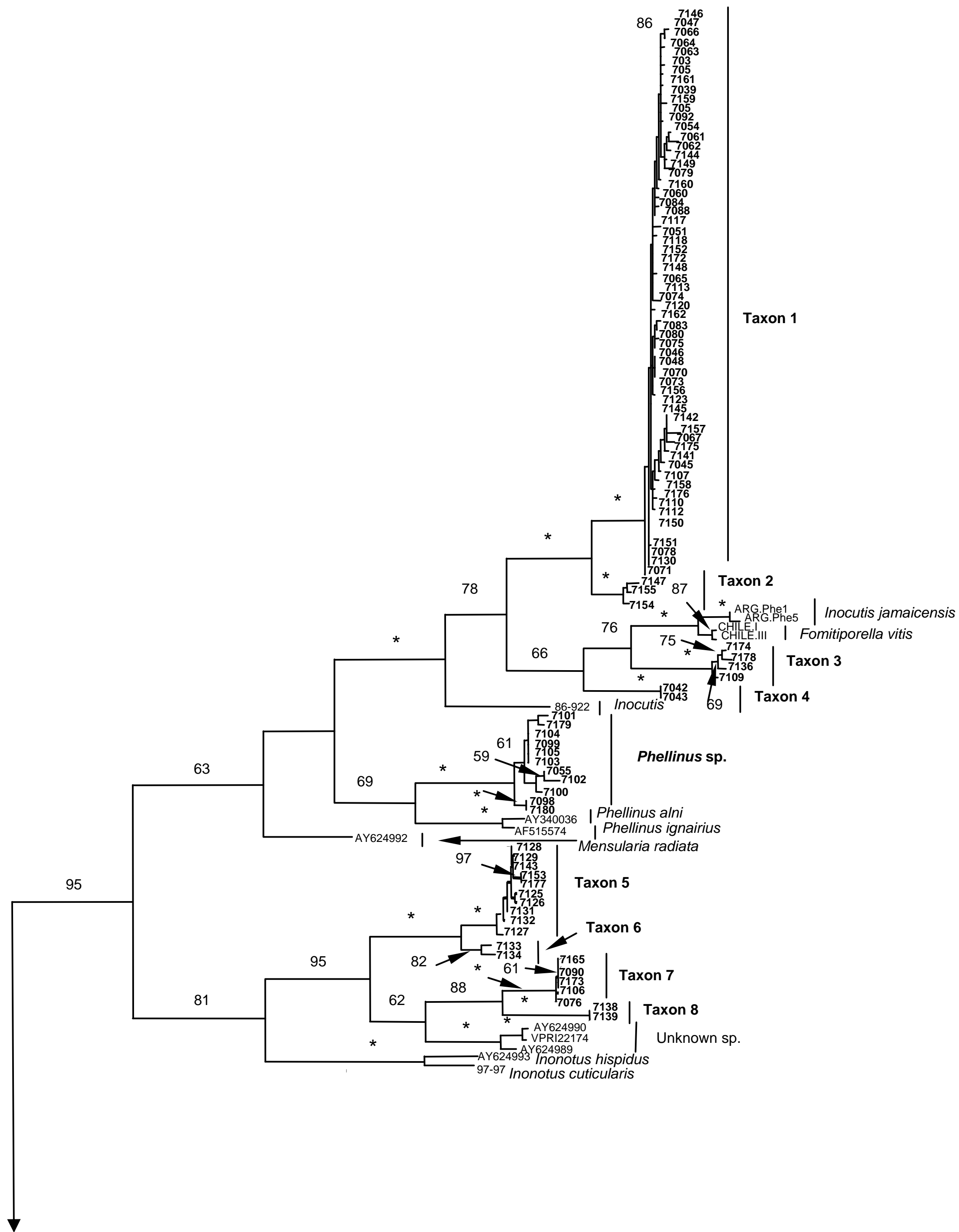


Fig. 3. One of 100 most parsimonious trees obtained from heuristic searches of the EF sequences (length: 466 steps; CI: 0.715; RI: 0.942; RC: 0.673) of the Botryosphaeriaceae isolates. Bootstrap support values (1000 replicates) are shown above the nodes and bootstrap values of 100 % are indicated by an asterisk (*). The outgroups used were *Cercospora penzigii* and *Cercospora beticola*. Isolates in bold print are isolates from this study.


Taxon 1
Taxon 2
Inocutis jamaicensis
Fomitiporella vitis
Taxon 3
Taxon 4
Inocutis
Phellinus sp.
Phellinus alni
Phellinus ignairius
Mensularia radiata
Taxon 5
Taxon 6
Taxon 7
Taxon 8
Unknown sp.
Inonotus hispidus
Inonotus cuticularis
ARG.Phe1
ARG.Phe5
CHILE.I
CHILE.III
86-922
AY340036
AF515574
AY624992
AY624990
VPRI22174
AY624989
AY624993
97-97

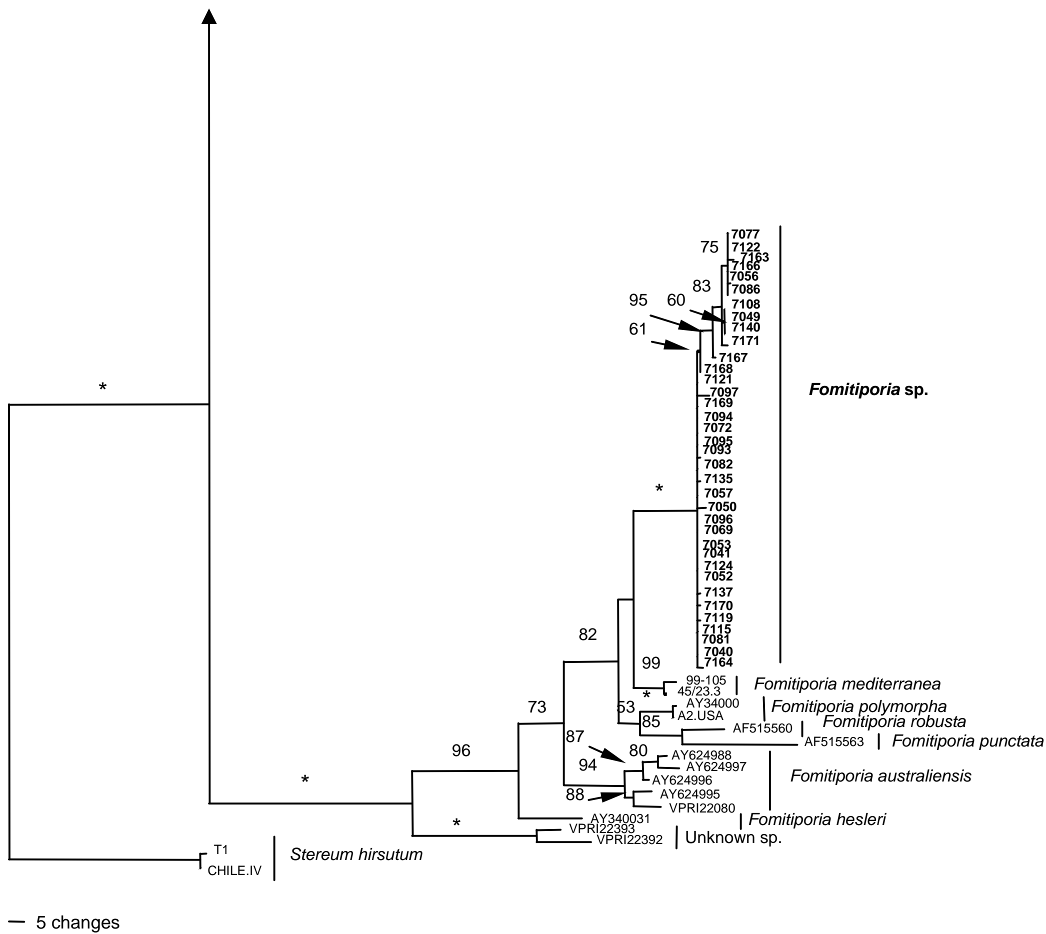


Fig. 4. One of 10 most parsimonious trees obtained from heuristic searches of the ITS sequences (length: 2050 steps; CI: 0.560; RI: 0.938; RC: 0.526) of the Basidiomycete isolates. Bootstrap support values (1000 replicates) are shown above the nodes and bootstrap values of 100 % are indicated by an asterisk (*). The outgroups used were *Stereum hirsutum* isolates T18 and Chile IV. Isolates in bold print are from this study.

CHAPTER 4

IDENTIFICATION OF TOXINS AND ENZYMES SECRETED BY BASIDIOMYCETE TAXA ISOLATED FROM ESCA DISEASED GRAPEVINES

ABSTRACT

The toxins and enzymes secreted by the ten different taxa of basidiomycetes identified from esca diseased grapevines in South African were determined. The presence of four toxins (4-hydroxy-benzaldehyde, sterehirsutinal, frustinol and frustulosine) was tested from extractions made from liquid broth cultures of the basidiomycete fungi. All of the basidiomycete isolates were able to produce 4-hydroxy-benzaldehyde, a toxin suspected of being involved in pathogenicity. The presence of enzymes involved in wood degradation was tested on specific media. All of the taxa produced manganese peroxidase, however, variable. The *Phellinus* sp. was an exception as all isolates within this taxon produced this enzyme. Laccase was produced by all taxa, except Taxon 8. All of the isolates of Taxa 1, 4, the *Fomitiporia* sp. and *Phellinus* sp. produced laccase consistently. Lignin peroxidase was consistently produced by the *Fomitiporia* sp. isolates and more variable production was found among the isolates of Taxa 1, 2, 7 and the *Phellinus* sp. All the basidiomycete isolates of the ten taxa were able to produce cellulase and none were able to produce xylanase. The enzyme tests showed that the basidiomycetes produced enzymes which are able to degrade cellulose and lignin, both of which are structural components of wood. The results showed that all ten basidiomycete taxa were able to secrete 4-hydroxy-benzaldehyde and enzymes involved in wood degradation, confirming their role as pathogens in esca.

INTRODUCTION

The basidiomycetes associated with grapevines showing esca, chlorotic leaf roll and "hoja de malvón" symptoms include *Fomitiporia mediterranea* M. Fischer, *Fomitiporia polymorpha* M. Fischer, *Inocutis jamaicensis* (Murrill) Gottlieb, J.E., Wright & Moncalvo, *Fomitiporella vitis* Auger, Aguilera & Esterio, *F. australiensis* M. Fisch., J. Edwards, Cunnington & Pascoe, *Inonotus* spp. and *Phellinus* spp. (Gatica *et al*., 2000; Fischer, 2002, Fischer and Kassemeyer, 2003; Fischer and Binder, 2004; Fischer, 2006). These fungi were all isolated from white wood rotting symptoms of grapevine wood. The leaf symptoms associated with these vines were variable and it is known that leaf symptoms of esca are influenced by various factors, not only by the fungi present in the wood.

Limited studies have been conducted to test the pathogenicity of the different basidiomycetes. Six months were needed for spongy wood decay to be observed in inoculation studies with *F. mediterranea* in Italy (Sparapano *et al*. 2000), whereas inoculation studies with *Phellinus* sp. (later identified as *Inocutis jamaicensis*) only showed leaf symptoms characteristic of 'hoja de malvón' on grapevines in Argentina after six years (Gatica *et al*., 2004).

The biological role of *Stereum hirsutum* (Willd.) Pers. and *Fomitiporia mediterranea* (then named *F. punctata*) have been investigated by identifying the toxins that are secreted by these fungi. *Stereum hirsutum* produced sterehirsutinal (Tabacchi et al. 2000), a compound of which the chemical structure is close to eutypine, the toxin secreted by *Eutypa lata* (Perrin-Cherioux *et al*. 2004). Sterehirsutinal was shown to strongly inhibit growth of grapevine callus (Perrin-Cherioux *et al*. 2004) at 500 μM (100 % inhibition) and at 100 μM (50 % inhibition) (Dubin *et al*., 2000). *Fomitiporia mediterranea* produced 4-hydroxy-benzaldehyde, and the new chromanone 6-formyl-2,2-dimethyl-4-chromanone (biogenetically related to eutypine) when grown in carrot broth for four weeks (Tabacchi *et al*., 2000). The phytotoxicity of the 4-hydroxy-benzaldehyde was confirmed by Tabacchi *et al*. (2000) on grapevine protoplasts and callus. However, nothing is known about the phytotoxicity of the other toxins that were isolated from the

basidiomycetes in this study. It is not known which toxins are secreted by the other *Fomitiporia* species and basidiomycete genera.

Plant cell walls are made up of cellulose (which gives strength and structure to the cell wall), matrix polysaccharides (made up of pectins, which prevents the cellulose network from collapsing), hemicelluloses (xylan, xyloglucan and callose, which are flexible and bound to the cellulose surface), lignin (giving mechanical strength and reducing the susceptibility of the plant walls to plant pathogens) and structural proteins, whose role is undetermined (Taiz and Zeiger, 2002). These structural barriers can be broken down by enzymes produced by basidiomycete fungi in grapevine, in order to use the components of the plant cell wall used for their own reproduction and growth (Fischer and Kassemeyer, 2003).

The white rot of grapevines caused by the basidiomycetes is due to the secretion of various extracellular enzymes that can degrade lignin (Germain *et al*., 2002; Kachlishvili *et al*., 2006). White rot fungi can produce four different types of oxidative enzymes involved in lignin degradation, namely lignin peroxidase, laccase, manganese-independent peroxidase and manganese-dependent peroxidase (Moreira *et al*., 1999; Overton *et al*., 2006). An initial study investigating the enzymes produced by the wood rotting fungi isolated from grapevines in the United States, showed that *Stereum hirsutum* produced laccase and *Fomes igniarius* produced both laccase and peroxidase (Chiarappa, 1959). The predominant species in Europe, previously named *F. punctata*, produced phenoloxidase, laccase and peroxidase, all of which are lignolytic enzymes (Mugnai *et al*., 1999).

The virulence of basidiomycete taxa and isolates depends on the toxins secreted, as well as the array of enzymes associated with cell wall degradation. Therefore, specific toxins and wood degrading enzymes were tested for selected isolates of the ten different basidiomycete taxa isolated from white rot symptoms of esca diseased vines in South Africa. Toxins that were tested for included frustinol, frustulosin, 4-hydroxy-benzaldehyde and sterehirsutinal. Frustulosin and frustulosinol have been shown to be produced by *Stereum frustulosum* (Nair and Anchel, 1975; Nair and Anchel, 1977). The presence of enzymes was also tested for and included lignin peroxidase, manganese peroxidase, laccase, cellulose, and xylanase.

MATERIALS AND METHODS

Toxin extraction

Two to five isolates from each of the taxa obtained in the phylogenetic analysis (Chapter 3) were chosen for toxin analysis. A total of 31 basidiomycete isolates were grown on 90 mm PDA Petri dishes for three weeks at 25 °C. Five plugs of 2 mm diameter of each isolate were used to inoculate 200 ml Potato dextrose broth (PDB, Biolab, South Africa) in 250 ml Erlenmeyer flasks. A control flask of PDB was also prepared and was not inoculated. After four weeks incubation at 25 °C in the dark, a volume of 50 ml was decanted from each flask.

The pH of this volume was adjusted to three and filtered through Whatman no. 1 filter paper with the aid of an extraction pump. The organic phase was extracted with 50 ml ethyl acetate (Sigma-Aldrich (Pty) LTD, Kempton Park, South Africa) and approximately 0.2 g of NaCl. The solution was mixed thoroughly and the layers were allowed to separate. The organic phase was removed and the volume recorded, prior to it being dried in a fumehood. The residue was resuspended in 1 ml methanol and then vortexed. One ml of water was added and centrifuged at 12 000 rpm for 10 minutes. The supernatant was then aliquoted into HPLC vials.

The toxin standards included sterehirsutinal, frustinol and frustulosine (obtained from E. Abou-Monseur, Institute of Chemistry, University of Neuchâtel) and 4-hydroxy-benzaldehyde (Sigma-Aldrich (Pty) LTD, Kempton Park, South Africa). Stock solutions for the standards were prepared with acetonitrile to obtain a concentration of 50 μg/ml for sterehirsutinal, frustulosinol and frustulosine and a concentration of 100 μg/ml for 4-hydroxy-benzaldehyde. These stocks were aliquoted into Eppendorf tubes and air dried. Two hundred μl acetonitrile was added to each of the standards and 100 μl of each was combined into an HPLC vial. Additionally, 500 μl of water was added to make a final concentration of 25 μg/ml. This was diluted to 2.5 μg/ml, 0.25 μg/ml and 0.025 μg/ml, respectively.

The extracts were analysed by liquid chromatography mass spectrometry (LCMS). A Waters API Quattro Micro triple quadruple mass spectrometer with an Alliance 2695 HPLC system (Waters, Milford, MA, USA) was used. The electrospray

ionization (ESI) source was used in the negative mode and the settings were as follows: capillary voltage at 3.50 kV; cone voltage at 15 V; source temperature at 100°C; desolvation temperature at 400°C; desolvation gas flow at 500 L/h; cone gas flow at 50 L/h. The following multiple reaction monitoring transitions were used for frustulosine: 201.5>90.8; frustulosinol: 203.2>188; 4-hydroxy-benzaldehyde: 121>91.8 and sterehirsutinal at 265.4>237. Separation was achieved on a Phenomenex Gemini C18 column (2.0 x 250 mm) at a flow rate of 0.3 ml/minute. The retention times for these compounds were 16.36 minutes for frustulosine; 14.41 minutes for frustulosinol; 13.32 minutes for 4-hydroxy-benzaldehyde and 17.68 minutes for sterehirsutinal. Mass Lynx NT software 4.1 was used for data processing.

Enzyme characterization

The 31 basidiomycete isolates were plated onto PDA in 90 mm Petri dishes and grown at 25°C for 2 weeks. Mycelial plugs of 2 mm diameter were made with a sterilized cork borer from the edge of the colonies on and placed on the media for the specific enzyme tests. Each isolate was plated out in triplicate and on one control plate for each enzyme assay performed. Each enzyme assay was repeated once.

Manganese peroxidase: The method used by Overton *et al*. (2006) was adjusted to include only two concentrations of Magnesium sulphate, namely at 80 ppm (80 mg $MnSO_4$/l) and at 100 ppm (100 mg $MnSO_4$/l). Control plates did not contain any magnesium sulphate. Mycelial plugs of the basidiomycetes were placed on the plates and grown at 25°C for 20 days, alternating with 12 hours of dark and 12 hours of cool fluorescent light. A positive result was indicated by the presence of black crystals on the media or when the media turned rust coloured on the outer side of the colony.

Lignin peroxidase: Mycelial plugs were placed onto minimal growth medium that contained 5 % maltose, 0.03 % o-anisidine (Sigma-Aldrich (Pty) LTD) and 1.4 % agar. Plates were incubated at 30°C for 14 days and then flooded with a solution of 50mM Na-tartrate buffer (pH 3), 50μM H_2O_2 and 2 mM veratryl alcohol (Sigma-Aldrich (Pty) LTD). Isolates which produced peroxidase also produced a purple halo (Conesa *et al*., 2000).

Laccase: This method was performed as described by Rigling (1995). Isolates were grown on tannic acid malt extract medium (TAM) made up of 1 % tannic acid (Merck, Gauteng, South Africa), 1.5 % malt extract (Difco, Le Pont de Claix, France) and 2 % bacto agar (Difco, Le Pont de Claix, France). The pH was adjusted to 4.5 with 1 M NaOH. The malt extract agar was autoclaved separately and cooled to $50^{o}C$ before mixed with the tannic acid. In the presence of laccase, the medium turned medium brown.

Cellulase and xylanase: The presence of these enzymes was tested according to the protocol in St. Leger *et al*. (1997). Mycelial plugs were placed onto minimal media containing 0.3 % $NaNO_3$, 0.1 % KH_2PO_4, 0.05 % $MgSO_4$ (at pH 6) supplemented with 0.1 % yeast extract (Biolab, South Africa) and 0.5 % CM-cellulose (medium viscosity) (Sigma-Aldrich (Pty) LTD) for the cellulose assay or 0.5 % birchwood xylan (Sigma-Aldrich (Pty) LTD) for the xylanase assay. Plates were stained with 1 mg/ml Congo red for 15 minutes and then destained with 1 M NaCl. The size of the cleared zone was measured and the ratio of zone size to colony diameter was calculated (Hankin and Anagnostakis, 1977).

RESULTS

Toxin analyses

No sterehirsutinal, frustilosine and frustulosinol were produced by any of the basidiomycete isolates (data not shown). The limits of detection were as follows: frustulosinol 0.5 ppm; Sterehirsutinal 0.125 ppm; 4-hydroxy-benzaldehyde 0.0125 ppm and frustulosine 0.125 ppm. Four-hydroxy-benzaldehyde was produced by all the isolates in relative low concentrations that ranged from 0.005 to 0.06 mg/L (Fig. 1). The level of 4-hydroxy-benzaldehyde was not related to a specific taxon and varied within each taxon. One isolate of Taxon 7 (STE-U 7165) had the highest secretion at 0.06 mg/L.

Enzyme characterization

Manganese peroxidase assays that tested positive showed black crystals that formed in the medium (Fig. 2 A - B), or the medium turned rust coloured (Fig. 2 D). Less commonly, a combination of the rust colour and the black crystals occurred (Fig. 2 C).

All isolates were capable of producing manganese peroxidase at 80 or 100 ppm except for Taxon 4 at 100 ppm (Table 1). The isolates of *Fomitiporia* sp. and Taxon 8 all secreted manganese peroxidase at 100 ppm and for the other taxa it was variable among the isolates. Taxon 4 produced very small amounts of this enzyme at 80 ppm (only two plates were positive) and was not able to produce this enzyme at higher concentrations. The isolates of the *Phellinus* sp. all produced manganese peroxidase at 80 ppm and at 100 ppm.

All of the *Fomitiporia* isolates were able to produce lignin peroxidase as indicated when the mycelium or medium turned purple in colour (Fig. 3). As shown in Table 1, an isolate in both Taxon 1 and 2 did not produce the enzyme whereas all the other isolates in these taxa did. No isolates in Taxa 3, 4, 5, 6 or 8 were able to produce this enzyme. *Phellinus* sp. and Taxon 7 did not produce lignin peroxidase except on one and three occasions respectively, showing partial secretion.

A positive result for laccase was the formation of a brown pigment in the medium (Fig. 4 A), varying from pale to dark (Fig. 4 B). All isolates of *Fomitiporia, Phellinus,* Taxon 1 and 4 were able to produce laccase (Table 2). Taxa 2, 3, 5, 6 and 7 produced varying amounts of this enzyme and Taxon 8 was not able to produce it.

Staining with Congo red, following destaining with NaCl, indicated a halo if cellulase was produced (Fig. 5). By measuring the size of the colony and the diameter of the halo, a ratio could be calculated indicating the level of production of the enzyme in comparison with the colony growth. All isolates were able to produce cellulase (Fig. 6). The different taxa produced different levels of cellulase and in the case of Taxon 3, all the isolates had a relatively high ratio, which could be a unique characteristic of this taxon. Most of the isolates were not significantly different and fell within the lower ratio group. No halos were observed for the xylanase test, indicating that none of the isolates were able to produce xylanase.

DISCUSSION

The basidiomycete isolates in this study all produced 4-hydroxy-benzaldehyde at low concentrations ranging from 0.005 mg/L to 0.06 mg/L. The different taxa could not be differentiated by the toxin levels produced. Our results are in line with previous studies that showed that four-hydroxy-benzaldehyde was previously found to be produced by *F. mediterranea*, *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum*, although the levels were not reported (Tabacchi *et al*., 2000). Poliart (2000) isolated 0.14 mg/L of p-hydroxy-benzaldehyde from *Phaeomoniella chlamydospora* and it was also isolated from *Phaeoacremonium aleophilum* and *F. mediterranea*, although, once again, the levels were not reported.

Various factors can influence toxin production in liquid media. These include the pH, carbon source and temperature of the medium (Schmidt *et al*., 1999; Strange, 2007). Some of these factors can also play a role in the low levels of toxin secreted in this study.

Four-hydroxy-benzaldehyde together with other hydroxy-benzaldehyde derivatives are thought to play a role in the phytotoxicity of the fungi causing esca, especially due to the presence of a hydroxy substituent on the aromatic ring and a vinylacetylenic chain (Tabacchi *et al*., 2000). In grapevine callus, p-hydroxy-benzaldehyde reduced growth by 20 % at 100 μM and 80% at 1000 μM (Poliart, 2000). Using grapevine leaf discs, no necrosis was observed after 48 h at 1 mM but 40 % of the leaf showed necrosis at 10 mM after 48 h. Poliart (2000) also tested p-hydroxy-benzaldehyde on tomato plants, which showed signs of wilting at concentrations of 1.381 mg/ml after 10 h and died after 24 h. Four-hydroxy-benzaldehyde has also been produced by other plant pathogens such as *Ceratocystis* sp. (Ayer *et al*., 1986), *Eutypa lata* (Jiménez-Teja *et al*., 2006), *Phoma exigua* (Cimmino *et al*., 2008) and *Diplodia seriata* (Venkatasubbaiah and Chilton, 1990; Venkatasubbaiah *et al*., 1991; Martos *et al*., 2008). *Phoma exigua* produced 1 mg/ L of 4-hydroxy-benzaldehyde (Cimmino *et al*., 2008), Venkatasubbaiah and Chilton (1990) reported that *B. obtusa* (*D. seriata*) produced 0.5 mg/ L and Venkatasubbaiah *et al*. (1991) showed that this compound caused necrotic lesions on leaves of various weed species. It is uncertain of how the levels produced in

this study will affect the vines. Further plant assays could confirm the phytotoxicity of this toxin.

No sterehirsutinal, frustulosinol and frustulosin were detected from the basidiomycetes in the present study. The absence of these toxins in the South African basidiomycete taxa indicates that our taxa do not produce the toxins produced by *Stereum hirsutum* and *Stereum frustulosum*. The genus *Stereum* falls within the Russulales, whereas the basidiomycete taxa that were found from esca diseased grapevines in South Africa fall within the Hymenochaetales.

The effect of the interaction between combinations of different toxins produced by the esca fungi remains unclear. Other phytotoxic compounds produced by *Ph. chlamydospora* and *P. aleophilum* include scytalone, isosclerone and pullulan which, when absorbed by detached leaves, caused foliar symptoms similar to esca diseased vines (Evidente *et al*., 2000; Sparapano *et al*., 2000; Surico, 2001; Bruno *et al*., 2007). Pullulan causes development of thin films in the mesophyll tissue, which makes it difficult for oxygen to permeate through the membranes and is therefore likely to contribute to brown wood streaking (Sparapano *et al*., 2000). Studies have shown that *F. mediterranea* produce pullulans and 4-hydroxy-benzaldehyde (Surico, 2001). Production of more than one toxin is advantageous to the fungus, as it increases overall toxicity (Tabacchi *et al*., 2000).

In the present study, the basidiomycete fungi produced three lignin-degrading enzymes namely lignin peroxidase, manganese peroxidase and laccase. White rot fungi such as *Funalia trogii, Lentinus edodes*, *Pleurotus dryinus* and *Pleurotus tuberrgium* produce xylanase, manganese peroxidase and laccase (Kachlishvili *et al*., 2006). White rot fungi secrete a diversity of extracellular enzymes which can degrade lignin. Few comparative studies have looked at the production levels of these enzymes (Kachlishvili *et al*., 2006). The production of laccase and peroxidase may assist the esca pathogens to degrade the wood, allowing competition between each of the pathogenic fungi and possibly detoxifying antimicrobial compounds produced by the plant (Bruno and Sparapano, 2006).

Manganese oxidation, which assists in the degradation of lignin (Overton *et al*., 2006), is a prerequisite for enzymes such as kinases, decarboxylases, dehydrogenases,

oxidases and peroxidases. It is also involved in photosynthetic oxygen evolution whilst associated with cation-activated enzymes (Taiz and Zeiger, 2002). In this study, the majority of the basidiomycetes were able to produce manganese peroxidase. Overton *et al*. (2006) found that *Fomitiporia punctata* produced dark brown pigmentation on control plates and on Mn-sulphate plates and so it was difficult to observe manganese oxidation. This was not observed in the current study, where no control plates indicated a brown pigmentation.

In this study, the majority of isolates were able to produce laccase to varying degrees. Only Taxon 8 did not produce laccase. In culture, laccase production is influenced by pH of the medium, the age of the fungus, the medium and the presence or absence of certain activators (Chiarappa, 1959). These factors could have played a role in the results obtained in this study. Laccase is detected by the conversion of tannin into a brown precipitate found in the mycelium (Bruno and Sparapano, 2006). The breakdown of lignin is a part of the infectious process, as the enzyme is secreted at the growing tips of hyphae (Rigling, 1995). *Fomitiporia* has been found to have laccase activity (Alessandro *et al*., 2000). *Phellinus* sp. (*Inocutis jamaicensis*) associated with 'hoja de malvón' of grapevines in Argentina, a disease with similar internal symptoms to esca, was found to produce tannic acid (Gatica *et al*., 2004), which is an inducer for laccase (Zhao and Kwan, 1999).

Lignin peroxidase was not produced by Taxa 3, 4, 5, 6 and 8. *Stereum hirsutum* also did not produce lignin peroxidase (Del Río *et al*., 2004), indicating that not all white rot species produce this enzyme. Taxa 3, 4, 5, 6 and 8 had fewer isolates in comparison with Taxon 1 and the *Fomitiporia* sp., the predominant taxa which produced lignin peroxidase. This indicates the possible role that lignin peroxidase plays in pathogenesis and wood rotting. The inability to produce lignin peroxidase could contribute to the low incidence of *Stereum hirsutum* on grapevines. Veratryl alcohol is an important co-factor that is synthesized in response to a manganese deficiency and this enables the correct functioning of lignin peroxidase (Mester *et al*., 1995). In this study, veratryl alcohol was used during staining to induce the functioning of the enzyme.

All taxa in this study were able to produce cellulase and no isolates were able to produce xylanase. Cellulase and xylanase break down pectic polysaccharides. Cellulase is

commonly produced by white rot fungi (Johansson, 1966; Martinez *et al*., 2005; Kachlishvili *et al*., 2006). Other white rot fungi, such as *Funalia trogii, Lentinus edodes*, *Pleurotus dryinus* and *Pleurotus tuberrgium*, have been shown to produce xylanase when grown under solid-state fermentation of wheat straw and beech tree leaves (Kachlishvili *et al*., 2006). Xylanase can be produced in small amounts by white rot fungi, but the current assay might not have been sufficiently sensitive to detect it (St. Leger *et al*., 1997).

CONCLUSIONS

All of the ten basidiomycete taxa produced 4-hydroxy-benzaldehyde. Four enzymes that can break down components of cell walls (primarily lignin and cellulose) were also detected. The specific role of 4-hydroxy-benzaldehdye is unclear and further studies should be performed to see if this toxin is able to produce foliar symptoms alone or only when combined with scytalone, isosclerone and pullulans produced by the esca fungi. Since the fungi occur in different combinations in the wood, it will be difficult to determine which fungus is responsible for the production of a specific toxin *in planta*. Further work should determine how these toxins and/ or enzymes work together to form foliar symptoms and wood rot.

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Table 1. The production of manganese peroxidase, lignin peroxidase and laccase by the selected basidiomycete isolates.

Taxon	STE-U Number	Magnesium sulphate[1] 80 ppm	Magnesium sulphate[1] 100 ppm	Lignin peroxidase[1]	Laccase[1]
1	7038	1	1	1	1
1	7058	1/0	0	1	1
1	7084	1/0	1/0	0	1
1	7141	1	1	1/0	1
1	7148	1	1	1	1
2	7147	0	1/0	0	1
2	7154	1/0	0	1	1
2	7155	1/0	1/0	1	0
3	7109	0	1/0	0	1/0
3	7136	1/0	1	0	1
3	7174	1	1	0	0
4	7042	0	0	0	1
4	7043	1/0	0	0	1
5	7126	1	1	0	1
5	7143	1/0	1/0	0	1/0
5	7153	1/0	1	0	1/0
6	7133	1/0	1/0	0	1/0
6	7134	1	1/0	0	1
7	7090	1/0	1/0	0	1/0
7	7106	1/0	1/0	0	1
7	7165	1/0	1	1/0	1/0
8	7138	1	1	0	0
8	7139	1/0	1	0	0
Fomitiporia sp.	7069	1	1	1	1
Fomitiporia sp.	7096	1	1	1	1
Fomitiporia sp.	7122	1/0	1	1	1
Fomitiporia sp.	7135	1	1	1	1
Fomitiporia sp.	7168	1	1	1	1
Phellinus sp.	7055	1	1	0	1
Phellinus sp.	7098	1	1	0	1
Phellinus sp.	7105	1	1	1/0	1

[1] If all six plates produced a positive result, 1 was denoted. If five or less plates were positive, 1/0 was denoted and 0 if no enzymes were produced.

Table 2. Number of plates which tested positive for laccase production according to the intensity of the brown discoloration produced.

Taxon	STE-U Number	Very pale	Pale	Pale-Medium	Medium	Medium - Dark	Dark	Total positive plates[1]
1	7038		2				4	1
1	7058					2	4	1
1	7084		3	3				1
1	7141						6	1
1	7148				1		5	1
2	7147		5				1	1
2	7154		1		1		4	1
2	7155							0
3	7109		3					1/0
3	7136	3	3					1
3	7174							0
4	7042		5		1			1
4	7043	3	3					1
5	7126	1	4		1			1
5	7143	1						1/0
5	7153	3						1/0
6	7133	1						1/0
6	7134	6						1
7	7090		1		1		1	1/0
7	7106		6					1
7	7165		3				2	1/0
8	7138							0
8	7139							0
Fomitiporia sp.	7069		6					1
Fomitiporia sp.	7096			3			3	1
Fomitiporia sp.	7122		6					1
Fomitiporia sp.	7135				1		5	1
Fomitiporia sp.	7168				6			1
Phellinus sp.	7055		1		5			1
Phellinus sp.	7098				6			1
Phellinus sp.	7105				6			1

[1] If all plates produced a positive result, 1 was denoted. If five or less plates were positive, 1/0 was denoted and 0 if no manganese peroxidase was produced.

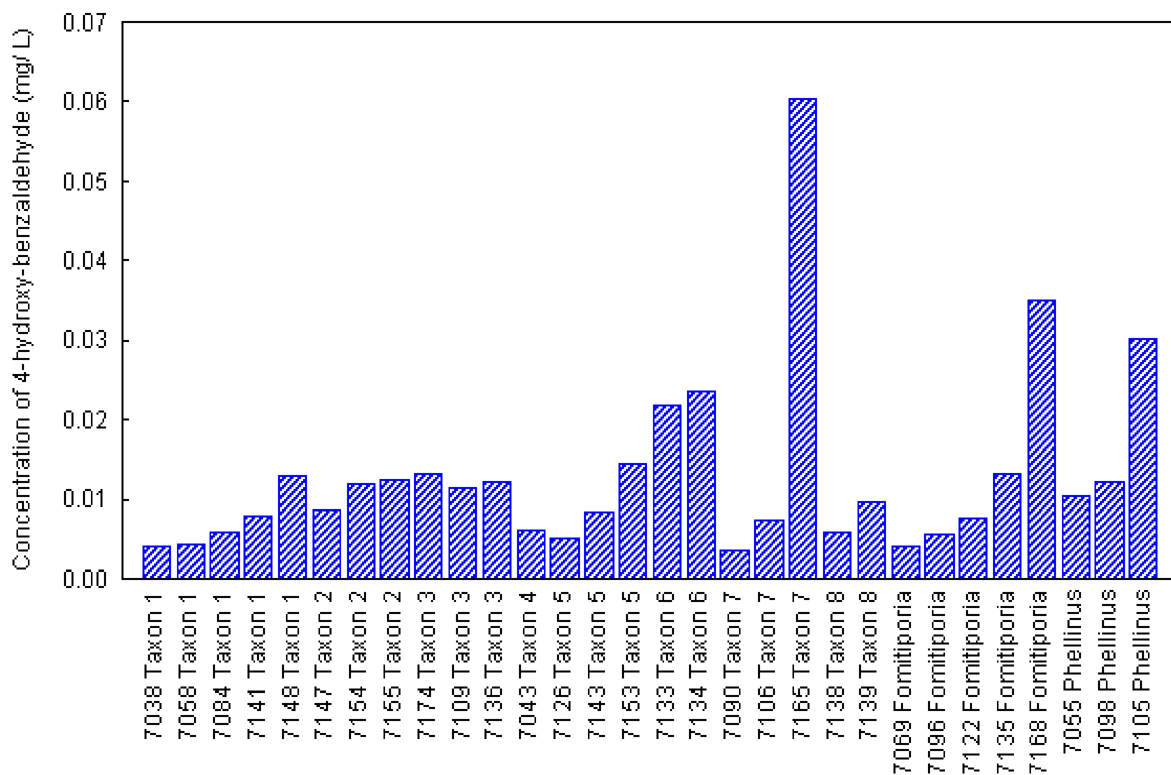

Fig. 1. Concentration of 4-hydroxy-benzaldehyde produced by selected basidiomycete isolates from the ten taxa.

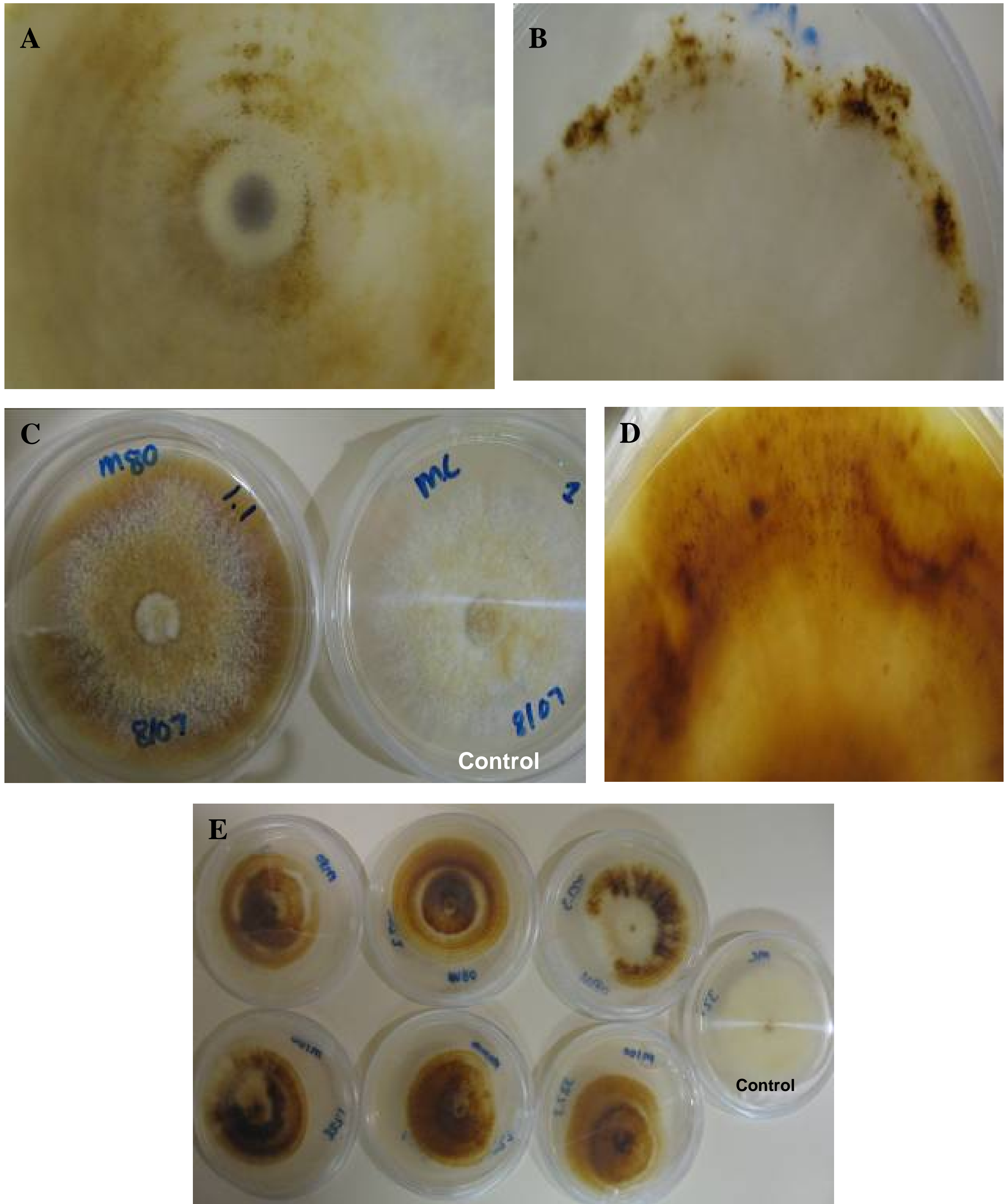

Fig. 2. Manganese peroxidase tests were positive if black crystals formed in the medium (A, B), the medium turned a rust colour (C) or had both the crystals and the rust colored medium (D). There was variation in results obtained by a number of isolates (E).

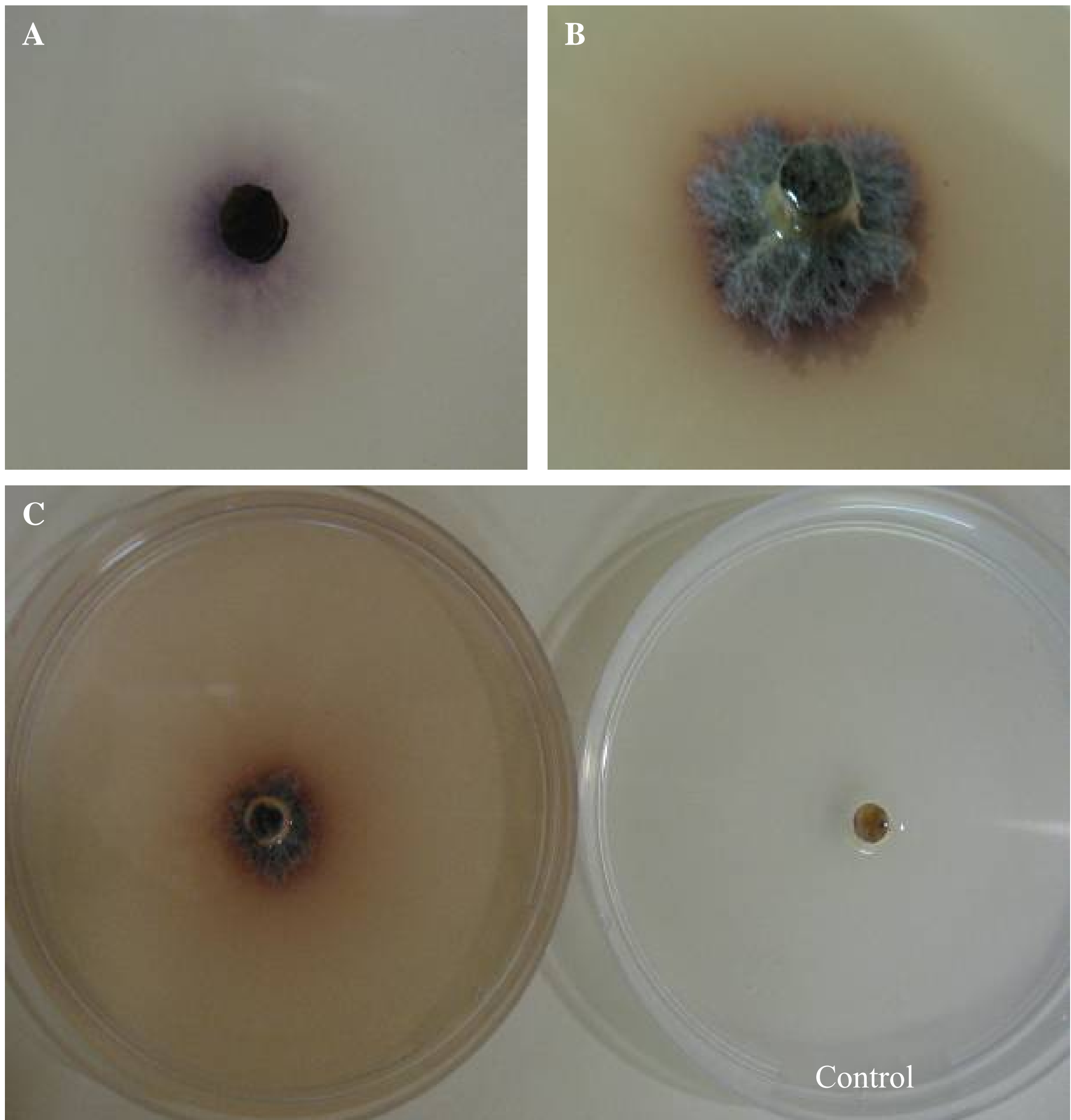

Fig. 3. Plates indicating the presence or absence of lignin peroxidase. A, B and C show the variation in purple coloration indicative of lignin peroxidase.

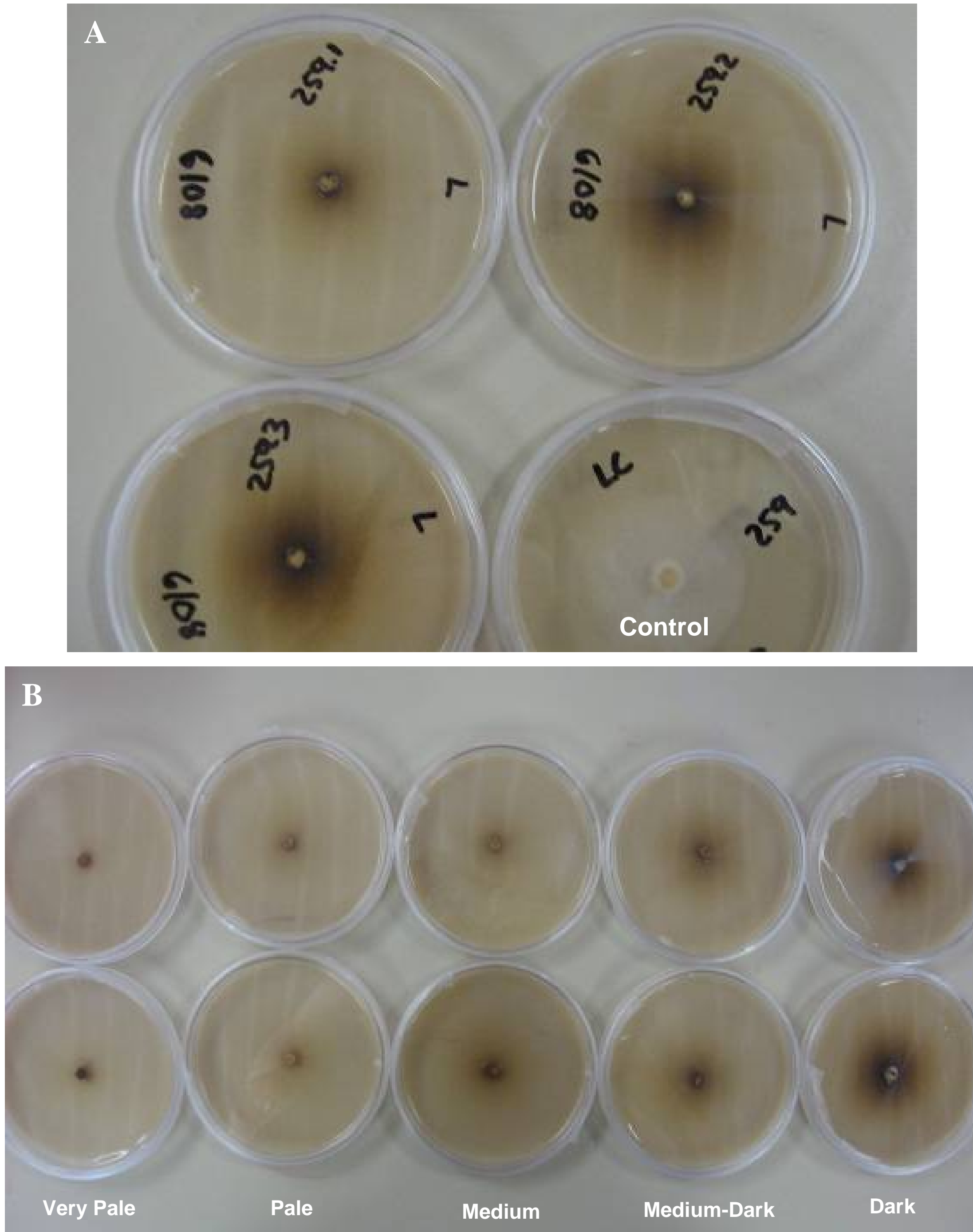

Fig. 4. Positive results for the laccase test are indicated by the medium turning brown (A). There is a variation in the secretion of laccase as shown in B.

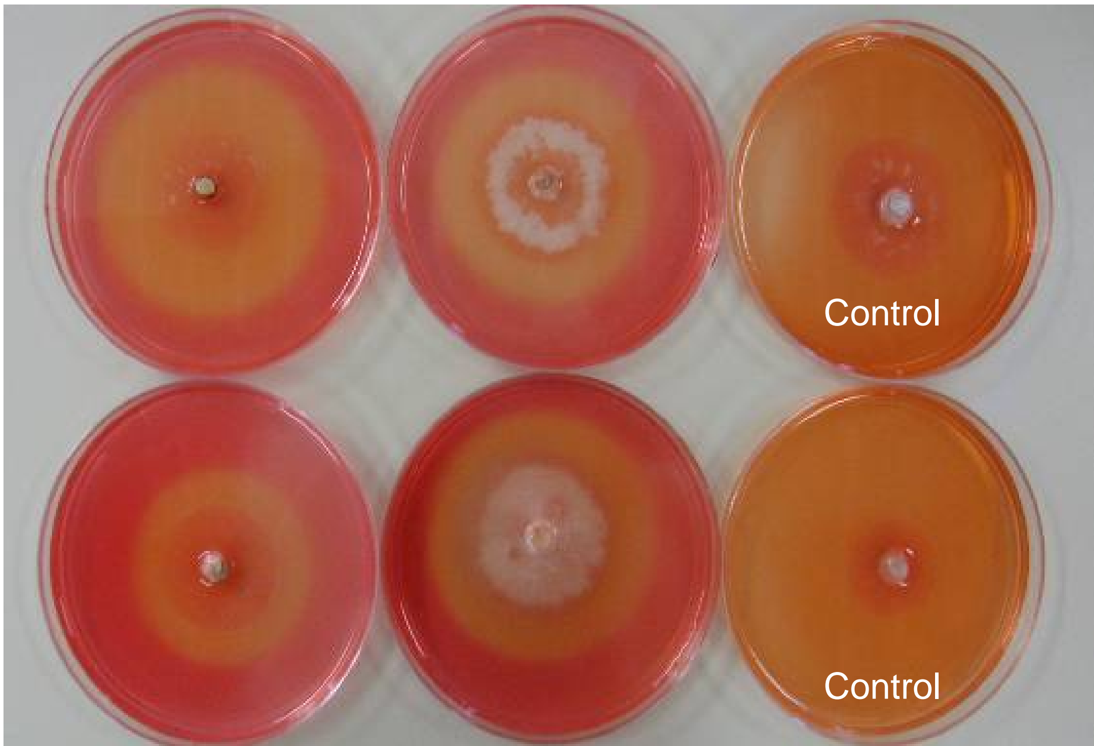

Fig. 5. Positive results for cellulase were determined after staining with Congo Red and destaining with 1 M NaCl and a halo was produced.

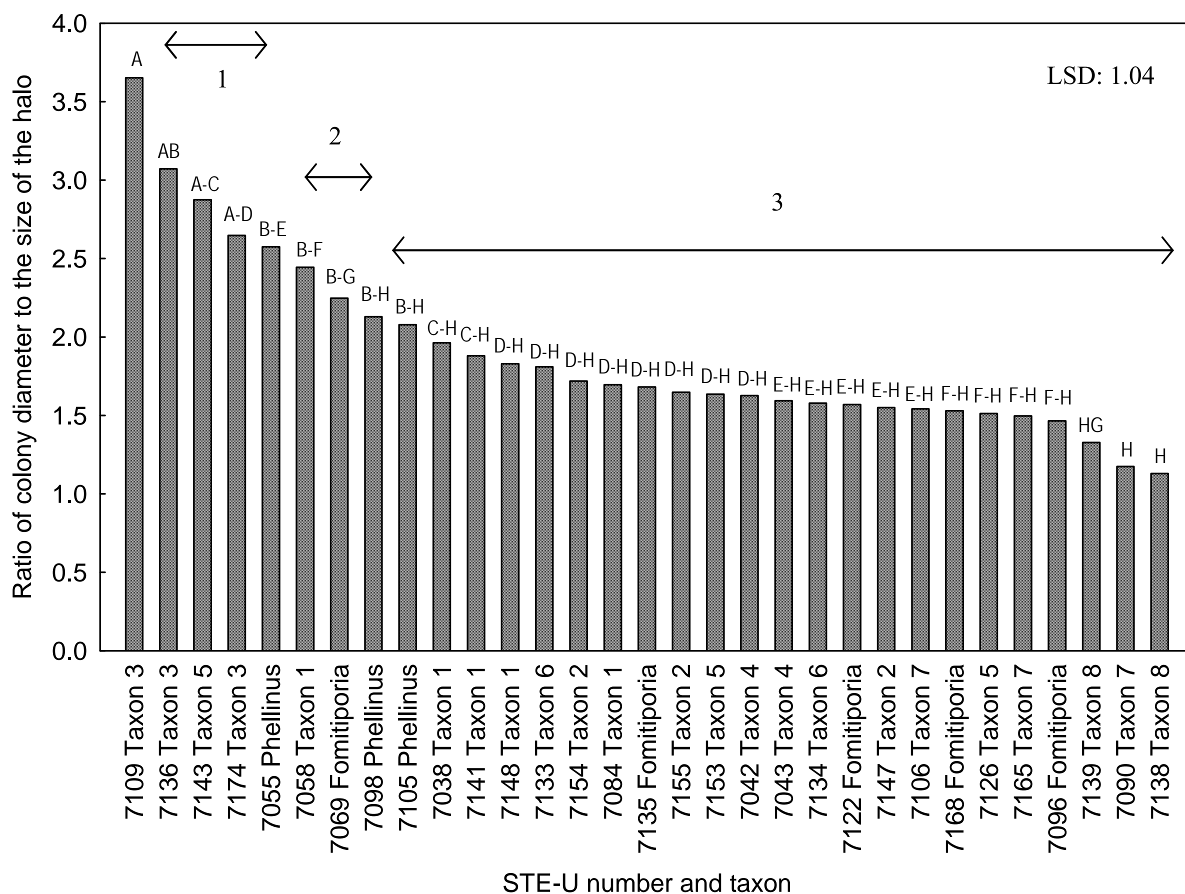

Fig. 6. The ratio of the colony diameter to the size of the halo produced by cellulase secretion where 1 indicates high, 2 medium and 3 low production of cellulase.