

**THE CHARACTERISATION OF BASIDIOMYCETES ASSOCIATED WITH ESCA
DISEASE IN SOUTH AFRICAN GRAPEVINES**

by

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Dissertation presented for the degree of
Doctor of Philosophy in the Faculty of AgriSciences at Stellenbosch University



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March 2015

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

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Date: 8 December 2014

Summary

Esca is a disease complex of grapevine that includes different foliar and vascular symptoms caused by various fungal pathogens. One of the distinguishing characteristics of the disease on mature vines is the white rot of the wood. Esca-related wood rot is caused by several lignicolous basidiomycetes from the order Hymenochaetales. The Hymenochaetales fungi associated with esca vary depending on geographic location. For example, in Europe and the Mediterranean grape-growing regions, *Fomitiporia mediterranea* is the prevalent species; in Argentina, *Inocutis jamaicensis*; in Chile "*Fomitiporella vitis*", and in Australia *Fomitiporia australiensis*. In the United States, *Fomitiporia polymorpha* has been associated with esca, though not consistently. A previous study identified ten different taxa belonging to the genera *Fomitiporella*, *Fomitiporia*, *Inocutis*, *Inonotus*, and *Phellinus* associated with esca in South Africa. The current study was tasked with characterising these taxa and assessing their epidemiology and pathogenicity. The study has characterised three novel species, *Fomitiporella viticola*, *Fomitiporia capensis* and *Phellinus resupinatus* from *Vitis vinifera* and a first report of *Inonotus setuloso-croceus* occurring on *Vitis vinifera* and *Salix* spp. worldwide and in South Africa. The sporulation of *F. viticola* was surveyed over two seasons. The pathogenicity of all ten taxa was tested on mature field grown vines and enzymes secreted by all ten taxa were assayed. This study aimed to add in the understanding of the esca complex disease in South Africa and contributed towards the wider knowledge regarding the ecology of the Hymenochaetales.

A novel *Fomitiporia* species, *F. capensis*, was described based on fruit body morphology and combined internal transcribed spacer rRNA ITS1-5.8S-ITS-2 (ITS) and large sub-unit (LSU) phylogeny, where it formed a clearly delineated and well-supported clade. Morphologically, *F. capensis* was similar to *F. punctata* in that both species essentially lack setae. *Fomitiporia capensis*, *F. punctata* and *F. aethiopica* produced similarly sized basidiospores, but differed in terms of host range, pore size and, possibly, fruiting body shape. Phylogenetically, *F. capensis* appeared to be related to *F. tenuis*, though morphologically the species differed significantly in that *F. tenuis* had smaller pores and smaller basidiospores. During all surveys conducted, *Fomitiporia capensis* was found to occur widely as throughout the Western Cape Province, though fruit bodies were scarce in comparison to mycelium isolated from symptomatic vines. Fruit bodies were also found in a vineyard in the Limpopo region in the north east part of the country.

Phellinus resupinatus was described based on fruit body morphology, ITS and LSU phylogenies. It formed a well-supported clade closely related to *Phellinus bicuspisidatus*, a species associated with white rot in oak trees in the United States. Morphologically, *P.*

resupinatus was characterised by its resupinate fruit body shape, straight, ventricose hymenial setae, and broadly ellipsoid hyaline basidiospores. It was only found on diseased grapevines in the summer rainfall regions of South Africa, mainly in the Northern Cape and Limpopo provinces.

Fomitiporella viticola was described from *Vitis vinifera* based on fruit body morphology and ITS phylogeny. It is characterised by a resupinate to effuse-reflexed fruit body with large, loosely spaced pores and fairly small yellowish-brown basidiospores. *Inonotus setulosus-croceus* was found occurring on *Salix* and *Vitis vinifera* and was identified based on fruit body morphology. The ITS region was sequenced from DNA isolated from cultures obtained from rotten wood or fruit bodies, and was matched to the Hymenochaetales species from *Vitis* previously classified as Taxon 7. The discovery of *Inonotus setulosus-croceus* on *Salix* validated the hypothesis that fruit bodies may occur on alternative hosts.

Fomitiporella viticola was often isolated from white rot on vines affected by esca and fruit bodies were often found on vines in the Western Cape Province. Twelve fruit bodies of *F. viticola* were monitored for sporulation weekly over two seasons lasting between winter and early summer. Levels of sporulation had a weak positive correlation with rainfall and a weak negative correlation with average temperature. Sporulation was found to occur throughout the entire monitoring period.

Little is known about the pathogenicity and aetiology of the Hymenochaetales taxa associated with esca in South Africa. All ten taxa were subjected to enzyme assays to determine which ligninolytic enzymes were secreted by each taxon. In addition, a field trial was undertaken to determine the pathogenicity of ten South African Hymenochaetales taxa associated with esca in grapevine. Twenty-seven fungal isolates and two negative controls were inoculated into mature grapevines and incubated for 24 months. The results of the enzyme assays indicated a difference in enzyme secretion between taxa and also among isolates of the same taxa. All isolates secreted cellulase and laccase, but there was a difference between isolates' ability to secrete manganese peroxidase and lignin peroxidase. The results of the pathogenicity trial showed that all of the isolates used were capable of causing the characteristic white rot symptom in the wood. There were also clear differences in susceptibility to white rot between the two cultivars tested. Cultivars also differed in which taxa proved pathogenic. On Shiraz, Taxon 6 (an *Inonotus* sp.), *Phellinus resupinatus* and *Inonotus setulosus-croceus* were significantly virulent. On Mourvèdre, however, Taxon 3, an *Inocutis* sp. and Taxon 2, a *Fomitiporella* sp. were significantly virulent. Cultivar differences could be due to various factors, including differences in host response to colonisation and

physical differences in wood structure, as well as the differences in enzyme secretion between taxa.

Opsomming

Esca is 'n siekte-kompleks op wingerd wat gekarakteriseer word deur verskeie blaar- en houtstptome. Die siekte word veroorsaak deur verskeie patogene. Een van die onderskeidende kenmerke van die siekte op ouer wingerd is die voorkoms van wit houtverrotting. Hout-verrotting wat met esca geassosieer word, word veroorsaak deur verskeie houtverrottende basidiomycete wat behoort aan die orde Hymenochaetales. Die Hymenochaetales spesie wat met esca geassosieer word, verskil na gelang van geografiese area. In Europa en die Mediterreense area, is *Fomitiporia mediterranea* die hoofspesie geassosieer met esca, terwyl *Inocutis jamaicensis* en "*Fomitiporella vitis*" hoofsaaklik in Argentië en Chile, onderskeidelik, voorkom. In Australië is *Fomitiporia australiensis* die hoofspesie, en in die Verenigde State is *Fomitiporia polymorpha* al vantevore met esca geassosieer. 'n Voorafgaande studie het ten verskillende taxa in die genera *Fomitiporia*, *Phellinus*, *Inocutis*, *Inonotus* and *Fomitiporella* gevind vanuit esca-geïnfekteerde wingerd. Die doel van hierdie studie was om hierdie taxa te karakteriseer, sowel as om hulle epidemiologie en patogenisiteit te ondersoek. Die studie het drie nuwe spesie gekarakteriseer, nl. *Fomitiporella viticola*, *Fomitiporia capensis* en *Phellinus resupinatus*. Die studie het ook 'n verdere spesie, *Inonotus setulosus-croceus*, vir die eerste keer op *Vitis vinifera* en *Salix* spp. in Suid Afrika en wereldwyd aangemeld. Die sporulasie van *F. viticola* is ondersoek gedurende twee seisoene. Die patogenisiteit van al tien taxa is ondersoek op wingerd in die veld, en ensiem-vrystelling deur al tien taxa is ondersoek. Hierdie studie het ten doel gehad om 'n bydrae te lewer tot verdere kennis van die aard van die esca siekte-kompleks in Suid Afrika, asook die breër kennis rakende die ekologie van die Hymenochaetales.

'n Nuwe *Fomitiporia* spesie, *Fomitiporia capensis*, is beskryf op grond van vrugliggaam morfologie en 'n gekombineerde rRNA ITS1-5.8S-ITS2 (ITS) en groot subeenheid (LSU) filogenie, waar dit 'n duidelike en goed ondersteunde groep gevorm het. Morfologies was *F. capensis* eenders aan *F. punctata* in dat albei spesies basies geen setae gehad het nie. *Fomitiporia capensis*, *F. punctata* en *F. aethiopica* het eenderse basidiospore gevorm, maar het verskil in terme van gasheer, porie-grootte en vrugliggaam vorm. *F. capensis* is waarskynlik filogeneties verwant aan *F. tenuis*, maar die spesie verskil in dat *F. tenuis* kleiner porieë en basidiospore het. Gedurende die studie is *F. capensis* in die hele Wes Kaap provinsie gevind. Vrugliggame was skaars in vergelyking met miselium wat vanuit simptomatiese wingerdstokke geïsoleer is. Vrugliggame is ook in 'n wingerd in Limpopo gevind.

Phellinus resupinatus is beskryf op grond van vrugliggaam morfologie en ITS en LSU filogenieë. Dit het 'n goed-ondersteunde groep gevorm wat naby verwant aan *P.*

bicuspidatus, 'n spesie wat geïssosieer word met wit vrot in eikebome in die Verenigde State. *P. resupinatus* word morfologies gekarakteriseer deur sy resupinate vrugliggaam-vorm, reguit-vormige ventricose setae en ellipsoid, hyaline basidiospore. Dit is skegs gevind op sieklike wingerdstokke in die somer-reënval areas in Suid Afrika, meestal in die Noord Kaap, maar ook in Limpopo.

Fomitiporella viticola is beskryf vanaf *Vitis vinifera* gebaseer op vrugliggaam morfologie en ITS filogenie. Dit word gekarakteriseer deur 'n resupinate tot effuse-reflexed vrugliggaam met groot, wyd-gespasieërde porieë en redelike klein gelerige basidiospore. *Inonotus setulosus-croceus* is op *Salix* en *Vitis vinifera* gevind en is geïdentifiseer deur middel van vrugliggaam-morfologie. Die ITS area is gekarakteriseer vanuit DNA wat geïsoleer is vanaf miselium kulture of vrugliggame, en is dieselfde as die Hymenochaetales spesie vroeër bekend as Taxon 7. Die ontdekking van *I. setulosus-croceus* op wingerd en *Salix* ondersteun die hipotese dat vrugliggame dalk op alternatiewe gasheer gevind kan word.

Fomitiporella viticola is gereeld geïsoleer uit wit vrot op wingerd geïffekteer deur esca en vrugliggame is algemeen gevind in die Wes Kaap. Twaalf vrugliggame van *F. viticola* is weekliks gemonitor vir sporulasie gedurende twee seisoene tussen winter en vroeë somer. Vlakke van sporulasie het 'n vlou positiewe korrelasie met reënval, en 'n swak negatiewe korrelasie met temperatuur gehad. Sporulasie het plaasgevind gedurende die hele moniterings-periode.

Min is bekend rakende die patogenisiteit en etiologie van die Hymenochaetales taxa geïssosieer met esca. Al tien taxa is onderwerp aan ensiem toetse om te bepaal watter ensieme deur elke taxon afgeskei word. 'n Veld-proef is ook onderneem om patogenisiteit te bepaal vir al tien taxa. Sewe en twintig swam-isolate en twee negatiewe kontroles is geïnkuleer in wingerdstokke en geïnkubeer vir 24 maande. Die resultate van die ensiem-toetse het aangedui dat daar 'n verskil in ensiem-sekresie tussen die verskeie taxa, asook tussen verskillende isolate binne dieselfde taxa, is. Alle isolate het cellulase en laccase afgeskei, maar daar was 'n verskil in isolate se vermoë om manganese peroxidase en lignin peroxidase af te skei. Die resultate van die patogenisiteitstoets het gedui daarop dat alle isolate wat in die toets gebruik word wel die vermoë het om die wit vrot te vorm. Daar was duidelike verskille in vatbaarheid teen wit vrot tussen die twee cultivars in die toets. Die patogeniese taxa het ook verskil tussen die twee cultivars. In Shiraz was Taxon 6 ('n *Inonotus* spesie), *Phellinus resupinatus* en *Inonotus setulosus-croceus* beduidend virulent. In Mourvèdre was Taxon 3 ('n *Inocutis* spesie) en Taxon 2, 'n *Fomitiporella* spesie, beduidend virulent. Cultivar-verskille kan as gevolg van verskeie faktore wees, insluitende verskille in gasheer-respons op kolonisering en fisiese verskille in hout-struktuur, sowel as die verskille in ensiem sekresie tussen taxa.

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CHAPTER 1

Hymenochaetales Oberw. fungi associated with esca related wood rots on grapevine

ABSTRACT

Esca disease is a problem on grapevines worldwide. This disease complex is characterised by several external and internal symptoms including, leaf-streaking, dieback, internal discolouration, and white rot. The causal organisms of esca are primarily *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. and Mugnai) Crous and W. Gams, several *Phaeoacremonium* W. Gams, Crous and M.J. Wingf. species and basidiomycete species from the order Hymenochaetales, the latter ones responsible for causing the white rot symptom. Basidiomycete species causing the wood rot symptom of esca differ among grapevine-growing areas worldwide. The South African grapevine industry is unique in having ten different basidiomycete taxa from five different genera associated with the disease complex. The Hymenochaetales are mainly associated with white rot on woody plants and there are several species that are economically important to the agricultural and forestry industries. Few Hymenochaetales species have been described from the African continent, though this study is an indication of previously unknown diversity in Southern Africa.

OVERVIEW OF THE SOUTH AFRICAN GRAPEVINE INDUSTRY

The South African grapevine industry was founded in the 1650's as part of the Dutch East India Company's programme to supply ships on the Spice Route from a way station based at the Cape. The first *Vitis vinifera* L. cuttings, consisting of the cultivars Hanepoot (Muscat d'Alexandrie), Muscadel and Chenin blanc, were imported to the Cape in 1655 and the first wine was produced in 1659 on Jan van Riebeeck's farm, Wynberg (Perold, 1927; Viall *et al.*, 2011). At that time, unfortified wine wasn't suitable for transport so local wine was produced for taverns that provided food and drink to soldiers and sailors. Winemaking only became a sustainable concern after the establishment of Constantia by Simon van der Stel in 1685 and the arrival of the French Huguenots in 1688, which created a demand for better quality Cape wines and provided farmers skilled in viticulture (Perold, 1927; Viall *et al.*, 2011).

The industry has overcome numerous challenges since first established, including the destruction of early vineyards by the wind and marauding Khoi herders during the mid 17th century, phylloxera in the 1880's, and the economic boycott during the 20th century (Viall *et al.*, 2011). Today, the grapevine industry takes up a total of 123,624 ha in the Western Cape, the Northern Cape, and the Northern provinces, making it the 12th largest grapevine production region in the world (Anonymous, 2014).

The wine industry, far removed from its start with a handful of cuttings, currently spans 99,680 ha in the Western Cape and Northern Cape Provinces, with the top five cultivars planted being Chenin blanc, Colombar, Cabernet Sauvignon, Shiraz, and Sauvignon blanc. The Northern Cape industry only represents about 5% of the total area planted to wine grapes (Floris-Samuels and Whitehead, 2013). 1,498,702 tons of grapes were crushed in 2013 equivalent to 1,097,200,000 litres of various types of wine, which approximately 57.4 % was exported. South Africa is currently the 9th biggest producer of wine in the world (Anonymous, 2014).

Grapes are a non-climacteric fruit and, as such, are highly perishable. The South African table grape export industry only came into existence with the expansion of the railway to the Hex River Valley after 1879, which created a way for farmers to get their goods to the harbour quickly and efficiently. After several seasons of trial and error, the Cape Fruit Syndicate was founded and, by 1892, had exported 1,900 cartons of fruit to Great Britain. Table grape exports grew to 12,000 cartons by 1899 after the adoption of more suitable cultivars like Waltham Cross (Viall *et al.*, 2011).

Currently, after the rapid expansion caused by the end of economic sanctions and deregulation of the deciduous fruit industry, the table grape industry takes up 15,484 ha, of which 4,787 ha are in the Northern Cape's Orange River growing area, 998 ha in the Northern Provinces (Limpopo and Mpumalanga), and the rest are situated in the warmer regions of the Western Cape. At the conclusion of the 2011 season, South African exports made up 47% of all table grape exports in the Southern Hemisphere. Exports in excess of 245,000 tons were achieved during the 2011/2012 season, the biggest harvest since the deregulation of the fruit export industry. The table grape industry makes up an important part of South African fruit exports (Anonymous, 2012).

Vineyard blocks in South Africa are expected to stay productive for longer than 20 years, although the expectation is generally closer to 15 years (Floris-Samuels and Whitehead, 2013).

Some of the most notable factors in this short longevity are the prevalence of grapevine diseases, mainly the leafroll viruses and grapevine trunk diseases.

A BRIEF INTRODUCTION TO GRAPEVINE TRUNK DISEASES

Grapevine trunk diseases include, Phomopsis, Botryosphaeria and Eutypa dieback, black foot, Petri disease, and esca, which affect vineyards in several ways causing an overall loss of longevity. They affect individual vine's longevity by causing the blockage of vascular tissue and the decline of structural wood, leading to gradual dieback of the arms and the eventual decline and death of the entire plant (Edwards *et al.*, 2001; Rumbos and Rumbou, 2001; Petit *et al.*, 2006; Calzarano *et al.*, 2009). This leads to a gradual loss in productivity per plant until it dies. Grape quality may be compromised due to uneven ripening (Mugnai *et al.*, 1999) and losses in grape quality will affect the alcohol content and the flavour components of wine (Mugnai *et al.*, 1999; Calzarano *et al.*, 2001, 2009; Pasquier *et al.*, 2013). In table grapes, where the appearance of clusters is its most important characteristic, yield losses may be due to cosmetic damage caused by uneven colouration. (Mugnai *et al.*, 1999).

Phomopsis cane and leaf spot and Phomopsis dieback are caused by several different *Phomopsis* (Sacc.) spp., mainly *Phomopsis viticola* (Sacc.) Bubák, in South Africa (Van Niekerk *et al.*, 2005). Phomopsis cane and leaf spot is characterised by longitudinal lesions, spotting and necrosis on green parts of the vine such as shoots, tendrils and bunch-rachises, and results in the eventual death of spurs and canes (Philips, 1998 and 2000). *Phomopsis* spp. are also often isolated from woody parts of vines and have been associated with cankers and pruning wounds. Other symptoms include vascular discolouration, bud mortality and lack of spring growth (Van Niekerk *et al.*, 2005; Úrbez-Torres *et al.*, 2013).

Eutypa dieback, caused by the diatrypaceous fungus *Eutypa lata* (Pers.) Tul. and C. Tul. and up to 10 other associated species (Trouillas *et al.*, 2009), was long considered the most important grapevine trunk disease in South Africa (Van Niekerk *et al.*, 2003). Botryosphaeria dieback, caused by several species in the Botryosphaeriaceae, a highly cosmopolitan ascomycete family of varying degrees of virulence, causes many of the same internal symptoms in grapevine wood, and the two diseases can be indistinguishable (Siebert, 2001; White *et al.*, 2011b). Botryosphaeriaceae spp. have been found in South African vineyards in large numbers (Van Niekerk *et al.*, 2004). Eutypa and Botryosphaeria cause similar patterns of internal

discolouration in affected vines, delayed budding and dieback of arms (Van Niekerk *et al.*, 2004, White *et al.*, 2011b). *Eutypa* sometimes results in a zig-zag pattern of growth caused by shortened internodes in green shoots and malformed, cup-shaped leaves, symptoms which are not seen in *Botryosphaeria* dieback (Petzold *et al.*, 1981; Munkvold *et al.*, 1994). These symptoms are the results of the production of secondary metabolites such as eutypine (Tey-Rulh *et al.*, 1991), and are expressed inconsistently due to various factors such as climate (Sosnowski *et al.*, 2007).

Black foot, caused by a complex of *Campylocarpon* Halleen, Schroers and Crous, *Neonectria* Wollenw. and *Ilyonectria* P. Chaverriand C. Salgado spp., affects young vines, both in the nursery and in the field causing an overall decline (Halleen *et al.*, 2004; Cabral *et al.*, 2012). Symptoms in nursery material are vascular streaking and reduced vigour, the latter presenting in affected plants once planted. Symptoms of vineyard infections include vascular streaking, abnormal root growth and root and rootstock necrosis (Halleen *et al.*, 2006). Vines between the ages of two and ten years are mainly affected by the disease (Gubler *et al.*, 2004). Young vines die soon after infection, but older vines may take up to a year to die (Gubler *et al.*, 2004).

Petri disease, also thought of as one of esca's related syndromes, is a major problem in South Africa, and is an important disease in nurseries (Halleen *et al.*, 2003). It was previously known as Black Goo or young grapevine decline, and affects nursery plants and young vines in the field (Fourie and Halleen, 2004). Petri disease is caused by *Phaeoconiella chlamydospora* and *Phaeoacremonium* species (Crous and Gams, 2000; Mostert *et al.*, 2006). Symptoms include wilting, decline and dieback in young plants and graft-failure in nursery grafted cuttings caused by blockage of xylem vessels by fungal colonisation or plant response to colonisation (Edwards *et al.*, 2001; Edwards *et al.*, 2007).

The sixth major trunk disease complex, esca, is far more common in South Africa than previously thought (White *et al.*, 2011b). Although certain symptoms of esca sometimes overlap with *Eutypa* and *Botryosphaeria* dieback, the disease has a distinct and complicated array of symptoms..

THE IMPORTANCE OF ESCA

The fungal grapevine disease complex, esca, was first described in detail by Ravaz in 1898 and after by Viala in 1926 in France. Interestingly, the term esca comes from a word for tinder, and people have been using bracket-shaped and resupinate fungi as tinder since the Neolithic era. The esca-industry was quite widespread in Europe until the end of the XIX century, with producers preparing esca that was used for anything from clothing to surgical dressings, and continued until the beginning of the First World War (Graniti, 2006). The name was given to the disease by Viala due to the association of *Phellinus igniarius* (L.) Quél., a highly sought-after esca fungus, with diseased grapevines (Graniti, 2006).

The disease has been the subject of extensive study in most grapevine growing regions of the world since the 1990's, when it became a prominent problem in Europe, coinciding with the banning of sodium-arsenite as fungicide treatment in the EU (Mugnai *et al.*, 1999; Surico, 2000). In 2000, Reizenzein *et al.* measured a 2.7% annual annually in vineyards showing foliar symptoms in Austria. The disease affected between 11 and 19% of vines in affected vineyards throughout Italy (Surico *et al.*, 2000) with a marked increase between results published in 2000 and 2006, where increases between 30% and 51% were found in surveyed vineyards (Surico *et al.*, 2006). Kuntzmann *et al.* (2010) estimated that up to 10% of plant material replacements in the Alsace region of France may be due to esca and Bruez *et al.* (2012) found esca and *Botryosphaeria dieback* symptoms on 0.9 and 8.2 % of French vines recorded in a survey of five different grapevine growing regions. They found an overall incidence of esca/*Botryosphaeria dieback* varying between 54 and 95%, depending on the region (Bruez *et al.*, 2012). Hofstetter *et al.* (2012) calculated the cost of replacing 1% of vines worldwide at 1.5 billion US Dollars annually. Replacement costs, yield loss, the costs of preventative control measures and increased labour and material costs linked to corrective measures (Siebert, 2001). Many studies have been conducted on effective preventative strategies since treatment mainly consists of removing infected material. Preventative strategies are generally focused on wound protection, as wounds caused by viticultural practices such as pruning and suckering are the main ports of entry for the grapevine trunk disease pathogens, including the esca fungi (Chapuis *et al.*, 1998; Epstein *et al.*, 2008; Fischer, 2009; Makatini *et al.*, 2012).

SYMPTOMATOLOGY OF ESCA

Esca includes an array of symptoms which have been observed and studied on grapevines in most grape-growing regions of the world (Chiarappa, 1959; Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Auger *et al.*, 2005; Fischer *et al.*, 2005; White *et al.*, 2011a). The definition of esca and the related symptoms have been an issue of debate during the past two decades.

After an extensive survey of esca-infected vineyards in South Africa, White *et al.* (2011b) described several types of symptoms associated with the disease under South African conditions. Externally, dieback was common. Apoplexy, the sudden death of an entire vine during hot weather was also observed, though not frequently. Leaf stripe symptoms were sometimes observed during the period between January and March. Berry symptoms were observed as discolouration and shrivelling, though black spots similar to Californian black measles were observed on a single occasion in one vineyards. Five internal symptom types were recorded, namely white rot, black and brown streaking, brown necrosis within white rot, V-shaped necrosis and a “central brown/red/black margin” (Fig. 1) (White, 2010; White *et al.*, 2011b). These external symptoms corresponded to Marais (1981), who reported dieback, decline, apoplexy, and leaf stripe symptoms appearing on afflicted vines.

According to Mugnai *et al.* (1999), esca may develop into two distinct syndromes. Chronic esca consists of leaf, berry and shoot symptoms coupled with wood discolouration and rot. Not all the symptoms occur in infected plants at the same time and symptoms such as leaf striping may not occur on the same plant for more than one year in a row (Bruno *et al.*, 2007). Acute esca refers to the rapid wilting of entire vines which results in mortality within a few days, also known as vine apoplexy.

Graniti *et al.* (2000) considered the esca disease complex to consist of 5 syndromes, namely brown wood streaking, Petri grapevine decline, young esca, white rot, and esca proper. “White rot” is defined as wood rot occurring due to infection by wood-rotting basidiomycetes. Fruit and leaf symptoms may or may not occur alongside the white rot syndrome. “Esca proper”, on the other hand, is defined as white rot occurring with brown wood-streaking and external symptoms. More recently, Surico (2009) proposed that the entire concept of esca be redefined, restricting use of the term “esca” to wood rot symptoms and suggesting the name “grapevine leaf stripe disease” for the phaeotracheomycosis complex with its tiger-stripe leaf patterning. The term “esca proper” would only be used when both wood-rot and leaf stripe symptoms occur on the same vines. Surico’s 2009 definition is contentious, because of the inconsistency of the

appearance of the leaf stripe symptoms. In Australia, esca is rarely associated with foliar symptoms (Pascoe and Cottral, 2000). In South America, the disease known as chlorotic leaf roll in Chile (Auger *et al.*, 2005) and ‘Hoja de Malvon’ in Argentina and Uruguay (Gatica *et al.*, 2000; Martinez, 2005), doesn’t present European leaf stripe symptoms at all, although chlorotic leaf symptoms may be due to photosynthetic disruption.

European leaf stripe symptoms, also known as “tiger–stripes”, are caused by disturbances in the photosynthetic processes of the plant which are activated by stress responses (Magnin-Robert *et al.*, 2011). The translocation of phytotoxins that are produced as secondary metabolites by the esca fungi are often thought to be the cause of this process (Mugnai *et al.*, 1999). Bruno *et al.* (2007) demonstrated that some metabolites produced by *Phaeoconiella chlamydospora* and *Togninia minima* (Tul. and C. Tul.) Berl. can cause leaf striping and black measles. Fungal metabolites have been demonstrated to cause disruption in the photosynthetic process (Manning *et al.*, 2009).

Esca, in its traditional sense, i.e. consisting of various combinations of external symptoms, white rot and wood discolouration, has been found to have several causal agents, mainly basidiomycetes and the phaeotracheomycosis fungi, *Phaeoconiella chlamydospora* and several *Phaeoacremonium* species (Chiarappa, 1959; Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Mostert *et al.*, 2006). The teleomorph of *Phaeoacremonium*, namely *Togninia* Berl., has also been found on grapevines (Eskalen *et al.*, 2005; Rooney-Latham *et al.*, 2005; Baloyi *et al.*, 2013) and presumably plays a part in the disease cycle. White rot seen in esca infected vines is always associated with the presence of a basidiomycete and the identity of the associated pathogen has been found to vary between different parts of the world, unlike the causal agents of the phaeotracheomycosis symptoms which are relatively consistent (Fischer, 2006). The fruit bodies of the basidiomycetes are rare and not easy to find on grapevines. When found, they are often in poor condition, making identification and formal descriptions difficult (Fischer, 2006). The usage of PCR-based methods to identify isolates made from symptomatic vines has revolutionised the study of the fungi associated with white rot.

BASIDIOMYCETES ASSOCIATED WITH ESCA

During early studies on the disease, Ravaz (1909) identified *Fomes igniarius* (L.) Fr. (later renamed *Phellinus igniarius* (L.) Quél.) based on fruit bodies found on vines in France, but was

unable to prove the pathogenicity of this organism. Vinet (1909) found fruit bodies of *Stereum hirsutum* (Willd.) Pers. on vines in France. Viala (1926) also found *S. hirsutum* associated with diseased vines in France, but was unable to subject the organism to conclusive pathogenicity trials. Chiarappa (1997) performed pathogenicity trials in California that proved that *P. igniarius* and not *S. hirsutum* was the cause of wood rot associated with esca. The extensive and seminal study by Larignon and Dubos (1997) connected *Phellinus punctatus* (P. Karst.) Pil. with wood rot in esca through isolation studies conducted in French vineyards. Today, it is generally accepted that this *P. punctatus* is synonymous to *Fomitiporia punctata* [Fr.: P. Karst] Murrill; (see Fiasson and Niemelä, 1984; Fischer, 1996). During an extensive survey of esca-infected vineyards in Italy, Cortesi *et al.* (2000) found only *F. punctata* on infected vines and concluded that it must be the main source of wood decay in esca. Fischer (2002) found that *F. punctata* strains collected from *Vitis* were different from strains collected from other woody hosts and, based on molecular data, mycelial growth and pairing tests renamed *F. punctata* strains from *Vitis* *Fomitiporia mediterranea* M. Fischer.

Today, *F. mediterranea* is the main wood rotting Basidiomycota associated with esca in Europe and the Mediterranean regions. In Australia, *Fomitiporia australiensis* M. Fisch., J. Edwards, Cunningt. and Pascoe has been associated with esca (Fischer *et al.*, 2005). In South America, the main wood rotting organism associated with local variants of the disease, “hoja de malvon” (Argentina) and chlorotic leaf roll (Chile) are *Inocutis jamaicensis* (Murrill) A.M. Gottlieb, J.E. Wright and Moncalvo and an unidentified species of *Fomitiporella* Murrill, respectively (Gatica *et al.*, 2004; Auger *et al.*, 2005; Lupo *et al.*, 2006). In North America, Chiarappa’s “*P. igniarius*” was widely associated with esca-related rot in the San Joaquin Valley of California (Chiarappa, 1959); however, *P. igniarius* in its strict sense has never been reported from North America (Fischer and Binder, 2004). *Fomitiporia polymorpha* M. Fischer has been associated with esca-related rot in California; though only on a single occasion (Fischer and Binder, 2004; Fischer, 2006). No further work has been published on the occurrence and cause of the white rot symptom of esca in the United States.

Esca has also been reported in South Africa in the past (Marais, 1981), and in recent years several pure cultures have been isolated from wood decay, a symptom which occurs often, though fruit bodies are seldom found (White *et al.*, 2011a). Fischer (2006) placed several of the South African isolates within *Fomitiporia* based on ITS phylogeny, but fruit bodies were not available at that time and no formal descriptions were made. White *et al.* (2011a) attempted to further identify some of the South African mycelial isolates through ITS phylogeny and found ten

discrete taxa falling under the order Hymenochaetales. The ten taxa included single *Fomitiporia* and *Phellinus* species, two *Fomitiporella* species, two *Inocutis* species and four *Inonotus* P. Karst. species (Fig. 2). One of the *Fomitiporella* species and the *Fomitiporia* species were isolated most frequently in the Western Cape province. The *Phellinus* species was isolated exclusively in the Northern Cape and Limpopo provinces (White *et al.* 2011a).

PATHOGENICITY OF BASIDIOMYCETES ON GRAPEVINES

Studies involving the pathogenicity of white rot basidiomycetes on grapevine and other hosts are rarely undertaken and the aetiology of the Hymenochaetales is poorly understood. There have been six trials of varying sizes and complexity involving esca and white rot on mature and young grapevines.

Chiarappa (1997) successfully performed inoculations of *Phellinus igniarius* on 7-year-old commercial vines and established *P. igniarius* (L.) Quél. as the main causal organism of the spongy decay symptom of the disease known as black measles in California.

In France, Larignon and Dubos (1997) inoculated a mycelial suspension of *F. mediterranea* (*P. punctatus* P. Karst.) on Cabernet Sauvignon cane segments, which were rooted for two months and grown in the glasshouse and the field for four months and a year, respectively. Larignon and Dubos (1997) also inoculated wooden blocks taken from healthy Cabernet Sauvignon vines by placing them in a culture tube containing *F. mediterranea*. Blocks were incubated for a year. The young vine inoculations of *F. mediterranea* showed brown vascular streaking, but the researchers were unable to re-isolate the basidiomycete from the inoculated plants. The wood blocks inoculated with *F. mediterranea* showed soft white rot after twelve months.

Sparapano *et al.* (2000) obtained white rot symptoms two years after inoculating *F. mediterranea* on 13-year-old Sangiovese vines in Italy. During further inoculations made by the authors on six- and nine-year-old Italia and Matilde vines, the first signs of white rot could be detected after six months. Inoculations were made by inserting colonised wooden toothpicks in holes drilled in grapevine arms and covered in cotton wool and paper tape.

Sparapano *et al.* (2001) included *Fomitiporia punctata* (P. Karst.) Murr. in a cross-inoculation trial with *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. and L. Mugnai and *Phaeomoniella chlamydospora* W. Gams, Crous, M.J. Wingf. and L. Mugnai on mature grapevines and found that *F. punctata* was able to cause limited, localised white rot within three years after inoculation.

Researchers in Argentina performed a limited experiment with an undescribed *Phellinus* sp. associated with the Argentine grapevine trunk disease, “hoja de malvón” (Gatica *et al.*, 2004). This species was later identified as *Inocutis jamaicensis* (Murrill) A.M. Gottlieb, J.E. Wright and Moncalvo (Lupo *et al.*, 2006). Five mature plants were inoculated with the “*Phellinus*” sp. by inserting mycelial plugs into 5mm holes drilled into various points on the grapevine trunks. Symptoms could only be detected after six to seven years.

In a Chilean trial, Diaz *et al.* (2013) inoculated a local *Inocutis* sp. on axenic plantlets incubated for 28 days, rooted 2 year old grapevines incubated for 15 months, grapevine shoots incubated for 60 days and detached grapevine shoots incubated for 14 days. All inoculations were via mycelial plugs inserted into holes of varying diameters bored in to plant material. The *Inocutis* sp. were associated with brown vascular discolouration in all the inoculations, but no white rot symptoms were observed in that study.

White rot in wood is caused by the degradation of lignin and cellulose within the cell-walls of woody plants. Lignin and cellulose degradation are effected by extracellular enzymes released by wood rotting fungi, which break up the complex components of the cell wall (Manion, 1981). Lignin is a complex compound to degrade, and only white rot basidiomycetes have been found to do it efficiently (Songulashvili, 2006). Three enzymes have been found to be essential for lignin degradation, namely a copper containing phenoloxidase, laccase and two heme-containing peroxidases, lignin peroxidase (LiP) and manganese-dependent lignin peroxidase (MnP) (Overton *et al.*, 2006; Songulashvili, 2006). According to Morgenstern *et al.* (2010), it is unlikely that ligninolytic processes would be possible without production of either lignin peroxidase or manganese peroxidase. Past trials involving enzymatic assays and basidiomycetes involved with esca have shown that *Fomes igniarius* produces laccase and peroxidases and *Fomitiporia punctata* (*F. mediterranea*) produces laccase and peroxidase (Chiarappa, 1997; Mugnai *et al.*, 1999).

AN INTRODUCTION TO THE HYMENOGYALES

The Série des Igniaries was first recognised as an entity by Patouillard in 1900 and was characterised by him and his successors as having brown hyphae and brown basidiomata with modified brown cystidia known as setae in the hymenium, simple-septate hyphae and a xanthochroic reaction when mounted in KOH. Many of the species were also associated with white rot in woody plants (Patouillard, 1900; Kühner, 1950; Donk, 1964; Oberwinkler, 1977).

Oberwinkler (1977) raised the Hymenochaetales to the rank of Order based on the same set of characteristics described by Patouillard and his successors, but it was only with the emergence of genetic studies that there was an indication that the Hymenochaetales might have to be expanded to include other polyporoid and even corticoid genera that lacked one or more of the abovementioned characteristics. Poroid *Oxyporus* (Bourdot and Galzin) Donk and *Trichaptum* Murrill and corticoid *Hyphodontia* J. Erikss. spp., *Basidioradulum radula* (Fr.) Nobles and *Schizopora paradoxa* (Schrad.) Donk were found to be closely related to the Hymenochaetales *sensu* Oberwinkler (Hibbett and Donaghue, 1995; Hibbett *et al.*, 1997). Further groups were also included later, including species from the agaricoid genera *Cantharellopsis* Kuyper, *Omphalina* Qué. and *Rickenella* Raitelh.(Redhead *et al.*, 2002). The morphological characteristics of genera now considered part of the Hymenochaetales are currently highly varied (Larsson *et al.*, 2006).

The poroid Hymenochaetales as described in Oberwinkler (1977), called Hymenochaetaceae in Binder *et al.* (2005) and Larsson *et al.* (2006) are characterised by imperforate parenthosomes and include the two large, morphologically diverse and economically important genera *Phellinus sensu lato* and *Inonotus sensu lato*. All *Phellinus* and *Inonotus s.l* species cause white rot on a variety of woody perennials (Wagner and Fischer, 2002). The division of species between *Phellinus* and *Inonotus* was initially based on hyphal mitism (dimitic vs. monomitic) and fruit body consistency, but many intermediate morphological forms have been reported over the years (Fiasson and Niemelä, 1984; Ryvarden and Gilbertson, 1994; Wagner and Fischer, 2001).

Fiasson and Niemelä (1984) did a multivariate analysis based on morphological and chemical characteristics of European poroid taxa and subdivided *Phellinus* and *Inonotus* into two families, the *Inonotaceae* consisting of *Inonotus sensu stricto*, *Inocutis* (Fiasson and Niemelä) and *Phylloporia* (Murr.) and the *Phellinaceae* consisting of *Phellinus sensu stricto*, *Fomitiporia* (Murr.), *Porodaedalea* (Murr.), *Fuscoporia* (Murr.), *Fulvifomes* (Murr.), *Onnia* (Karst.), *Inonotopsis* (Parm), *Ochroporus* (J. Schroet.) and *Phellinidium* (Kotl.). The subdivision of *Phellinus s.l* and *Inonotus s.l* was supported by the nuLSU study of Wagner and Fischer (2001). The Wagner and Fischer (2002) study of *Phellinus s.l* and *Inonotus s.l* showed that the two genera are polyphyletic in origin and confirmed the status of all of the above, with the exception of *Phellinidium* which remained uncertain. Larsson *et al.* (2006), also working with the nuLSU, were still unable to find a satisfactory resolution in terms of related subclades within the Hymenochaetaceae (Fig. 3).

THE ECONOMIC IMPACT OF *Phellinus S.L* AND *Inonotus S.L*

Phellinus and *Inonotus* in the loose sense are large genera, and are of economic importance worldwide (Wagner and Fischer, 2002; Larsson *et al.*, 2006). Many species have been used in traditional medicine (Dai *et al.* 2010). Recent research has been conducted on fungal exopolysaccharides and other substances that may be beneficial in the medical, biotechnology and processing areas (Larsson *et al.*, 2006).

Inonotus baumii (Pilát) T. Wagner and M. Fischer has been used in Asian medicine for centuries and is also known as sanghuang in China and Korea and meshimakobu in Japan (Parmasto and Shih, 2012). There have been several studies demonstrating the potential use of exopolysaccharides secreted by this species to treat diabetes in mice (Hwang *et al.*, 2005; Cho *et al.*, 2007), inhibit pulmonary inflammation in rats (Jang *et al.*, 2004) and as a free-radical scavenger (Shon *et al.*, 2003) and anti-proliferative agent *in vitro* (Sun *et al.*, 2014).

Likewise, *Phellinus rimosus* (Berk.) Pilát, which is widely used in traditional medicine in India (Ajith and Janardhanan, 2007), has been found to secrete substances shown to be antioxidants and anti-hepatotoxic *in vitro* (Ajith and Janardhanan, 2002; Lakshmi *et al.*, 2004) and some substances that show cytotoxic and antitumour characteristics (Ajith and Janardhanan, 2003). Crude extracts of *P. rimosus* have also been demonstrated as having antibacterial properties against such common bacteria as *Escherichia coli* (Migula) Castellani and Chalmers, *Staphylococcus aureus* Rosenbach and *Salmonella* Lignieres spp. (Sheena *et al.*, 2003).

Inonotus obliquus (Ach. ex Pers.) Pilát is one of the most sought-after medicinal mushrooms and have historically been used in folk medicine in Eastern Europe and the Middle or Near East. Its exopolysaccharides have been shown to have various beneficial effects such as anti-tumour activity *in vitro* (Kim *et al.*, 2006) and anti-proliferative activity on human tumour cells (Ma *et al.*, 2013; Ning *et al.*, 2014), anti-oxidative properties *in vitro* (Cui *et al.*, 2005; Lee *et al.*, 2007), anti-inflammatory properties (Ma *et al.*, 2013) and anti-hyperglycemic properties in mice (Sun *et al.*, 2008).

Although most of the poroid Hymenochaetales are saprophytes, forming an integral part in the ecosystem as decayers of natural woody substrates, there are a large number of species that are aggressive primary pathogens of wood, causing white rot in economically important woody plants such as forestry trees and perennial agricultural crops like grapevine, citrus and

kiwi fruit (Fischer, 2000; Elena and Paplomatas, 2002; Elena *et al.*, 2006). While wood rot is a process that is slow to affect crop-bearing plants, decay in forestry trees affect not only the quality of wood harvested, but also the pulping qualities of wood for other purposes (Reis and Libby, 1960; Blanchette, 1982). *Porodaedalea pini* (Brot.) Murrill is one of the most important forestry pathogens, causing highly destructive pocket rot on conifers such as various *Pinus* spp. It can also form heart rot and cankers on *Abies* spp. (Larsen *et al.*, 1979; Lewis and Lindgren, 1999).

Fuscoporia weirii (Murrill) Aoshima is one of most important pathogens on Douglas fir (*Pseudotsuga menzies*), and causes aggressive laminated root rot in conifers (Holah *et al.*, 1993; Hansen and Goheen, 2000). Douglas fir is the most important timber species grown in the Pacific Northwest of the United States and infections by *F. weirii* cause disruptions in second growth stands of Douglas fir, and major losses in the long term as the disease only becomes apparent 50 years after establishment (Manion, 1981).

Phellinidium noxium (Corner) G.H. Cunningham is a devastating pathogen on many tree species in Asia and the Pacific, and causes brown root rot resulting in decline on forestry and fruit trees, as well as ornamental plants (Ann *et al.*, 2002)

Fomitiporia mediterranea is the most prominent member of the Hymenochaetales in viticulture and affects vineyards in the Europe and Mediterranean grapevine growing countries (Fischer, 2006). It has several other hosts, including kiwi fruit, *Olea* spp. and *Citrus* spp. (Elena and Paplomatas, 2002; Fischer, 2002, Elena *et al.*, 2006) as well as tree species such as *Quercus* spp., *Acer negundo* and *Laurus nobilis*. *Fomitiporia mediterranea* causes quite severe disease on *Citrus* in Greece, with trees declining and cankers forming on affected branches (Elena *et al.*, 2006). Its main economic impact is made by its role in the esca disease complex in Europe, which has been causing severe economic losses during the past three decades and will be discussed in the pages that follow.

Inocutis jamaicensis can be found on native plants in Uruguay and Argentina. It is also associated with serious stem rot, heartwood decay and lesions on *Eucalyptus globulus* in Uruguay and is the Basidiomycete associated with “hoja de Malvon” in Argentina and Uruguay (Martinez, 2005; Lupo *et al.*, 2006).

ECOLOGY AND EPIDEMIOLOGY OF HYMENOGYSALES

Despite the economic impact of Hymenochaetales such as *Phellinus s.l.* and *Inonotus s.l.*, little is known about their ecology and epidemiology. Certain species, such as *F. weirii* spread via an asexual state by root to root contact in infected forests (Hansen and Goheen, 2000). Many species spread via basidiospores. Cortesi *et al.* (2000) and Fischer (2002) used the high diversity of somatic incompatibility to demonstrate that *F. mediterranea* (*F. punctata*) infects grapevine via basidiospores.

Infection by members of the Hymenochaetales can be through naturally occurring wounds, such as in the case of *Phellinus torulosus* (Pers.) Bourdot and Galzin infecting trees via fire or frost scars (Panconesi *et al.*, 1994). Infection can also occur through man-made pruning wounds, which has been hypothesized for *F. mediterranea* on grapevine (Cortesi *et al.*, 2000; Graniti *et al.*, 2000). In a casual study presented at a conference, Fischer found fruit bodies of *F. mediterranea* sporulating between 190 to 250 days in a year (Fischer, 2008). This kind of life-strategy, also observed in common polypores such as *Ganoderma applanatum* (Pers.) Pat., is thought to increase the likelihood of a spore finding a suitable substrate, according to Rockett and Kramer (1974).

There have been very few studies on sporulation of the Hymenochaetales. Yohem (1982), studying *Inonotus weirianus* (*Phellinus weirianus*), a causal agent of destructive heart rot in walnut trees, reported the use of spore traps. In that study, spore traps consisting of microscope slides were set up underneath fruit bodies between mid-January and mid-February in Arizona, USA. The author reported spores adhering to the slide-surfaces during this period, though no other information was reported and the aim of the study is unclear.

Fischer (2008) measured sporulation by affixing slides to fruit bodies of *F. mediterranea* in the field. The author reported that sporulation of *F. mediterranea* under German conditions was largely dependent on average daily temperatures and relative humidity, requiring conditions with temperatures higher than 10°C and a relative humidity higher than 80%. He also reported increased spore deposit after periods of rain.

Using microscope slides as spore traps is a technique commonly seen in grapevine trunk disease research in France, the United States and South Africa (Larignon and Dubos, 1997; Eskalen and Gubler, 2001; Úrbez-Torres *et al.*, 2010; Van Niekerk *et al.*, 2010). Slides are covered in petroleum jelly and left in the field for a set period of time, after which traps are removed and processed. Spores are collected by washing traps with water, which is filtered and plated out. Úrbez-Torres *et al.*, (2010) and Van Niekerk *et al.* (2010) also used volumetric spore

traps with discs sprayed with petroleum jelly and divided into segments representing set hours of exposure time. Úrbez-Torres *et al.*, (2010) analysed traps by counting Botryosphaeriaceae spores directly under a microscope. To compensate for the diversity of species involved in their study, Van Niekerk *et al.* (2010) plated spore suspensions onto water agar and allowed spores to germinate before transferring the germinated spores to potato dextrose agar for easy identification. These methods of processing are largely dependent on the ability of spores to germinate under laboratory conditions, an ability often absent from members of the Hymenochaetales (pers. comm. Michael Fischer). Rockett and Kramer (1974) noted that basidiospores have a lower rate of viability than spores of other types of fungi and presumably there are still more factors involved in basidiospore viability that need to be studied.

In the studies of Eskalen and Gubler (2001) and Rooney-Latham *et al.* (2005), the sporulation of *Phaeoacremonium inflatipes* and *Phaeomoniella chlamydospora* in California was found to be directly correlated to rainfall events. Úrbez-Torres *et al.*, (2010) found higher levels of sporulation of the Botryosphaeriaceae to be directly related to rainfall and overhead irrigation in various parts of California. Under South African conditions, Van Niekerk *et al.* (2010) found rainfall, RH and temperature to be the most important weather variables involved in the sporulation of the Botryosphaeriaceae and *Phomopsis* spp. Several studies investigating the relation between South African climatic conditions and the sporulation of esca disease pathogens are currently being conducted and will contribute to the further understanding of this phenomenon.

HYMENOCHAETALES IN AFRICA

While the European and Asian Hymenochaetales have been collected and studied in large numbers over the years, very little work has been done in dealing with the African species (Ryvarden, 1998). There are published flora of Zimbabwe, Malawi and East Africa (Ryvarden and Johansen, 1980; Morris, 1990; Masuka and Ryvarden, 1992) and some species were recorded by David and Rajchenberg (1992) in West Africa. Several novel *Fomitiporia* species have been described from sub-Saharan Africa lately, notably *F. aethiopica* Decock, Bitew and G. Castillo and *F. tenuis* Decock, Bitew and Castillo from the East Africa and *F. nobilissima* Decock and Yombiyeni and *F. ivindoensis* Decock, Amalfi and Yombiyeni from Gabon (Decock *et al.*, 2005; Ipulet, 2007 in Amalfi *et al.*, 2010; Amalfi *et al.*, 2010). *Fomitiporia robusta* (P. Karst.) Fiasson and Niemelä and *F. sublaevigata* (Cleland and Rodway) Y.C. Dai were also

reported from East Africa (Decock *et al.* 2005). Yombiyeni *et al.* (2011) also reported a new species of *Phellinus* s.s., *P. gabonensis* Decock and Yombiyeni from Gabon. Roberts and Ryvardeen (2006) recorded a large collection of species from Cameroon, including eight *Phellinus* s.l. spp. and *Phylloporia spathulata* (Hook.) Ryvardeen, representing the poroid Hymenochaetales. Given the diversity of African flora and the inaccessibility of the terrain in many places, all the endemic Hymenochaetales may never be recorded, but work done during the past three decades has made significant progress in that direction.

RATIONALE AND SCOPE OF THE STUDY

During a pilot study on esca causal agents in South Africa, ten different basidiomycete taxa belonging to the order Hymenochaetales were isolated from grapevines showing symptoms of esca disease (White *et al.*, 2011a). This relatively high number is in stark contrast to the rest of the grapevine growing world where there is usually no more than one dominant species involved in causing white rot in diseased vines (Fischer, 2006).

Several of the taxa isolated in South Africa are novel. Taxonomic novelties are described in Chapter 2, Chapter 3 and Chapter 4. A first report of *Inonotus setuloso-croceus* (Clel. andand Rodw.) P.K. Buchanan andand Ryvardeen occurring on grapevine in South Africa is discussed in Chapter 4. Spore-trapping studies were conducted in order to determine parts of the lifecycle of a species and to establish whether this species may be of concern during pruning and suckering season. The results of this study are discussed in Chapter 4.

There is very little information available regarding the pathogenicity and virulence of Hymenochaetales taxa. All ten taxa were inoculated into mature vines under field conditions to test for pathogenicity. All ten taxa were also used to determine what kinds of hydrolytic enzymes are produced by each taxon in order to better understand pathogenicity. The results of this study are discussed in Chapter 5.

AIMS OF THE STUDY

The aim of this study was to determine the taxonomy and aetiology of basidiomycete fungi that are associated with esca disease of grapevine in South Africa. More specifically, the aims and objectives of this study were:

1. Describe taxonomic novelties within the Hymenochaetales that were found to be associated with esca infected vines in South Africa.

2. Conduct pathogenicity studies on field-grown vines with the newly isolated taxa.
3. Investigate the epidemiology of these Hymenochaetales using spore-trapping experiments.

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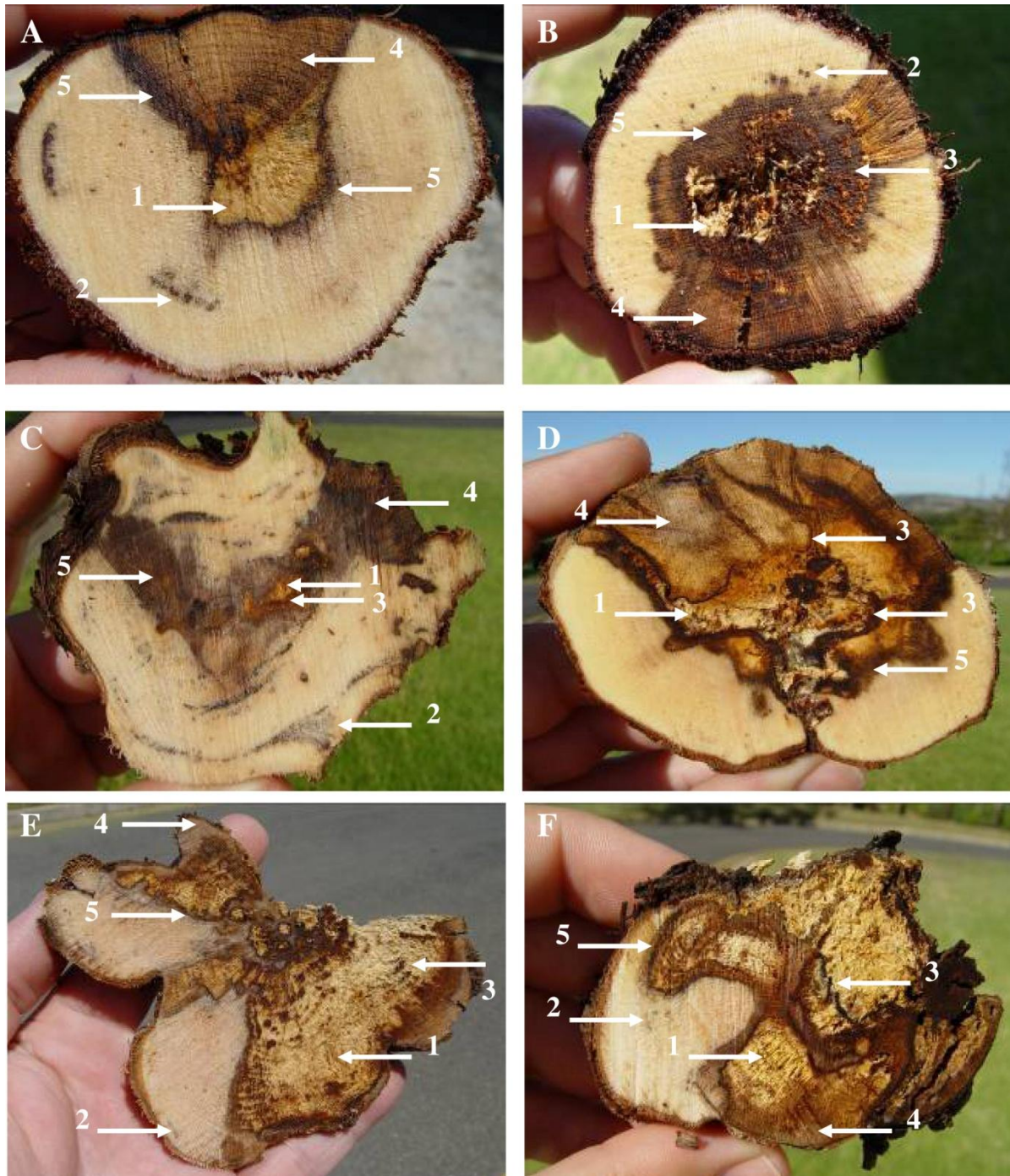
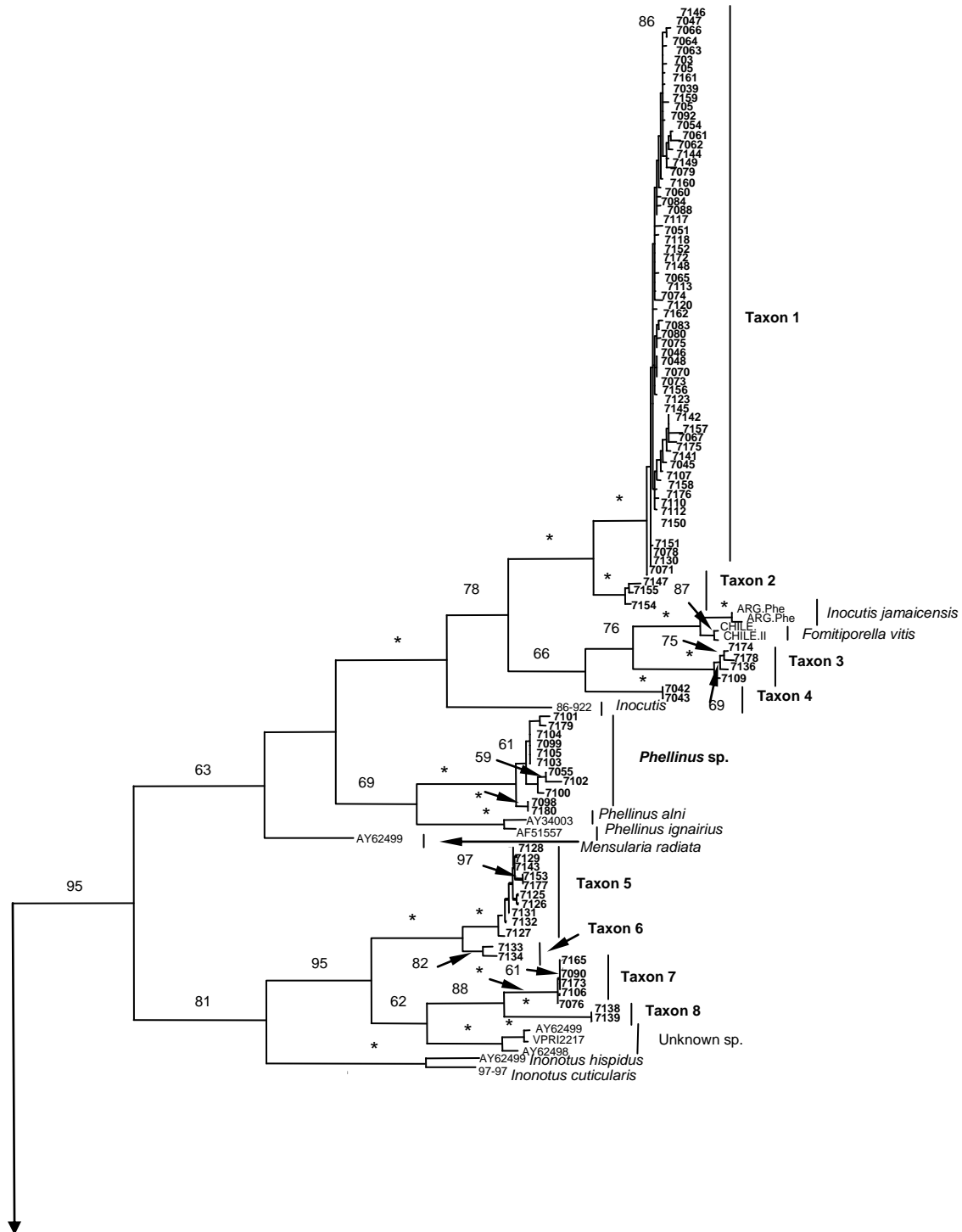


Figure 1. A - F. Cross sections showing the internal symptoms and symptom types found in diseased vines. 1.White rot; 2. Black and brown wood streaking; 3. Brown necrosis within white rot; 4. Sectorial brown necrosis; 5. Central brown/ red/ black margin. From White (2010).



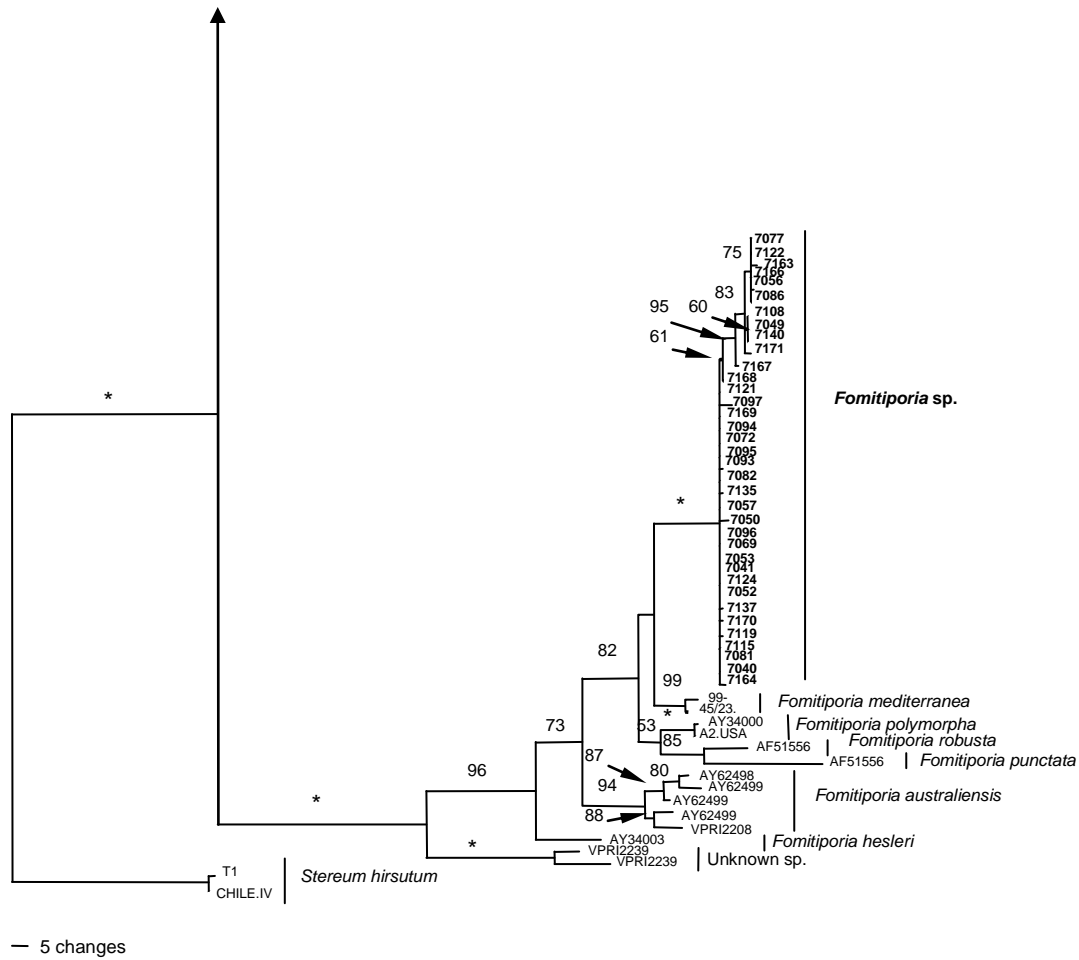


Figure 2. One of 10 most parsimonious trees obtained from heuristic searches of the ITS sequences (length: 2050 steps; CI: 0.560; RI: 0.938; RC: 0.526) of the Basidiomycete isolates. Bootstrap support values (1000 replicates) are shown above the nodes and bootstrap values of 100 % are indicated by an asterisk (*). The outgroups used were *Stereum hirsutum* isolates T18 and Chile IV. From White *et al.*(2011a).

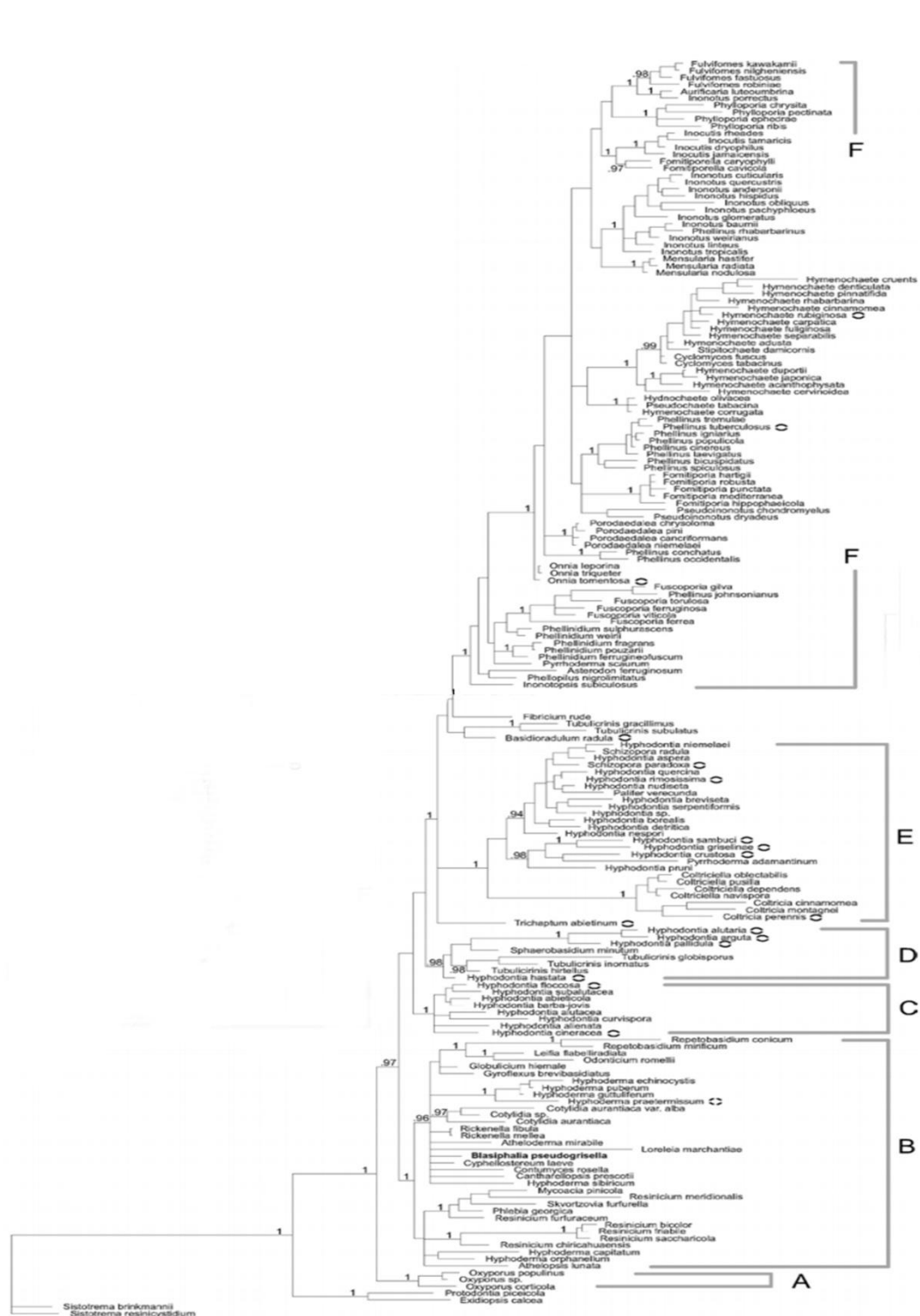


Figure 3. Phylogenetic relationships of Hymenochaetales inferred from 5.8S and nuclLSU DNA sequences with Bayesian analysis. A 50% majority rule consensus cladogram Bayesian posterior probabilities ≥ 0.94 are shown above internodes; branch lengths reflect estimated number of changes per site. Closed horizontal parentheses indicate that the species has dolipores with continuous parenthesomes. Broken horizontal parentheses indicate presence of the perforated parenthesome type. A. Oxyporus clade. B. Rickenella clade. C. Kneifiella clade. D. Hyphodontia clade. E. Coltricia clade. F. Hymenochaetaceae clade. (Larsson *et al.*, 2006)

CHAPTER 2

A novel *Fomitiporia* species associated with esca on grapevine in South Africa¹

ABSTRACT

Esca disease of grapevine is a complex trunk disease, which includes several foliar and wood symptoms. Among them, white rot has been found to be caused by various basidiomycetes within the order Hymenochaetales. During recent surveys of esca-associated pathogens in South African vineyards, several unidentified basidiomycetes were isolated from white rot occurring in diseased vines. A new *Fomitiporia* species, *F. capensis*, is described here based on morphological characteristics and combined ITS and LSU phylogeny. Morphologically, *F. capensis* is similar to *F. punctata* in that both species essentially lack setae. *Fomitiporia capensis*, *F. punctata* and *F. aethiopica* produce similarly sized basidiospores but differ in terms of host range, pore size and, possibly, fruit body shape. Phylogenetically, *F. capensis* appears to be related to *F. tenuis*, though morphologically the species differ significantly in that *F. tenuis* has smaller pores and smaller basidiospores. *Fomitiporia capensis* was found to occur widely as vegetative mycelium throughout the Western Cape Province, though fruit bodies were scarce in comparison. A vineyard with fruit bodies was also found in Limpopo in the north east of the country. Fruit bodies were found growing on the underside of the cordon of living vines displaying external symptoms typically associated with esca, or general decline and dieback symptoms along with internal white rot.

INTRODUCTION

The fungal grapevine disease complex, esca, has been known since ancient times and consists of an array of symptoms which have been observed and studied on grapevines in different parts of the world (Chiarappa, 1959; Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Auger *et al.*, 2005; Fischer *et al.*, 2005; White *et al.*, 2011a). Graniti *et al.* (2000) considered the esca disease complex to consist of 5 syndromes, namely brown wood streaking, Petri grapevine decline, young esca, white rot and esca proper. "White rot" is defined as wood rot occurring due

¹ This chapter was originally published as Cloete, M., Fischer, M., Mostert, L., and Halleen, F. 2014. A novel *Fomitiporia* species associated with esca on grapevine in South Africa. *Mycological Progress*, 13(2): 303-311.

to infection by wood-rotting basidiomycetes. Fruit and leaf symptoms may or may not occur with the white rot syndrome. “Esca proper”, on the other hand, is defined as white rot occurring with brown wood-streaking.

Esca has been found to have several causal agents, mainly basidiomycetes and the phaeotracheomycosis fungi, *Phaeoconiella chlamydospora* W. Gams, Crous, M.J. Wingf. & L. Mugnai and several *Phaeoacremonium* W. Gams, Crous & M.J. Wingf. species (Chiarappa, 1959; Larignon and Dubos, 1997; Mugnai *et al.*, 1999; Mostert *et al.*, 2006). White rot seen in esca infected vines is always associated with the presence of a basidiomycete and the identity of the associated pathogens has been found to vary among different parts of the world (Fischer, 2006). The fruit bodies of these basidiomycetes seem to be rare and are not easy to find on grapevines, making identification and formal descriptions difficult.

During early studies on esca, Ravaz (1909) identified *Fomes igniarius* (L.) Fr. (later renamed *Phellinus igniarius* (L.) Quél.) based on fruit bodies found on vines in France??, but was unable to prove the pathogenicity of this organism. Vinet (1909) found fruit bodies of *Stereum hirsutum* (Willd.) Pers. on diseased vines in France and Viala (1926) confirmed the latter but was also unable to verify this species as the causal pathogen. Chiarappa (1959) was able to prove experimentally that an organism that he identified as *P. igniarius* was the cause of wood rot associated with esca. Later studies connected *Phellinus punctatus* (P. Karst.) Pil. (currently known as *Fomitiporia punctata* [Fr.: P. Karst.] Murrill; see Fiasson and Niemelä, 1984; Fischer, 1996) with wood rot in esca (Larignon and Dubos, 1997). During an extensive survey of esca-infected vineyards in Italy, Cortesi *et al.* (2000) encountered only *F. punctata* on infected vines and concluded that it was the main source of wood decay in esca. Fischer (2002) found that *F. punctata* strains collected from *Vitis* were different from strains collected from other woody hosts and based on molecular data, mycelial growth and pairing tests renamed *F. punctata* strains from *Vitis* and some other hosts as *Fomitiporia mediterranea* M. Fischer.

Fomitiporia mediterranea remains the main wood rotting basidiomycete associated with esca in Europe, but the basidiomycetes involved in the complex have been found to differ geographically (Fischer, 2006). In Australia, *Fomitiporia australiensis* M. Fischer *et al.* has been associated with esca (Fischer *et al.*, 2005). In South America, the main wood rotting organism associated with local variants of esca, known as “hoja de malvon” in Argentina and chlorotic leaf roll in Chile, are suspected to be *Inocutis jamaicensis* and a species of *Fomitiporella*, respectively (Gatica *et al.*, 2004; Auger *et al.*, 2005; Lupo *et al.*, 2006). In North America,

Chiarappa's "*P. igniarius*" was widely associated with esca-related rot in the San Joaquin Valley of California (Chiarappa, 1959); however, *P. igniarius* apparently does not exist in North America (Fischer and Binder, 2004). Instead, *Fomitiporia polymorpha* M. Fischer has been associated with esca-related rot in California; based only on a single observation (Fischer and Binder, 2004; Fischer, 2006).

Esca has also been observed in South Africa in the past (Marais 1981) and in recent years although fruit bodies are seldom found, several mycelial cultures have been isolated from wood decay, a symptom which occurs often (White *et al.*, 2011a). Fischer (2006) placed several of the South African isolates within *Fomitiporia* based on ITS phylogeny, but fruit bodies were not available at that time and no formal conclusions were made. White *et al.* (2011b) attempted to further identify some of the South African mycelial isolates through ITS phylogeny and found an unprecedented variety consisting of ten discrete taxa, including a taxon appearing to be positioned within *Fomitiporia*. This taxon comprised appr. 25% of all the basidiomycete isolates found by White *et al.* (2011b).

Since 2006 several fruit bodies identified as belonging to this unknown taxon have been found on symptomatic vines between the ages of 10- and 40-year-old. The vast majority of mycelial isolates and fruit bodies were distributed in a small part of the south western region of the Western Cape Province, from Riebeeck Kasteel in the north to the Hemel-en-Aarde valley in the south, and Darling in the west to Robertson in the east. Two further fruit bodies belonging to this species were also later found in Limpopo province in the northeast of South Africa. Unlike the rest of the material, no isolations were made from symptoms in that vineyard and mycelium was obtained directly from fruit bodies for DNA isolation.

This chapter describes a new species of *Fomitiporia* based on a combined ITS and LSU phylogeny and morphological characteristics gleaned from several isolates and fruit bodies found on *Vitis vinifera* L. in the Western Cape and Limpopo, South Africa.

MATERIALS AND METHODS

Fungal material and culturing

Thirty-six mycelial isolates from *Vitis vinifera* were identified during a previous study (White *et al.*, 2011b) as a putative novel *Fomitiporia* species. Fungal strains are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch (STE-U) and

stored on PDA (Potato Dextrose Agar, Biolab, Midrand, SA) slants in sterile water at 15°C and spores?? in tubes filled with 70% glycerol at -85°C. Prior to DNA extraction, cultures were grown on PDA at 25°C under daylight conditions. Growth studies were conducted with three isolates on MEA (Malt extract agar Biolab, Midrand, SA??) at intervals of 5°C between 5°C and 45°C. Growth was measured after a period of seven days and cardinal temperatures for colony growth were obtained.

Comparative microscopy of fruit bodies

Sections of fruit bodies were mounted in water or Melzer's reagent and were studied at 500x or 1250x under phase contrast optics. A maximum of 25 observations was recorded for measurements of basidiospores.

Pictures of sections mounted in Melzer's reagent were taken at 400x and 1000x magnification using a Nikon Eclipse E600 compound microscope with a Nikon DMX1200C digital camera attachment.

DNA isolation, PCR amplification and sequencing

DNA was isolated from cultured mycelium grown for 21 days on PDA plates using CTAB buffer (Damm *et al.*, 2008). Approximately 700 bp of the ITS region was amplified using the primer pair ITS1 and ITS4 (White *et al.*, 1990). PCR and cloning were done according to the protocol in White *et al.* (2011b). Cloning was necessary to allow the sequencing of a single copy of the gene region (Clark and Anderson, 2004). The LSU region was amplified using the primer pair LR0R and LR5 (<http://www.biology.duke.edu/fungi/mycolab/primers.htm>) and PCR products were cloned using an instAclone PCR cloning kit (Fermentas Life Sciences) according to the manufacturer's instructions. Products were purified using an MSB Spin PCRapase kit (Invitex, Germany) and sequenced using ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA). The products were then analyzed on an ABI Prism 3130XL DNA sequencer (Perkin-Elmer, Norwalk, CN).

Phylogenetic analysis

Sequences were edited using Geneious Pro 3.5.6 (2007, Biomatters Ltd., Auckland, New Zealand) and consensus sequences were run through the Basic Local Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to obtain a preliminary identification. Reference sequences were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>) to build

representative combined ITS and LSU alignments for phylogenetic analyses. Selection of reference sequences was based on several publications, including Dai *et al.* (2001), Fischer (2002), Decock *et al.* (2005), Fischer *et al.* (2005), Fischer (2006), Decock *et al.* (2007), Dai *et al.* (2008) and Amalfi *et al.* (2010, 2012), and included all relevant and available taxa, ie., with main emphasis on species occurring on grapevine in Africa. *Phellinus uncisetus* Robledo Urcelay & Rajchenb. MUCL46231 and MUCL47061 were selected as outgroup. The sequences were automatically aligned using MAFFT v6 (Kato *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) with the heuristic search option, and 10 random taxon additions for all the datasets. Tree bisection and reconstruction were used as the branch swapping algorithms. 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.

Bayesian analyses were conducted for the ITS and LSU partitions of the alignment using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Modeltest 3.6 was used for selecting the optimal model of sequence evolution for each clade alignment. The likelihood and prior settings for each partition were changed accordingly in MrBayes. Markov chains were initiated from a random tree and run 2.000.000 generations, keeping one out of every 100 generations. Convergence among chains was monitored by examining plots of log-likelihood values and observing when the values of the four chains reached a plateau. The average deviation of split frequencies was 0.003292 and the potential scale reduction factors (PSRF) were 1.00 for each parameter. The first 1250 generations (burn-in) were discarded and the remaining generations were used to calculate the 50% majority-rule tree and the posterior probability for the individual branches.

RESULTS

Thirty-six isolates belonging to the new species, described here as *Fomitiporia capensis*, were found in vineyards across the Western Cape and two isolates in a vineyard in Limpopo (Table 1). *Fomitiporia capensis* is clearly separated by molecular means from all the other species of *Fomitiporia* included in our analyses (Fig. 1).

Morphology***Fomitiporia capensis* M. Fisch., M. Cloete, L. Mostert, F. Halleen sp. nov. MycoBank****MB800998 Figs. 2 a-e**

Basidiomata perennia, resupinata; superficies pororum luteobrunnea ad brunnea, pori circulares, 4-6 in quoque millimetro; systema hypharum dimiticum, omnia septa fibulis egentia; hyphae skeletales luteobrunneae, 3.0 – 4.0 μm latae, hyphae generativae hyalinae, septatae, 2.5 – 4.0 μm latae; sporae ellipsoideae ad subglobosae, hyalinae, crassitunicatae, amyloideae, 6.5-7.5 x 5.5-7 μm ; setae hymeniales minimae.

Holotypus FH 183, in Pretoria (PREM60818), collectus a F. Halleen, in *Vitis vinifera*, cv. Chenin blanc in Wellington (Western Cape), July 2007.

Etymology: *capensis* refers to the geographic origin of the first isolations of the species, the Cape region in South Africa.

Specimens examined: FH 43 (corresponds to sequences STEU-7049 and 7050), FH 183 (STEU-7093, PREM60818), FH 184 (STEU-7094), MP 5 (STEU-7489).

Habitat. Fruit bodies usually occur on living, but primarily declining vines in the uppermost part of trunk where the trunk and cordons meet on cordon-trained vines, on the underside of the cordon. On untrained vines, fruit bodies often occur just above soil level, but also on the underside of arms.

Fruit bodies are resupinate, inseparable, woody hard, perennial; in the holotype (FH 183) with blackish crust in upper part, but crust inconsistently present in other specimens; up to 5 mm thick; margin inconspicuous in some specimens, more obvious in others, yellowish-brown.

Pore surface yellowish brown – rusty brown; pores more or less circular, in the holotype (FH 183) somewhat irregularly arranged, 4-6 per mm, dissepiments thick, entire.

Tube layer not stratified in most fruit bodies, indistinctly stratified in MP5, up to 5 mm thick; darker than pore surface, dark reddish brown.

Context grayish - yellowish, up to 2 mm thick; darkening with KOH.

Hyphal system dimitic; hyphae parallel in hymenophoral trama; septa without clamp connections; skeletal hyphae golden brown, very rarely branched, 3 – 4 μm wide; generative hyphae hyaline, thin-walled, regularly branched, simple septate, 2.5 – 3.5 (4) μm wide.

Setae essentially absent; one single seta found in the hymenium of specimen FH 43, straight and subulate, 25 x 7 μm .

Spores ovoid–subglobose, appearing slightly thick-walled, some with distinct apiculus, hyaline, smooth, mostly dextrinoid, (6) 6.5–7.5 (8) x (5) 5.5–7 (7.5).

Crystals common in the hymenial layer of some specimens, rhomboid, up to > 20 μ long. The optimal temperature for growth of mycelial colonies was 25°C.

Remarks: Morphologically, *F. capensis* is very similar to *F. punctata* (boreal species occurring on many genera of deciduous host plants) and *F. aethiopica* Decock, Bitew & G. Castillo (species of Eastern Africa, occurring on deciduous trees; Decock *et al.*, 2005). These three taxa are characterized by large spores and absence of setae; pores are slightly smaller in *F. punctata*. So far, no cushion-shaped fruit bodies have been found for *F. capensis*.

Phylogeny

During analysis of the combined ITS and LSU dataset, 29 characters were excluded as missing or ambiguous and 1619 characters were included, of which 1251 were constant, 119 were parsimony-uninformative and 249 were parsimony informative. The heuristic search on the dataset resulted in 60 most parsimonious trees with the same topology (length 621, CI=0.692, RI=0.861, RC=0.596). *Fomitiporia capensis* formed a well supported clade with 100% bootstrap support and a probability of 1 (Fig. 1). Immediately basal to *F. capensis*, *F. tenuis* and *F. hippophaeicola* formed equally well supported clades.

DISCUSSION

An overview summarising some characters of *Fomitiporia capensis* and related species is given in Table 2 with emphasis on species included in our phylogenetic analysis. While this table contains species known from Africa as well as species occurring on grapevine, it is far from complete since the number of taxa within *Fomitiporia* has significantly increased within the last decade. This formerly small genus now forms the largest group within *Phellinus* s.l. Clearly, emphasis of new descriptions is on non-European locations such as Africa and Asia (www.mycobank.org). There have been several descriptions of novel *Fomitiporia* species in Africa recently (Decock *et al.*, 2005; Amalfi *et al.*, 2010), greatly expanding knowledge of the genus and its occurrence on the continent. The novel South African species, *Fomitiporia capensis*, is one of several basidiomycetes within the Hymenochaetales associated with esca symptoms on *Vitis vinifera* in South Africa (White *et al.*, 2011b).

Fomitiporia capensis is morphologically similar to *F. aethiopica* since both species essentially lack setae and both pores and basidiospores are within the same size range. In the holotype specimen of *F. capensis* (FH 183), a contrast between colouration of the pore surface and tube layer was observed, a phenomenon which was also described for *F. aethiopica* by Decock *et al.* (2005). The differences between the former and latter species are the thin shape of the fruit body of *F. capensis* and the fact that it seems to be perennial whereas the fruit body of *F. aethiopica* may be annual sometimes. As for the host range of the two species, data are limited. Currently, *F. aethiopica* is known only from non-defined “deciduous trees”, whereas *F. capensis* has been found on *Vitis vinifera* only. *Fomitiporia mediterranea* and *F. punctata* are also morphologically similar but differ in their distinctly smaller pore size than that observed in *F. capensis* (Ryvarden and Gilbertson, 1994; Fischer, 2002).

Fomitiporia capensis forms a well-supported and clearly delineated clade in a combined ITS and LSU phylogeny based on bootstrap support and Bayesian probability (Fig. 1). It is phylogenetically closest to another African species, *Fomitiporia tenuis* Decock, Bitew & Castillo, however the morphological differences between the two species are significant. *Fomitiporia tenuis* is characterised by having very small pores and much smaller basidiospores than those of *F. capensis* (Table 2). Another genetically related taxon is *Fomitiporia hippophaeicola* (H. Jahn) Fiasson & Niemelä, forming pileate or effused-reflexed fruit bodies on *Hippophaë rhamnoides* and *Eleagnus* in Europe and Asia (Jahn, 1976; Ryvarden and Gilbertson, 1994). Based on phylogeny, *F. capensis* is not closely related to other species occurring on grapevine, namely *F. mediterranea*, *F. australiensis* and *F. polymorpha*. Although all the other individual species formed separate clades, the combined ITS and LSU phylogeny failed to distinguish between *F. mediterranea* and *F. pseudopunctata*. These two species are closely related by molecular analysis using the ITS, LSU and *tef1* regions (Amalfi *et al.*, 2010), but can be separated by sequences of the *rpb2*-gene (Amalfi, pers. comm.). Morphologically, *F. pseudopunctata* has abundant setae and can thus be easily distinguished from *F. mediterranea* (David *et al.*, 1982). *Fomitiporia capensis* has only been isolated from grapevines diagnosed with esca. Its potential virulence as a primary wood rotting agent is currently being tested on *Vitis vinifera*. Other fungi isolated from esca affected vines in South Africa can include *Phaeoacremonium* spp., *Phaeoconiella chlamydospora*, *Phomopsis* spp., Botryosphaeriaceae spp. and *Eutypa* spp. (Van Niekerk *et al.*, 2011; White *et al.*, 2011b). The full host range of *F. capensis* is unknown, as only grapevines were sampled in the present study. During sampling of a wider geographical range of South African vineyards, its distribution was limited to the main

wine-growing region of the Western Cape Province and a single vineyard in Limpopo province in the North of the country. Its common occurrence in a geographically small area of the Western Cape may be due to the fact that this area is the oldest wine producing area in South Africa. It could also be that *F. capensis* originates from the natural vegetation of the Western Cape and also finds the climatic conditions ideal in this region. Investigation of the occurrence of *F. capensis* on other hosts may shed more light on the host range and ecology of *F. capensis*.

There is a large discrepancy between the number of mycelial cultures found through isolations on vines showing symptoms of esca and the number of fruit bodies of *F. capensis* collected during surveys (Table 1). Fruit bodies seem to occur rarely under South African conditions; however, the frequent presence of the species in many vineyards would appear to suggest that fruit bodies are formed often enough to effectively spread infection via basidiospores (Fischer, 2000). Little is known about the exact infection process of vines with the esca related basidiomycetes, though it has been assumed to be by basidiospores entering the plant through wound sites such as pruning wounds (Cortesi *et al.*, 2000; Lupo *et al.*, 2006). If it is the case that infection occurs through basidiospores, it follows that fruit bodies would have to be present to release such spores.

The apparent scarcity of fruit bodies may be due to several factors. Fruit bodies of *F. capensis* have been found to occur underneath the grapevine cordon, in the shade and sometimes underneath the bark, making them difficult to spot especially if vines are trained close to the ground. It may also be likely that fruit bodies simply don't form often on *Vitis vinifera*, which in fact might represent a secondary host for *F. capensis*. While this idea is in some contrast with the high number of mycelia recovered from affected vines, further studies should include sampling from woody hosts close to vineyards to establish if fruit bodies might be present on them.

This study demonstrated the existence of a distinct new *Fomitiporia* species associated with the grapevine disease esca in South Africa based on morphological and phylogenetic data.

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Table 1. Isolation details of *Fomitiporia capensis* isolates collected during this study.

Collection number (STE-U)	Location	Grape cultivar	Age of vineyard (Years)	GenBank accession number (ITS)	GenBank accession number (LSU)
7040	Stellenbosch	Sauvignon blanc	23		
7041	Stellenbosch	Sauvignon blanc	23		
7049	Paarl	Hanepoot	22	JX297429	JX280898
7050	Paarl	Hanepoot	22	JX297430	JX280899
7052	Paarl	Chenin blanc	18		
7053	Paarl	Chenin blanc	18		
7056	Stellenbosch	Hanepoot	Unknown		
7057	Stellenbosch	Malbec	Unknown		
7069	Stellenbosch	Unknown	15		
7072	Stellenbosch	Unknown	15		
7077	Stellenbosch	Sauvignon blanc	25		
7081	Stellenbosch	Sauvignon blanc	25		
7082	Stellenbosch	Sauvignon blanc	25	JX297431	JX280900
7086	Klaas voogds	Red globe	10		
7093	Wellington	Chenin blanc	20	JX297432	JX280901
7094	Wellington	Chenin blanc	20	JX297433	JX280902
7095	Wellington	Chenin blanc	20	JX297434	JX280903
7096	Franschoek	Chenin blanc	40	JX297435	JX280904
7097	Somerset Wes	Sauvignon blanc	16	JX297436	JX280905
7108	Constantia	Sauvignon blanc	18	JX297437	JX280906
7115	Durbanville	Chenin blanc	26		
7119	Durbanville	Sauvignon blanc	23		
7121	Durbanville	Sauvignon blanc	23		
7122	Durbanville	Sauvignon blanc	23		
7124	Darling	Chenin blanc	23		
7135	Grabouw	Chardonnay	15		
7137	Botrivier	Chenin blanc	Unknown		
7140	Riebeeck Wes	Chenin blanc	19		
7163	Franschoek	Cab. Sauvignon	14		
7164	Franschoek	Cab. Sauvignon	14		
7166	Hermanus	Chardonnay	21		
7167	Hermanus	Chardonnay	Unknown		
7168	Hermanus	Chardonnay	21	JX297438	JX280907
7169	Wellington	Cab. Sauvignon	13		
7170	Wellington	Cab. Sauvignon	Unknown		
7171	Somerset West	Tinta Barroca	28	JX297439	JX280908
7486	Modimolle	Piobella	27		
7489	Modimolle	Piobella	27	JX297428	JX280909

Table 2. Morphological characters of fruit bodies of *Fomitiporia capensis* and related taxa, with main emphasis on African species and species occurring on *Vitis vinifera*.

Taxon	Distribution	Known substrate	Shape fruit body	Consistency	Pores/mm	Pore surface	Mitism fruit body	Setae	Spores	Literature
<i>Fomitiporia capensis</i>	South Africa	<i>Vitis</i>	resupinate, thin	perennial	4-6	yellowish brown – rusty brown	dimitic	+/- absent	6.0-8.0 x 5.0-7.5 µm	This paper
<i>F. punctata</i>	mostly boreal species of Europe	deciduous trees	resupinate, cushion-shaped	perennial	6-8	grayish brown	dimitic	absent	6.5-8.5 x 5.5-7 µm	Ryvarden & Gilbertson, 1994; Decock et al., 2007
<i>F. pseudopunctata</i>	Southern Europe	deciduous trees	resupinate, cushion-shaped	perennial	6-8	yellowish brown – greyish brown	dimitic	abundant	6.5-7.5 x 5.5-7 µm	David et al., 1982; Amalfi et al., 2010
<i>F. mediterranea</i>	Europe, Near East	<i>Vitis</i> , deciduous trees	resupinate, cushion-shaped	perennial	5-8	yellowish brown – pale brown	dimitic	very rare	5.5-7.5 x 4.5-6.5 µm	Fischer, 2002
<i>F. australiensis</i>	Australia	<i>Vitis</i> , <i>Dodonaea</i>	resupinate - pileate	perennial	3-5	grayish brown – ferruginous	dimitic	absent	6-8 x 5-6.5 µm	Fischer et al., 2005
<i>F. aethiopica</i>	Eastern Africa	deciduous trees	resupinate, cushion-shaped	seasonal (to perennial)	5-6	grayish orange – golden brown	dimitic	absent	6.0-8.8 x 5.5-7.2 µm	Decock et al., 2005
<i>F. tenuis</i>	Central and eastern Africa	deciduous trees (liana)	resupinate, thin	no data	10-11	brown – brownish grey	dimitic	rare	4.8-6.0 x 4.3-5.5 µm	Decock et al., 2005
<i>F. gaboensis</i>	Central Africa	deciduous trees	pileate	perennial	6-8	brownish, greyish	dimitic	absent	4.0-6.0 x 3.5-4.8 µm	Amalfi et al., 2010
<i>F. ivindoensis</i>	Central Africa	deciduous trees	cushion-shaped, pileate	perennial	8-10	honey-colored – cinnamon brown	dimitic	absent	4.2-5.5 x 3.5-5.0 µm	Amalfi et al., 2010
<i>F. nobilissima</i>	Central Africa	deciduous trees	pileate	perennial	6-8	greyish brown	dimitic	absent	4.0-6.0 x 3.5-5.0 µm	Amalfi et al., 2010
<i>F. punicata</i>	China	deciduous trees	effused-reflexed - pileate	perennial	4-6	yellowish brown – cinnamon brown	dimitic	absent	5.0-7.5 x 4.0-6.5 µm	Dai et al., 2008
<i>F. bannaensis</i>	Asia	deciduous trees	resupinate	perennial	8-10	yellowish brown	dimitic	abundant	4.2-5.2 x 3.8-4.8 µm	Dai et al., 2001

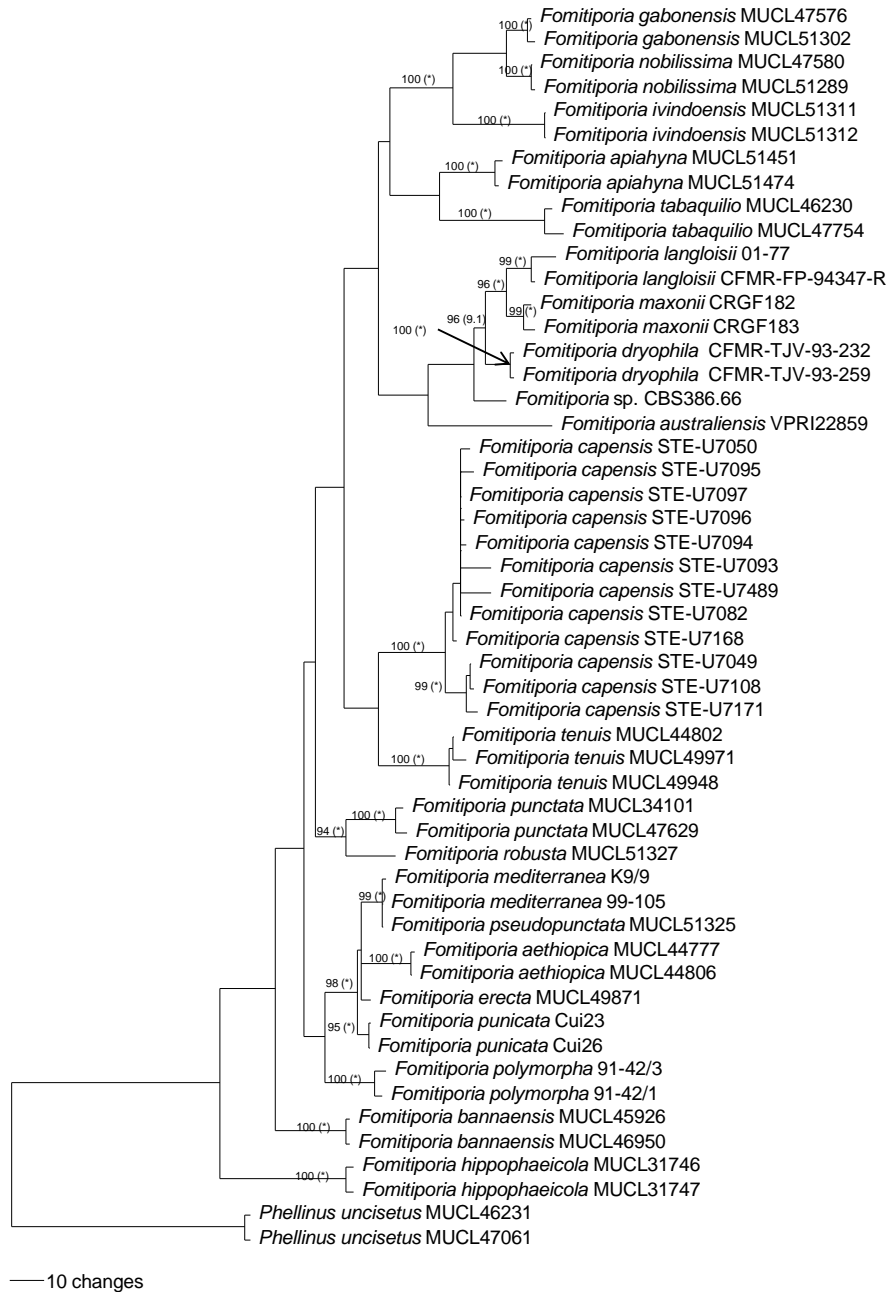


Fig. 1. A combined ITS and LSU phylogeny of *Fomitiporia capensis* sp. nov. (length 621, CI=0.692, RI=0.861, RC=0.596). *Fomitiporia capensis* formed a well supported clade with 100% bootstrap support and a probability of 1.

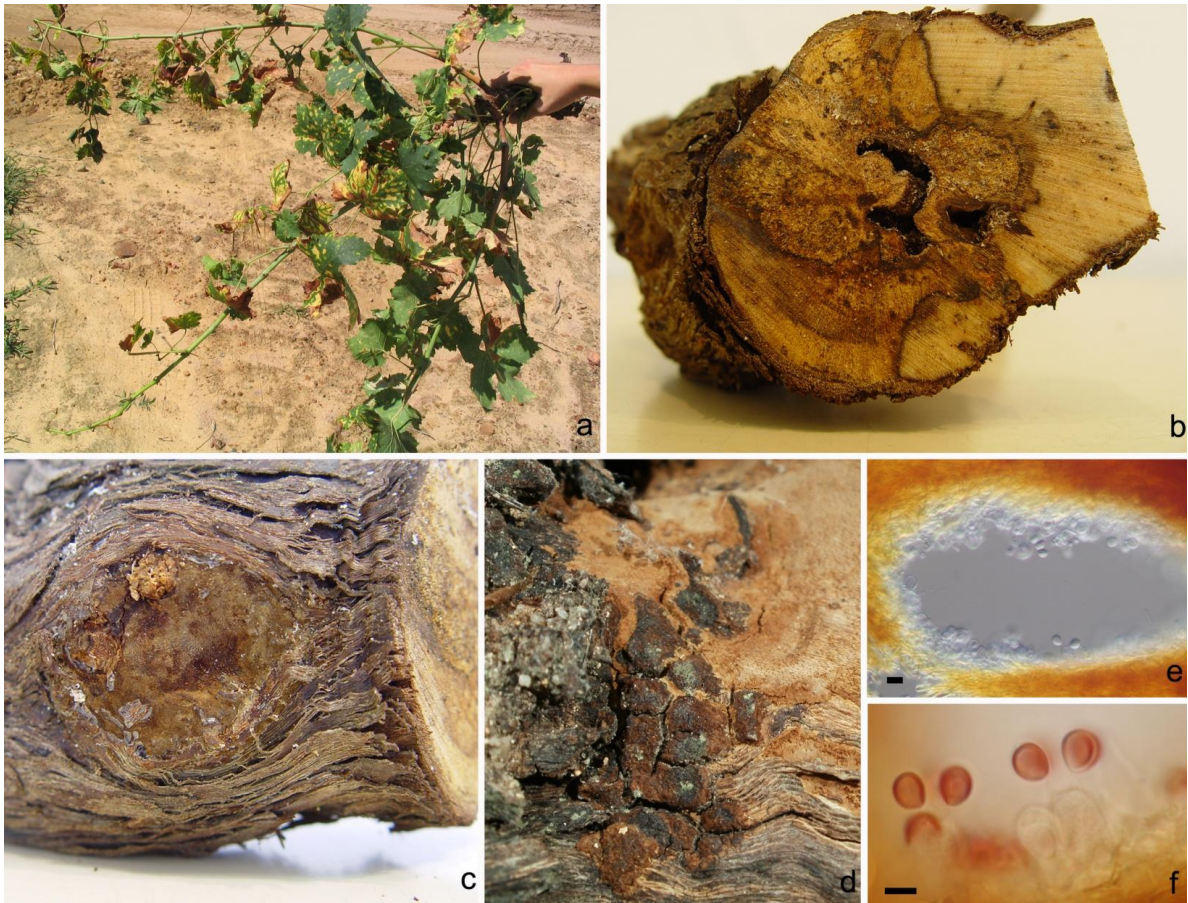


Fig. 2. a-c: Leaf streaking symptoms and internal wood rot on *Vitis vinifera* cv. Chenin blanc associated with a fruit body of *Fomitiporia capensis* FH 183 located on the trunk of the vine. d: Blackish crust forming on the margin of FH 183. e: Hymenial features of *F. capensis*. Scale bar indicates 10 μm . f: Basidiospores of FH 183 showing dextrinoid reaction characteristic of the genus. Scale bar indicates 10 μm .

CHAPTER 3

A new species of *Phellinus sensu stricto* associated with esca on grapevine in South Africa

ABSTRACT

A new species of *Phellinus sensu stricto* was isolated from diseased *Vitis vinifera* in the Northern Cape and Limpopo provinces of South Africa. *Phellinus resupinatus* is described here based on fruit body morphology, internal transcribed spacer (ITS) and large-subunit (LSU) phylogenies. *Phellinus resupinatus* forms a well-supported clade closely related to *Phellinus bicuspidatus*, a species associated with white rot in oak trees in the United States. Morphologically, *P. resupinatus* is characterised by its resupinate fruit body shape, straight, ventricose hymenial setae and broadly ellipsoid hyaline basidiospores. It has been isolated from with esca-diseased grapevines in the summer rainfall regions of South Africa and is found on the uppermost trunk of declining vines showing symptoms of white rot.

INTRODUCTION

The former genus *Phellinus* Quél. has a worldwide distribution and is one of the largest and most economically significant genera in the Hymenochaetales (Basidiomycetes; Oberwinkler, 1977). The genus in its wide sense comprises more than 150 taxa (Larsen & Cobb-Pouille, 1990) but has been divided into several smaller generic units based on morphological and molecular studies (Murrill, 1907; Fiasson and Niemelä, 1984; Ryvar den, 1991; Fischer, 1996; Wagner and Fischer, 2002; Larsson *et al.*, 2006). *Phellinus sensu stricto* (type: *Phellinus igniarius* L.:Fr. Quél) contains approximately a dozen species worldwide (Sell, 2008; Tomsovsky *et al.*, 2010; Cui and Decock, 2013). *Phellinus* s.s. is characterized by pileate, effused-reflexed or resupinate fruit bodies with a crust developed on the pileus surface. The hyphal system is strictly dimitic, the spores are hyaline and of ellipsoid to subglobose shape. *Phellinus* s.s. species are only known to occur on deciduous trees. Host specificity can be an important diagnostic character in some species such as *Phellinus populicola* Niemelä and *Phellinus tremulae* (Bondartsev) Bondartsev & P.N. Borisov which are found on *Populus* spp., and *Phellinus cinereus* (Niemelä) M. Fisch. and *Phellinus laevigatus* (Fr.) Bourdot & Galzin on *Betula* (Jahn, 1963; Niemelä, 1972, 1975; Ryvar den and Gilbertson, 1994; Dai, 1999; Tomsovsky *et al.*, 2010).

While the majority of *Phellinus* s.s. species are of European origin, several taxa have been described from other geographic areas; for example, *Phellinus arctostaphyli* (Long) Niemelä, *Phellinus bicuspidatus* Lombard & M.J. Larsen and *Phellinus spiculosus* (W.A. Campb. & R.W. Davidson) Niemelä are primarily found in North America (for an overview, see Larson and Cobb-Pouille, 1990), and *Phellinus caribeo-quercicolus* Decock & Herrera (Central America; Decock et al., 2006), *Phellinus chaquensis* (Iaconis & J.E. Wright) J.E. Wright & J.R. Deschamps (South America; Wright & Blumenfeld, 1984), and *Phellinus orienticus* Parmasto are more prevalent in Asia (Parmasto, 1985). In Africa, approximately 30 species of *Phellinus* s.l. have been reported from East Africa (Ryvarden and Johansen, 1980; Ryvarden, 1998) and 14 from a lowland rain forest in Cameroon (Roberts and Ryvarden, 2006). Among these, no species belonging to *Phellinus* s.s. have been recognised. Recently, *Phellinus gabonensis* Decock & Yombiyeni has been described as a new species of *Phellinus* s.s. primarily from rain forests of Gabon in West Africa (Yombiyeni et al, 2011). In the current study, only non-African species came out as closely related to *P. gabonensis* by means of nuLSU sequences; other African collections fell into *Fomitiporia* Murr., *Inonotus* P. Karst. or *Fuscoporia* Murr.

Members of *Phellinus* s.s. cause white rot symptoms in the wood of a wide variety of deciduous trees such as *Acer*, *Betula*, *Salix*, *Quercus* etc. (Jahn, 1963, 1981; Niemelä, 1972, 1975; Gilbertson and Ryvarden, 1987; Ryvarden and Gilbertson, 1994; Fischer, 1995; Fischer and Binder, 2004; Sell, 2008). Species such as *P. tuberosus* (Baumg.) Niemelä and *P. alni* (Bondartsev) Parmasto have been found to occur on fruit trees such as *Prunus* spp. and *Malus* spp. (Parmasto, 1976; Niemelä, 1977; Fischer, 1995; Sell, 2008). Damage caused by these organisms is considered economically insignificant though. Historically, *P. igniarius* has been associated with the esca disease complex on grapevine in California (Chiarappa, 1959, 1997); however, no fruiting structures were ever documented and thus, the identity of this species was not confirmed. Furthermore, *P. igniarius* has been recently found not to occur in North America (Fischer and Binder, 2004; Fischer, 2006).

The esca disease complex is one of the most important grapevine diseases, found in all grape-growing regions of the world (Mugnai et al., 1999; White et al., 2011a). The disease is characterised by several symptoms, both external and internal. External symptoms may include decline, foliar tiger stripes and black measles of the berries. Vascular streaking observed in the wood is mainly caused by *Phaeoacremonium* W. Gams, Crous & M.J. Wingf. spp. and *Phaeomoniella chlamydospora* W. Gams, Crous, M.J. Wingf. & Mugnai. The white rot symptom is caused by several basidiomycete species (Chiarappa, 1959; Cortesi et al., 2000; Crous et al., 1996; Larignon and Dubos, 1997; Mugnai et al., 1999; Crous and

Gams, 2006; Mostert *et al.*, 2006; White *et al.*, 2011b). The basidiomycete species linked to the white rot tend to vary among grape-growing regions of the world (Fischer, 2006) and include, *Fomitiporia mediterranea* M. Fisch. (Europe and Near East), *F. australiensis* M. Fisch., J. Edwards, Cunningt. & Pascoe (Australia), *F. polymorpha* M. Fisch. (North America) and *Inocutis jamaicensis* (Murrill) A.M. Gottlieb, J.E. Wright & Moncalvo (South America) (Fischer, 2002; Fischer and Binder, 2004; Fischer *et al.*, 2005; Martinez, 2005). Several distinct taxa have been found to occur in South Africa, all of them undescribed with the exception of the recently discovered *Fomitiporia capensis* M. Fisch. *et al.* (White *et al.*, 2011a; Cloete *et al.*, 2014).

This study describes a novel *Phellinus* s.s species from grapevine in the Northern Cape and Limpopo provinces of South Africa and Namibia. The species was isolated from grapevines showing severe symptoms of the grapevine trunk disease complex, esca, and a number of related fruit bodies were found on the trunks of affected vines, both dead and alive. The species could not be assigned to any known taxon of *Phellinus* s.s. and was described by morphological means as well as by comparing the sequences of the nuclear large subunit and the internal transcribed spacer region to those from other species of *Phellinus* s.s. originating from different regions and hosts.

MATERIALS AND METHODS

Fungal material and culturing

Eleven mycelial isolates from *Vitis vinifera* from the Orange River production area of the Northern Cape were identified during a previous study (White *et al.*, 2011a) as a putative novel *Phellinus* species. Six fresh isolates were made from fruit body material collected from the Orange River sites during 2010. Samples from grapevines showing symptoms of esca were collected from Limpopo province of South Africa and southern Namibia in 2011. Isolations were made by plating triple sterilised symptomatic grapevine material or flame-sterilised fruit body material on Potato Dextrose Agar (PDA, Biolab, Midrand, SA). Fungal mycelial isolates are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch (STE-U) and stored on PDA slants, in sterile water at 15°C, and in tubes with 70% glycerol at -85°C. Prior to DNA extraction, cultures were grown on PDA at 25°C under daylight conditions. Growth studies were conducted with three isolates on MEA (Malt extract agar) at intervals of 5°C between 5°C and 45°C. Growth was measured after a period of seven days and cardinal temperatures for colony growth was obtained.

Comparative microscopy of fruit bodies

Fruiting bodies sections were mounted in water and Melzer's reagent, and studied at 500x and 1250x under phase contrast optics. Ten observations were recorded for measurements of basidiospores and hymenial setae, respectively.

Pictures of sections mounted in Melzer's reagent were taken at 400x and 1000x magnification using a Nikon Eclipse E600 compound microscope with a Nikon DMX1200C digital camera attached.

DNA isolation, PCR amplification, and Sequencing

DNA was isolated from cultured mycelium grown for 21 days on PDA plates using a CTAB based extraction method (Damm *et al.*, 2008). The LSU and ITS1-5.8S-ITS2 regions were amplified using the primer pairs LR0R and LR5 (<http://www.biology.duke.edu/fungi/mycolab/primers.htm>) and ITS1 and ITS4 (White, 1990), respectively. PCR products were cloned using an insTAclone PCR cloning kit (Fermentas Life Sciences) according to the manufacturer's instructions. All PCR products were purified using an MSB Spin PCRapase kit (Invitex, Germany) and sequenced using ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA) on an ABI Prism 3130XL DNA sequencer (Perkin-Elmer, Norwalk, CN).

Phylogenetic analysis

Sequences were edited using Geneious Pro 3.5.6 (2007, Biomatters Ltd., Auckland, New Zealand) and consensus sequences were run through the Basic Local Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to obtain a preliminary identification. Reference sequences were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>) to build a representative LSU and ITS alignment for phylogenetic analyses. Selection of reference sequences was based on several publications, including Decock *et al.* (2006), Wagner and Fischer (2002) and Yombiyeni *et al.* (2011). Sequences were automatically aligned using MAFFT v6 (Kato *et al.*, 2002) and further manual alignment was performed using Sequence alignment editor v2.0a11 (Rambaut, 2002). Maximum parsimony analysis was performed with 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. Bayesian analyses were conducted for

the ITS and LSU alignment using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Modeltest 3.6 was used for selecting the optimal model of sequence evolution for each clade alignment. The likelihood and prior settings for each partition were changed accordingly in MrBayes. Markov chains were initiated from a random tree and run 2.000.000 generations, keeping one out of every 100 generations. Convergence among chains was monitored by examining plots of log-likelihood values and observing when the values of the four chains reached a plateau. The average deviation of split frequencies was 0.001724 for the LSU alignment and 0.006634 for the ITS alignment and the potential scale reduction factors (PSRF) were 1,00 for each parameter in both alignments. The first 2500 generations (burn-in) were discarded and the remaining generations were used to calculate the 50% majority-rule tree and the posterior probability for the individual branches in both alignments.

RESULTS

Seventeen isolates (comprising of 11 isolates identified during the previous study by White *et al.* 2011a and six isolated during the current study) of the novel *Phellinus* species were found in vineyards in the Orange River area of the Northern Cape. Three isolates were from the Limpopo province, and one from Namibia (Table 1). The 20 isolates formed a well-supported group separated from other *Phellinus* species based both on the ITS (Fig. 1) and LSU (Fig. 2) phylogenies. Phylogenies were not combined due to the lack of availability of certain reference isolates on public databases.

Morphology

***Phellinus resupinatus* M. Fisch., M. Cloete, L. Mostert, F. Halleen sp. nov. MycoBank MB 810783, Fig. 3 a-d**

Holotypus “Kanon Eiland 1”, in Stellenbosch (STE-U7769-KE1), collectus a F. Halleen, in *Vitis vinifera* Sultana, 2011.

Etymology: *resupinatus* refers to the attachment of the fruit bodies to the substrate, which is resupinate to cushion-shaped.

Specimens examined: “Keboes” (somewhat irregular appearance) (STE-U7179), MP 1 (STE-U7484), Keboes plant 1 (STE-U7771), Kanon Eiland P1 (STE-U7769)

Habitat: Fruit bodies rare, formed on the uppermost parts of living or dead trunks or underneath the cordon. Vines were declining and all showed distinct white rot inside.

Fruit bodies are resupinate to cushion-shaped (MP1), firmly attached to the host surface, woody hard, perennial; up to 6 mm thick in total; with distinct sterile yellowish margin, up to 5 mm wide, (in MP1); no margin in other specimens.

Pore surface dark yellowish – pale brownish; bright reddish brown in active specimens; cracked in dry specimens (“Kanon Eiland”, “Keboes”); pores more or less circular to angular, very small, (6) 7-8 (9)/mm; dissepiments thin, entire.

Tube layer stratified, with up to three layers (in MP1); 2-4 mm thick, of the same colour or slightly darker than pore surface.

Subiculum very thin, up to 1 mm, grayish brown; darkening with KOH.

Hyphal system dimitic; hyphae subparallel in hymenophoral trama; septa without clamp connections; skeletal hyphae golden brown, essentially aseptate, very rarely branched, slightly thick-walled, 2-4 μm wide; generative hyphae hyaline, most evident in subiculum, thin-walled to slightly thick-walled, rarely septate, rarely branched, 2-3.5 μm wide.

Setae: hymenial, scattered, not evident in all sections; most apparent in Keboes 1 and Kanon Eiland, in one single section of MP 1, straight and more or less ventricose, 12 - 25 x 5-8 μm .

Spores: broadly ellipsoid, hyaline, non-dextrinoid; (4) 4.5 – 5 (5.5) x 3 –3.5 (4) μm ; very rare in MP 1.

Optimal temperature for growth of mycelial colonies: 35°C, with an average radial growth of 68.2 mm per week. Isolates had an average radial growth of 65.4 mm and 64.1 mm at 30°C and 40°C, respectively.

Remarks: *Phellinus resupinatus* is apparently restricted to the summer rainfall areas of South Africa. Our collections have been derived from the Northern Cape and Limpopo; one isolate has been collected from southern Namibia.

Phylogeny

Analysis of the ITS dataset included 834 characters, of which 376 were constant, 97 parsimony-uninformative, and 361 parsimony informative. The heuristic search resulted in two most parsimonious trees with tree scores length 1010, CI=0.712, RI=0.823 and RC=0.586.

Analysis of the LSU dataset included 899 characters, of which 741 were constant, 37 parsimony-uninformative, and 121 parsimony informative. The heuristic search resulted in 77 most parsimonious trees with tree scores length 284, CI=0.701, RI=0.859 and RC=0.602.. *Phellinus resupinatus* formed a well-supported clade in both analyses with bootstrap support value of 100% (ITS and LSU) and a posterior probability of 1 (ITS) and 0.99 (LSU) (Figs. 1 and 2). *Phellinus bicuspidatus* grouped basal to *P. resupinatus* in both the ITS and LSU phylogenies. In the ITS phylogeny, *P. resupinatus* and *P. bicuspidatus* grouped with *Phellinus* s.s. species *P. gabonensis*, *P. caribaeo-quercicolus* and *P. castanopsidis*.

DISCUSSION

Systematics

Phellinus sensu lato had traditionally been distinguished from *Inonotus s.l.*, the other large genus in Hymenochaetales, by means of comparing the hyphal mitism and fruit body consistency of species. This approach has been demonstrated to be inconsistent due to a high level of morphological overlap and the existence of intermediate morphological forms (Fiasson and Niemelä, 1984; Corner, 1991; Wagner and Fischer, 2001, 2002). Several other genera have since been found to accommodate species within *Phellinus s.l.* such as *Fomitiporia*, *Fomitiporella* Murr., *Phellinidium* (Kotl.), *Fuscoporia*, *Fulvifomes* Murr. (Fiasson and Niemelä, 1984) and *Porodaedalea* (M. Lars., Lomb. & Aho); for an overview see Wagner and Fischer (2001, 2002).

Based on a phylogenetic study of the nuclear large subunit (LSU), Wagner and Fischer (2002) found the composition of *Phellinus* s.s. to be inconclusive. While nine European species, including *P. igniarius* and *P. alni* and the non-European species, *P. arctostaphyli*, formed a highly supported clade, the other non-European taxa *Phellinus bicuspidatus* and *P. spiculosus* had only low support for inclusion in *Phellinus* s.s.. Decock *et al.* (2006) described a novel species, *P. caribaeo-quercicolus* Decock & S. Herrera, closely related to *P. bicuspidatus*, as belonging to *Phellinus* s.s. and Yombiyeni *et al.* (2011) found *P. gabonensis*, another close relative to *P. bicuspidatus*. In the current study, the new species was most closely related to *P. bicuspidatus*. *Phellinus resupinatus* together with *P. bicuspidatus* grouped together with *P. gabonensis*, *P. castanopsidis* B.K. Cui & Decock (Cui and Decock, 2013) and *P. caribaeo-quercicolus* in the LSU phylogeny. *Phellinus bicuspidatus* and *P. resupinatus* form a subclade basal to *P. gabonensis*, *P. castanopsidis*

and *P. caribaeo-quercicolus*, which form a separate subclade also basal to *Phellinus* s.s. based on both the LSU and ITS phylogenies.

Phellinus resupinatus and *P. bicuspidatus* differ morphologically in that the former has smaller pores and straight, ventricose hymenial setae compared to the latter's eponymous short, bicuspid setae. *Phellinus bicuspidatus* also has a monomitic hyphal system which sets it apart from the other four species (Lombard and Larsen, 1985). Although the group comprising of *P. caribaeo-quercicolus*, *P. castanopsidis*, *P. gabonensis*, *P. bicuspidatus* and *P. resupinatus* is clearly delineated from *Phellinus* s.s. based on ITS and LSU phylogeny, previous publications have classified these species as part of *Phellinus* s.s (Wagner and Fischer, 2002; Decock *et al.*, 2006; Yombiyeni *et al.*, 2010) and there is still no compelling morphological evidence that suggests otherwise. Taking this into consideration, it is interesting to note that the non-European species mentioned above, i.e *P. bicuspidatus*, *P. gabonensis*, *P. caribaeo-quercicolus*, and *P. castanopsidis* are considered *Phellinus* s.l. in Cui & Decock (2013).

Ecology

During an exploratory study on Hymenochaetales associated with esca in South Africa, White *et al.* (2011a) found nine different taxa occurring on grapevine in the Western Cape area of South Africa. In the Northern Cape Orange River area, one of South Africa's biggest table grape production areas, only *Phellinus resupinatus* was found on diseased grapevines during the exploratory and current studies. During the current study, the species was isolated as mycelial cultures from severe white rot symptoms and fruit bodies were also found in vineyards. In the Limpopo table-grape-growing region, fruit bodies of *P. resupinatus* have since been found on diseased vines where fruit bodies of another species, *Fomitiporia capensis*, were also found (Cloete *et al.*, 2014). A single isolate of *P. resupinatus* was found randomly in southern Namibia on grapevine by a visiting colleague (pers. comm. M. Fischer). From the total number of basidiomycete isolates collected from diseased vines in South Africa, *P. resupinatus* represents 12%, which could be due to the relative lower number of vines sampled outside of the Western Cape.

The disparity between the taxa found in the different areas of South Africa in White *et al.* (2011a) may be explained by differences in climate and ecosystem. The Western Cape has a significant diverse natural ecosystem and a Mediterranean climate, whereas the Northern Cape and Limpopo have higher average temperatures, summer rainfall and a less diverse

natural ecosystem. The occurrence of *P. resupinatus* in the Northern Cape coincides with the higher optimum growth temperature of 35 °C in comparison with the optimum of 25 °C for *F. capensis* (Cloete *et al.*, 2014), the species more commonly found in the Western Cape. Ryvarden (1998) lists differences in climate as well as a different set of potential hosts for wood inhabiting fungi as reasons why there were only a small number of polypores in common between Africa and Europe. The same example may possibly apply to these areas in South Africa. Ryvarden (1998) also mentions dimitic hyphal construction as a possible reason why his review found a higher number of diverse *Phellinus s.l.* species reported in Africa compared to *Inonotus s.l.* species, as the soft annual fruit bodies traditionally associated with the latter might be better adapted to temperate-boreal climates. Once again, this idea may be applied to the South African esca situation as several genera belonging to *Inonotus s.l.* (Chapter 4) are found only in the Western Cape. While the Western Cape has a harsh, dry summer, winters are mild and rainy. In the Northern Cape and Limpopo areas, as well as in southern Namibia, conditions in winter are dry. The morphology of the fruit body, amongst other factors, may assist this species further in surviving under these circumstances where the Western Cape *Inonotus s.l.* species might not have thrived. The exact host range for *P. resupinatus* is presently unknown as no specimens have yet been found on indigenous or introduced flora, but it is a possibility that *P. resupinatus* may occur on other hosts than *Vitis*.

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Table 1. *Phellinus resupinatus* isolates considered during this study.

Specimen number	Collection number ^a (STE-U)	Grapevine cultivar	Location ^b	GenBank accession number ITS	GenBank accession number LSU	Isolated by
FH196	7099*	Sultana	Kanon Eiland, NC			White <i>et al.</i> (2011a)
FH334	7179*	Sultana	Keboes, NC			White <i>et al.</i> (2011a)
FH197	7100	Chenin blanc	Keimoes, NC			White <i>et al.</i> (2011a)
FH200	7101	Colomino	Keimoes, NC			White <i>et al.</i> (2011a)
FH203	7102	Colomino	Keimoes, NC			White <i>et al.</i> (2011a)
FH207	7103	Sultana	Marchand, NC			White <i>et al.</i> (2011a)
FH209	7104	Sultana	Marchand, NC			White <i>et al.</i> (2011a)
FH210	7105	Sultana	Marchand, NC			White <i>et al.</i> (2011a)
FH62	7055	Prime seedless/R99	Marken, LP	KM523247	KM523254	White <i>et al.</i> (2011a)
FH194	7098	Sultana	Kanon Eiland, NC	KM523244	KM523250	White <i>et al.</i> (2011a)
FH337	7180	Sultana	Prieska, NC			White <i>et al.</i> (2011a)
KS1	7771*	Sultana	Keboes, NC	KM523245	KM523249	Cloete <i>et al.</i>
KS2	7772*	Sultana	Keboes, NC			Cloete <i>et al.</i>
KS4	7773*	Sultana	Keboes, NC			Cloete <i>et al.</i>
KS5	7774*	Sultana	Keboes, NC			Cloete <i>et al.</i>
KE1	7769*	Sultana	Kanon Eiland, NC	KM523246	KM523251	Cloete <i>et al.</i>
KE3	7770*	Sultana	Kanon Eiland, NC			Cloete <i>et al.</i>
MP1	7484*	Piobella	Modimolle, LP		KM523252	Cloete <i>et al.</i>
MP4	7488*	Piobella	Modimolle, LP			Cloete <i>et al.</i>
MP6	7490*	Piobella	Modimolle, LP			Cloete <i>et al.</i>
NAM	7775	Unknown	Namibia	KM523248	KM523253	Cloete <i>et al.</i>

a. An * indicates cultures that were isolated from fruit bodies

b. NC denotes Northern Cape, LP denotes Limpopo Province

Table 2. Morphological characters of fruit bodies of *Phellinus resupinatus* and related taxa, with emphasis on African species and species forming resupinate fruit bodies.

Taxon	Distribution	Known substrate	Shape fruit body	Consistency	Pores/mm	Pore surface	Hyphal system	Setae	Spores	Reference
<i>Phellinus resupinatus</i>	South Africa	<i>Vitis</i>	resupinate, cushion-shaped	perennial	8-9	dark yellowish – bright reddish brown	dimitic	present, 12-25 x 5-8 µm	4.0-5.5 x 3.0-4.0 µm	This study
<i>P. bicuspidatus</i>	North America	<i>Quercus</i>	resupinate	perennial	5-7	brownish	monomitc	present, 16-27 x 8-14 µm	4.0-6.0 x 3.0-4.0 µm	Lombard & Larson, 1985
<i>P. gabonensis</i>	Western Africa (Gabon)	deciduous trees (<i>Sacoglottis</i>)	resupinate, cushion-shaped	perennial	6-8	yellowish brown – dark brown	dimitic	abundant, 14-28 x 5.5-12.5 µm	4.3-5.5 x 3.5-4.5 µm	Yombiyeni et al., 2011
<i>P. igniarius</i>	Europe	deciduous trees	effused-reflexed, pileate	perennial	5-6	pale brown – dark brown	dimitic	present, 14-17 x 4-6 µm	5.0-6.5 x 4.5-6.0 µm	Niemelä 1975; Fischer & Binder, 2004
<i>P. caribaeo-quercicolus</i>	Cuba	<i>Quercus cubana</i>	resupinate, pileate	perennial	6-7	grayish-brown	dimitic	present, 15-34.5 x 5.3-11 µm	4.5-6.0 x 3.5-4.7 µm	Decock et al., 2006
<i>P. laevigatus</i>	Europe	deciduous trees	resupinate	perennial	8-10	reddish brown	dimitic	present, 13-30 x 6-9 µm	4.0-5.0 x 3.0-4.0 µm	Niemelä, 1972; Ryvarden & Gilbertson, 1994
<i>P. orienticus</i>	Asia	<i>Betula</i>	resupinate	perennial	6-10	pale brown – dark brown	dimitic	present, 15-18 x 5-6 µm	3.0-4.2 x 2.5-3.1 µm	Parmasto, 1985
<i>P. lundellii</i>	Europe	deciduous trees	resupinate, effused-reflexed	perennial	5-6	reddish brown	dimitic	abundant, 17-30 x 4-7 µm	4.5-6.0 x 4.0-5.0 µm	Niemelä, 1972
<i>P. castanopsidis</i>	China	<i>Castanopsis</i>	resupinate	annual	5-8	grayish brown - fuscous	dimitic	abundant, 21-33 x 10-14 µm	5.0-6.0 x 4.5-5.0 µm	Cui & Decock, 2013

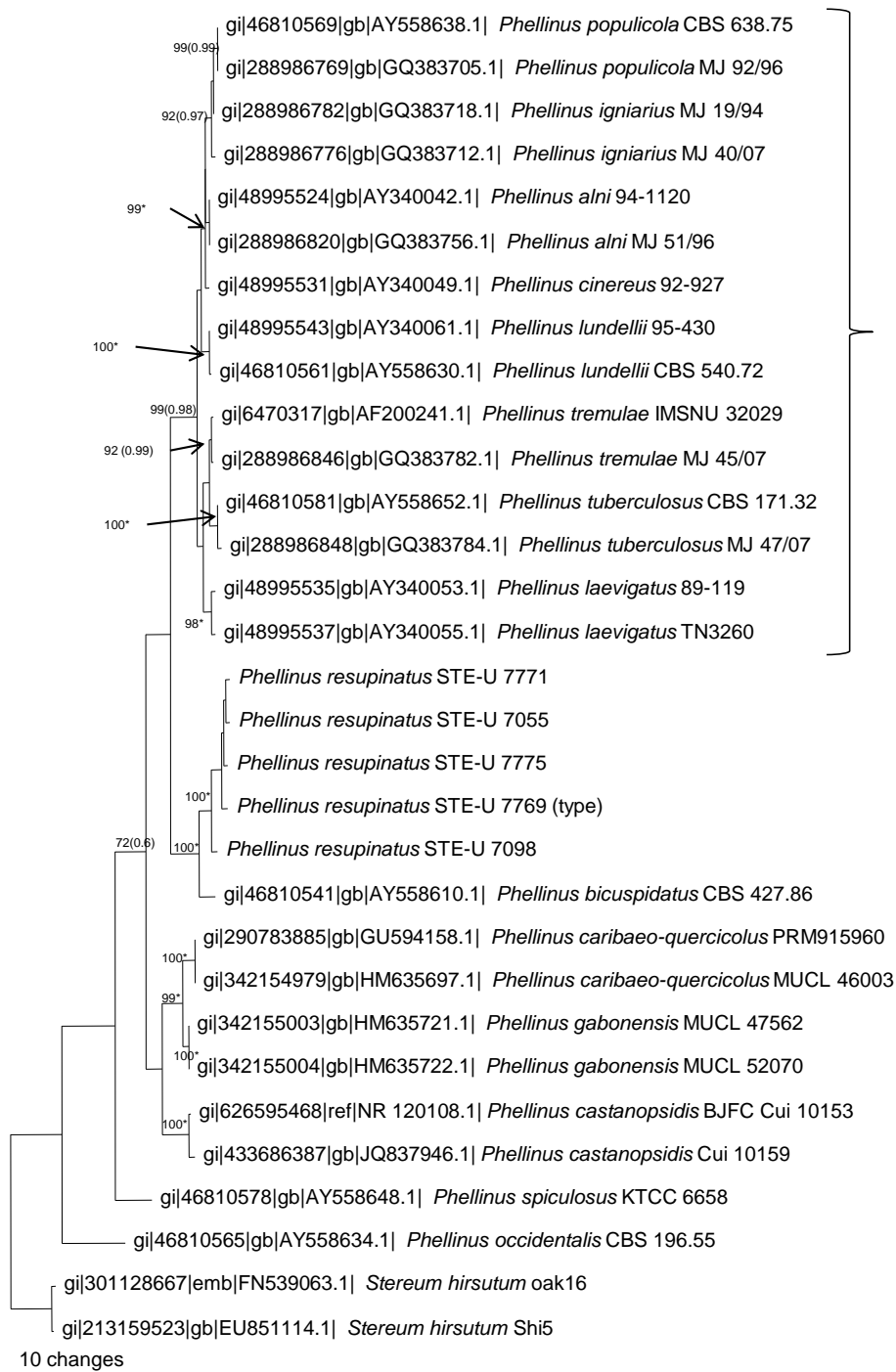


Figure 1. An ITS phylogeny of *Phellinus resupinatus* sp. nov.. Bootstrap values and probability values are indicated. Probability values of 1.0 are indicated by an asterisk. *Phellinus resupinatus* forms a well-supported clade with 100% bootstrap support and a probability of 1.0. *Phellinus sensu stricto* is indicated by a bracket.

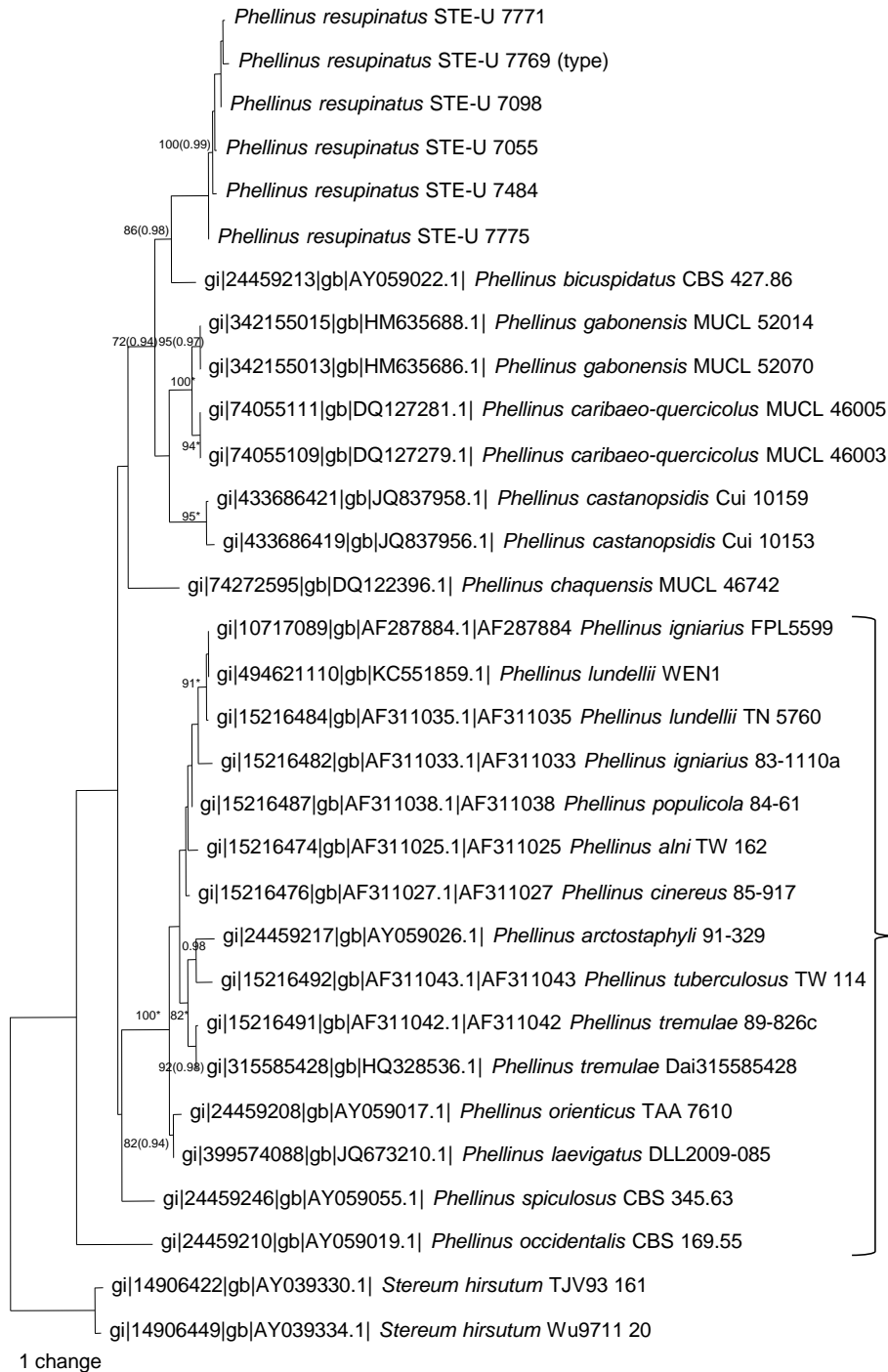


Figure 2. An LSU phylogeny of *Phellinus resupinatus* sp. nov.. Bootstrap values and probability values are indicated. Probability values of 1.0 are indicated by an asterisk. *Phellinus resupinatus* forms a well-supported clade with 100% bootstrap support and a probability 1.0. *Phellinus sensu stricto* is indicated by a bracket.

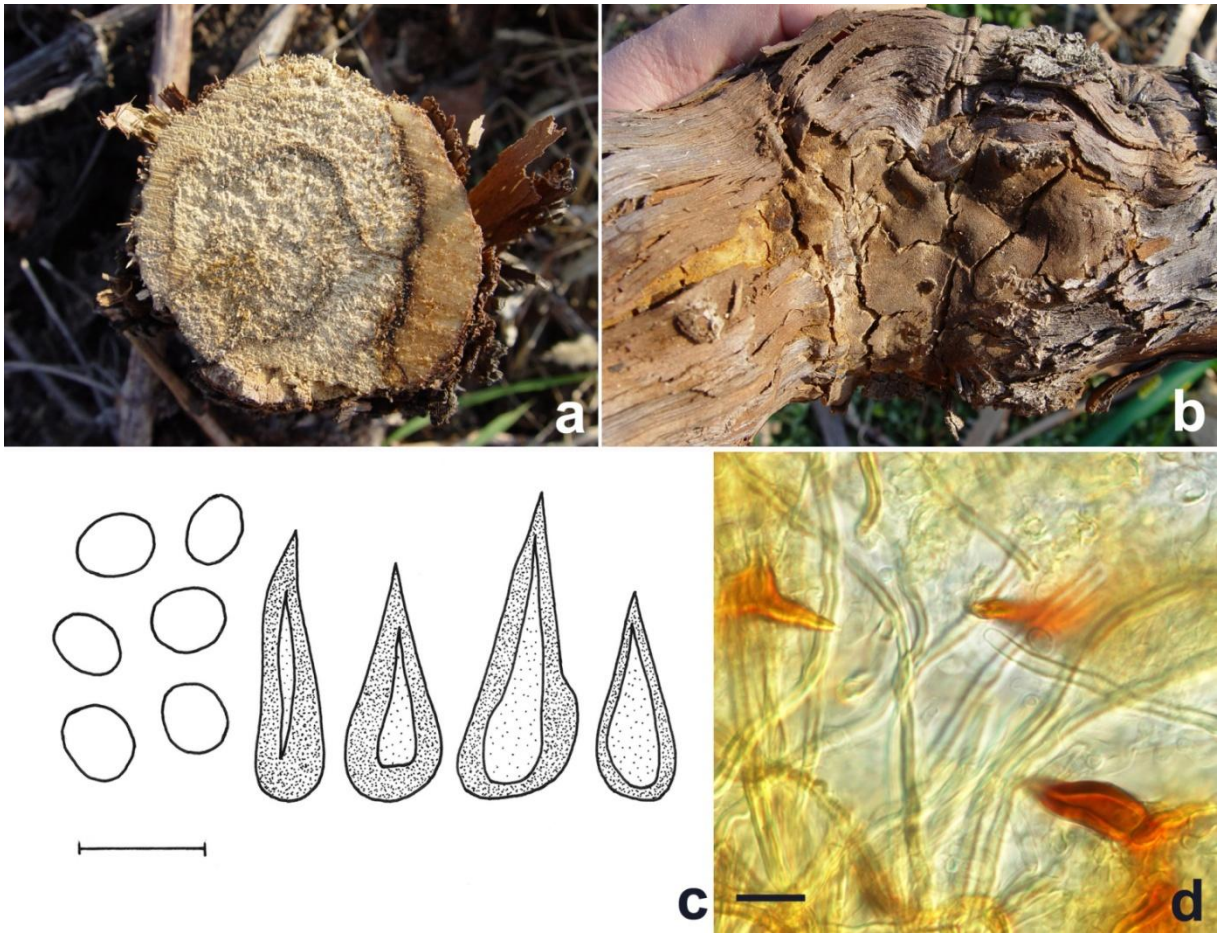


Figure 3.a. White rot symptoms on a vine infected with *Phellinus resupinatus*. b. Fruit body of *P. resupinatus*. c. Basidiospores and hymenial setae of *P. resupinatus*. d. Hymenial setae and skeletal hyphae of *P. resupinatus*. Scale bars indicate 10µm.

CHAPTER 4

Diversity and epidemiology in *Inonotus s.l.* and *Fomitiporella* spp. associated with the esca disease complex in South African vineyards

ABSTRACT

White rot of esca-diseased grapevine in South Africa is associated with ten different basidiomycete taxa, all within the Hymenochaetales. Eight of these taxa fall within *Inonotus*, *Inocutis* and *Fomitiporella*. The majority of these taxa have not been described, since fruit bodies have not been found on *Vitis* or other hosts. This study reports one taxonomic novelty, *Fomitiporella viticola* from *Vitis vinifera* and provides a first report of *Inonotus setulosus-croceus* occurring on *Salix* and *Vitis vinifera* in South Africa. The internal transcribed spacer region (ITS1-5.8S-ITS2) of the nuclear ribosomal DNA (rDNA) was sequenced from DNA isolated from cultures obtained from grapevine rotted wood or from fruit bodies. During this study, *Fomitiporella viticola* was often isolated from white rot on esca-affected vines and fruit bodies were often found on vines. Twelve fruit bodies of *F. viticola* were monitored for sporulation weekly over two seasons between winter and early summer using spore traps. Levels of sporulation had a weak positive correlation with rainfall and a weak negative correlation with average temperature. Sporulation was found to occur throughout the entire monitoring period.

INTRODUCTION

Inonotus P. Karst. *sensu lato* is one of the two largest genera in the Hymenochaetales, along with *Phellinus* Quél. *s.l.* (Fiasson and Niemelä, 1984; Wagner and Fischer, 2002). In the past, the two genera were differentiated by mitism of the hyphal system and fruit body consistency. The genus *Phellinus* included species with perennial fruit bodies and a dimitic hyphal system, while *Inonotus* included species with annual fruit bodies and a monomitic hyphal system. These definitions of the generic concepts of *Phellinus* and *Inonotus* have been discredited on several occasions, as intermediate forms are relatively common and transitional forms occur (Fiasson and Niemelä, 1984; Fischer, 1996; Wagner and Fischer, 2001, 2002).

Fiasson and Niemelä (1984), based primarily on morphological characters, proposed the recognition of several genera within *Inonotus* and *Phellinus*. *Fomitiporia* Murr., *Phylloporia* Murr., *Porodaedalea* Murr., *Ochroporus* J. Schroet., *Phellinidium* Kotl., *Phellinus*,

Fuscoporia Murr., and *Fulvifomes* Murr. were subdivided from *Phellinus*. *Inonotus* was split into *Inonotus* with *Inonotus hispidus* (Bull.) P. Karst. as type specimen and *Inocutis* Fiasson & Niemelä to include the former *Inonotus rheades* (Pers.) P. Karst. complex. Wagner and Fischer (2002) demonstrated that *Inonotus* has to be defined in a wider sense, using sequences of the nuc-LSU rDNA. Their study demonstrated a close relation between *Inocutis*, *Inonotus*, *Fomitiporella* Murr., *Phylloporia*, *Fulvifomes* and *Mensularia* Lázaro Ibiza.

The genera *Inocutis* and *Fomitiporella* have a shared historical relation to the esca disease complex on grapevine. *Inocutis jamaicensis* (Murrill) A.M. Gottlieb, J.E. Wright & Moncalvo has been associated with the Argentine form of esca, “hoja de malvon”, as well as with stem rot of *Eucalyptus globulus* in Uruguay and Argentina (Martinez, 2005; Lupo *et al.*, 2006). Auger *et al.* (2003) associated a species by the name of *Fomitiporella vitis* with esca in Chile; however, this species was not described morphologically and phylogenetic studies done by White *et al.* (2011) suggested that it is more likely to be another *Inocutis* species and not a *Fomitiporella*. *Fomitiporella* can be distinguished by perennial fruit bodies (resupinate or effused-reflexed), a dimitic hyphal system and darkly pigmented ellipsoid to globose non-dextrinoid basidiospores (Wagner and Fischer, 2002). South African vineyards, widely affected by the esca disease complex, are host to a diversity of basidiomycetes falling within the Hymenochaetales. There are currently ten different taxa associated with the white rot symptom characteristic of mature esca (White *et al.*, 2011). Two species have been described as *Fomitiporia capensis* M.Fisch. *et al.* and *Phellinus resupinatus* M. Fisch. *et al.* (Cloete *et al.*, 2014; Chapter 2; Chapter 3). The rest eight taxa fall within *Inonotus s.l.*

The aim of this study was to find fruit bodies and describe any of the *Inonotus s.l.* and *Fomitiporella* spp. associated with esca diseased vines in South Africa. Fruit bodies of *Fomitiporella viticola*, previously classified as Taxon 1 (White *et al.*, 2011), were regularly found on affected vines in the Western Cape during this study and the previous one by White *et al.* (2011). A study of the spore release patterns on twelve of these fruit bodies was undertaken to determine whether inoculum is available during pruning and desuckering season. Grapevine trunk diseases are known to infect vines during these periods; therefore further knowledge regarding the range of airborne pathogens during these seasons is valuable.

Fruit bodies of the other seven taxa have not been found on grapevines, therefore attempts were made to find fruit bodies on alternative hosts.

MATERIALS AND METHODS

Fungal material and culturing

Twenty seven mycelial isolates from *Vitis vinifera* were identified by White *et al.* (2011) and classified in eight taxa within *Fomitiporella*, *Inonotus* and *Inocutis*. During the current study, fruit bodies representing the novel *Fomitiporella* sp. were collected from vineyards in the Stellenbosch area. Two fruit bodies identified as an *Inonotus* sp. were collected from *Salix* in Stellenbosch and Somerset West in the Western Cape during the current study. Fungal strains from White *et al.* (2011) and the current study are maintained in the culture collection of the Department of Plant Pathology at the University of Stellenbosch (STE-U) and stored on PDA (Potato Dextrose Agar, Biolab, Midrand, SA) slants, sterile water at 15°C, and in tubes filled with 70% glycerol at –85°C. Prior to DNA extraction, cultures were grown on PDA at 25°C under daylight conditions. Growth studies were conducted with three isolates on MEA (Malt extract agar) at intervals of 5°C between 5°C and 45°C. Growth was measured after a period of seven days and cardinal temperatures for colony growth were obtained.

Comparative microscopy of fruit bodies

Sections of fruit bodies were mounted in water and Melzer's reagent and studied at 500x and 1000x under phase contrast optics. A maximum of 25 observations was recorded for measurements of basidiospores. Pictures of sections mounted in Melzer's reagent were taken at 400x and 1000x magnification using a Nikon Eclipse E600 compound microscope with a Nikon DMX1200C digital camera attachment.

DNA isolation, PCR amplification and sequencing

DNA was isolated from fruit bodies found on *Salix* or cultured isolates grown for 21 days on PDA plates using a CTAB-based extraction protocol (Damm *et al.*, 2008). Approximately, 700 bp of the ITS region was amplified using the primer pair ITS1 and ITS4 (White *et al.*, 1990). PCR and cloning were done according to the protocol described by White *et al.* (2011). Products were purified using an MSB Spin PCRapase kit (Invitex, Berlin, Germany) and sequenced using ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA) in a ABI Prism 3130XL DNA sequencer (Perkin-Elmer, Norwalk, CN).

Phylogenetic analysis

Sequences were edited using Geneious Pro 3.5.6 (2007, Biomatters Ltd., Auckland, New Zealand) and consensus sequences were run through the Basic Local Alignment Search

Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to obtain a preliminary identification. Reference sequences were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>) to build representative combined ITS alignments for phylogenetic analyses including ex-type specimens. *Phellinus igniarius* strains 85–625 and TN5758.1 (Fischer, 2002) were selected as outgroup.

The sequences were automatically aligned using MAFFT v6 (Katoch *et al.*, 2002) and further manual alignment was performed using Sequence alignment editor v2.0a11 (Rambaut, 2002). Maximum parsimony analysis was performed using PAUP (Phylogenetic Analysis Using Parsimony) v4.0b10 (Swofford, 2003) using the heuristic search option with 10 random taxon additions for all the dataset. 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.

Bayesian analysis was conducted for the ITS alignment using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Modeltest 3.6 was used for selecting the optimal model of sequence evolution for each clade alignment. The likelihood and prior settings for each partition were changed accordingly in MrBayes. Markov chains were initiated from a random tree and run 2.000.000 generations, keeping one out of every 100 generations. Convergence among chains was monitored by examining plots of log-likelihood values and observing when the values of the four chains reached a plateau. The average deviation of split frequencies was 0.004712 and the potential scale reduction factors (PSRF) were 1.00 for each parameter. The first 1250 generations (burn-in) were discarded and the remaining generations were used to calculate the 50% majority-rule tree and the posterior probability for the individual branches.

Spore trapping studies

Spore trapping was conducted over two six month periods between June and November during 2010 and 2011. Two vineyards, a 30-year-old cvr. Pinotage and a 25-year-old experimental table grape block consisting of mixed cultivars, at the Nietvoorbij experimental farm in the Stellenbosch area were chosen for monitoring. Twelve fruit bodies identified as *Fomitiporella viticola*, based on morphological and molecular studies, were monitored over both seasons. The spore traps used were based on the method developed by Eskalen and Gubler (2001) and consisted of glass slides covered with Petroleum Jelly spray (Interlock systems, Groenkloof, South Africa) affixed to the bottom of fruit bodies (Fig. 1a). Spore traps were positioned approximately 3cm from fruit bodies to allow for the movement of air and

water around the fruit body. Slides were exchanged weekly. Spores were counted on uncovered slides at 20x magnification using a Nikon Eclipse E600 compound microscope. Spore identification on the slide was made based on comparison with spore slides from other *Fomitiporella capensis* fruit bodies. Spore counts were done in a single microscopic field at the point of highest spore concentration on the slide, the point underneath the fruit body. Spore counts for both vineyard sites were subjected to single factor analysis of variance in Excel 2010 (Microsoft, WA, USA). Spore counts were compared to several climatic parameters for the Stellenbosch area, recorded by the ARC Infruitec-Nietvoorbij, including total rainfall, average relative humidity (RH), and average temperature over the period during which the slides were in the field. The calculation of Pearson's correlation coefficient (r) between spore counts and weather variables was performed in Excel 2010.

RESULTS

Twenty-five mycelial isolates from White *et al.* (2011) and four from the current study, representing vineyards all over the Western Cape are listed in Table 1, in addition to fruit bodies recovered from *Vitis vinifera* and *Salix*. Fruit bodies obtained from *V. vinifera* could be assigned to one single morphotype and are described as follows:

Taxonomy

***Fomitiporella viticola* M. Fisch., M. Cloete, L. Mostert, F. Halleen sp. Fig 1 b–d**

Holotypus: “Kanonkop” (STE-U7073), collectus a Francois Halleen, *Vitis vinifera*, cultivar Chenin blanc 2011.

Specimens examined: Stellenbosch “Kanonkop” STE-U7073; Klawer “Uitkoms”; Stellenbosch “Remhoogte”; Stellenbosch “P3” STE-U7799, “P5” STE-U7800, “P7” all from *Vitis vinifera*; Taxon 1 (White *et al.*, 2011).

Habitat: Fruit bodies scattered, usually formed on uppermost part of living or dead trunks or underneath the cordon. Associated vines all with distinct sign of white rot inside.

Fruit bodies are resupinate to effused–reflexed with a more or less tomentose blackish margin.; they are firmly attached to the substrate. Effused–reflexed specimens have a black, crust-like pileus with a “burnt” appearance during summer. Fruit bodies are variable in length, up to 8 cm, up to 1 cm thick, maximum 2.5 cm wide and annual to perennial.

The pore surface is dark reddish–brown to brown; pores are more or less angular, dissepiments thin to moderately thick; often of irregular arrangement; approximately 3–4 (5) per mm.

Tube layer is not stratified in examined specimens, up to 8 mm thick, slightly darker than pore surface. Context is very thin, up to 2 mm, reddish brown to almost black.

Hyphal system is dimitic; septa are without clamps; skeletal hyphae are bright golden brown, essentially non-branched, rarely septate, thin- to thick-walled, (2)3–4 μm wide, arrangement (sub)parallel in trama; generative hyphae are hyaline, more or less thin-walled, rarely septate, 2–3 μm wide.

Spores are numerous in all specimens; shape is ellipsoid – ovoid, (4.5) 5 – 5.5 (6) x (3) 3.5 – 4 (4.5) μm (on the average slightly larger in specimen “Remhogte”), slightly yellowish–brown, non–dextrinoid, sometimes appearing slightly thick–walled.

No setae have been detected. Optimum temperatures for growth are between 30 and 35 °C.

Remarks: Species of *Fomitiporella* are generally considered perennial. Based on the examined specimens, this characteristic is unclear. Observations on tube layer, hyphal system and spore discharge (below) are inconclusive in this respect. In general, the condition of fruit bodies varies in relation to collecting season. Additional specimens, preferably collected during the winter rainfall season, need to be studied for further details.

The available collections are somewhat similar to *Inocutis* morphologically, but can be distinguished from *Inocutis* by forming non-pileate fruit bodies. A comparative list of selected *Fomitiporella* species can be found in Table 2. *Fomitiporella viticola* can be distinguished from other species of the genus by the size and spacing of its pores, having 3–4 (5) pores/mm compared to 5–6 and 10–12 pores/mm in the cases of *Fomitiporella cavicola* and *Fomitiporella caryophylli*, respectively.

***Inonotus setuloso–croceus* (Ciel. & Rodw.) P.K. Buchanan & Ryvarden Fig. 1.e–f**

Examined specimen: Somerset West “Vergelegen”, covering the inside of woodpecker holes, from living *Salix* tree.

Fruit bodies are resupinate and soft when fresh, rapidly becoming brittle and hard upon drying. Fruit bodies are presumably annual. Fruit bodies smell fungi–like when fresh and less pleasant when dry.

The pore surface is dark reddish–brown and pores are small and angular. There are (4) 5–6 pores/mm. Dissepiments are thick and entire. Upon drying pores become rectangular with

thinner dissepiments. The tube layer indistinctly stratified, less than 1 cm thick and dark yellowish–brown.

The context is not visible in in the examined samples.

The core has scattered strands of yellowish mycelium and is otherwise dark reddish–brown.

Spores are numerous and are ellipsoid, hyaline and dextrinoid. Spore size is 7–9 x (4.5) 5 (5.5) μm .

Setae / setal hyphae are numerous and hyphoid / tramal, of varying lengths between 24 – > 100 x 6 – 10 μm .

Remarks: While most characters as described above fit well with the description of *I. setuloso-croceus* provided in Ryvarden (2005), there is some divergence in pore size. The reported pore size is 6-8/mm from the Australian and Japanese material (Ryvarden 2005), but only 5-6/mm in South African specimens. Genetic material may be needed in order to study the matter further.

Phylogenetic analysis

Analysis of the ITS dataset included 959 characters, of which 324 were constant, 135 were parsimony–uninformative and 500 were parsimony informative (Fig. 2). Tree scores were Length=2204, CI=0.508, RI=0.837, RC=0.425. *Fomitiporella viticola* formed a well–supported clade with a bootstrap value of 100% and a posterior probability of 1. Immediately basal to *Fomitiporella viticola*, Taxon 2 formed a clade with a bootstrap value of 100 and a posterior probability of 1. *Fomitiporella viticola* and Taxon 2 were closely related, but distinct from *Fomitiporella caryophylli*. The two isolates of Taxon 4 formed a well–supported clade (100% bootstrap and posterior probability of 1) closely related to *Fomitiporella* sp. “Oe.”, an unidentified, unpublished species from *Olea europaea* in Ethiopia lodged only on GenBank. Taxon 3 formed a well–supported clade (100% bootstrap and posterior probability of 1) and was closely related to *Inocutis jamaicensis* and an unidentified species from *Vitis* sp. in Argentina, distinct from other *Inocutis* species.

Within the *Inonotus* section of the phylogeny, Taxon 5 and Taxon 6 formed a well–supported clade (100% bootstrap and posterior probability of 1) somewhat related to, but distinct from Taxon 7 and Taxon 8. Taxon 8 formed a well–supported clade very closely related to, but distinct from American strains of *Inonotus rickii* D.A. Reid / *Ptychogaster cubensis* Pat. *Inonotus obliquus* formed a well–supported clade (100% bootstrap and posterior probability of 1) basal to the Taxa 5–8 and *Inonotus rickii* isolates. The DNA isolated from the fruit body from *Salix*, morphologically identified as *Inonotus setuloso–croceus*, formed a well–supported clade (100% bootstrap and posterior probability of 1) with the isolates of Taxon 7.

Spore trapping

During 2010 and 2011, spore traps were in the field for 23 weeks between the start of June and the end of November, covering the period from the start of winter to early summer. The Western Cape is situated in a winter rainfall area and has a Mediterranean climate with mild wet winters and dry, hot summers.

There were no significant differences between spore counts from both vineyard sites during 2010 ($P=0.29$) and 2011 ($P=0.13$) (Table 3 and 4). Spore counts from both vineyards were therefore combined, given the close physical proximity of the sites and the statistical similarity of the spore counts. During both years, spores were available throughout the entire period of monitoring, though spore counts declined over time, with fewer spores being counted on average at the end of the monitoring period (Fig. 3, Fig. 4). In 2010 spore counts were fairly stable throughout the season, with the exception of a peak occurring during September (Fig. 3). In 2011 (Fig. 4) there was a sharper decline in spore counts towards summer than in 2010.

During 2010, a negative correlation ($r^2=-0.11$) was observed between spore count and average relative humidity, while during 2011, a stronger positive correlation was recorded between both ($r^2=0.54$) (Fig. 5). A weak positive correlation ($r^2=0.06$) between spore count and total rain during 2010 and a positive correlation ($r^2=0.39$) during 2011 were observed (Fig. 6). A weak negative correlation ($r^2=-0.04$) between spore count and average temperature during 2010 and a negative correlation ($r^2=-0.46$) during 2011 were observed (Fig. 7).

DISCUSSION

Fomitiporella viticola is the most commonly isolated Hymenochaetales from esca-affected vines in South Africa. It was only found in the Western Cape, but it was found in every grape-growing area from this particular region (White *et al.*, 2011). Morphologically, it differs quite significantly from related species such as *Fomitiporella caryophylli* (Racib.) T. Wagner & M. Fisch. and *Fomitiporella cavicola* (Kotl. and Pouzar) T. Wagner & M. Fisch. *Fomitiporella viticola* presents much larger and looser spaced pores than both of the aforementioned species, including the genetically most closely related species, *F. caryophylli*.

Phylogenetic analysis indicated that there are three species of *Fomitiporella* associated with grapevine in South Africa. Taxon 2, which is closely related to Taxon 1, was only found in the Oudtshoorn grape-growing area of the Western Cape, a region with a distinct climate compared to the Stellenbosch area where Taxon 1 is dominant. Taxon 4, which was only isolated from one vineyard in Stellenbosch (currently removed), is closely related to an

unnamed, unpublished *Fomitiporella* species from *Olea europaea* in Ethiopia found in GenBank. It might be advisable to search for fruit bodies on *Olea* in future research.

Taxon 8, closely related to American isolates of *Inonotus rickii* (Pat.) / *Ptychogaster cubensis*, was only isolated from a single vineyard in Botrivier, a coastal grapevine-growing area in the Overberg region. *Ptychogaster cubensis* is the anamorph of *Inonotus rickii*, forming copious chlamydospores and a pseudo-fruit body on hosts such as *Quercus phellos* L. and *Myrica* L. in the United States and *Celtis australis* L. in the Mediterranean region, (Davidson *et al.*, 1942; Ramos *et al.*, 2008; De Simone *et al.*, 2011). Hosts of the same genus or species might be a good alternative to finding fruit bodies in the Western Cape.

Fomitiporella viticola is the dominant species associated with esca in South Africa. As such, its epidemiology is important to the grapevine industry. Little is known about the epidemiology of most of the Hymenochaetales, and even less about *Fomitiporella* species. Yohem (1982) reported that slides set up underneath fruit bodies of *Inonotus weirianus* (Bres.) T. Wagner and M. Fischer for one month during winter had spores adhering to the surface of the slides. Fischer (2009) found that *Fomitiporia mediterranea* sporulated between 190 and 250 days per year, with sporulation dependent on daily average temperatures and spore deposit increasing slightly after rain. Buller (1922, in Rockett and Kramer, 1974) found the perennial fruit bodies of the polypore *Ganoderma applanatum* (Pers.) Pat. to continuously sporulate for up to six months. In the present study, spore trapping studies were conducted to coincide with pruning and suckering in the vineyard from winter until early summer, respectively. *Fomitiporella viticola* was found to sporulate continuously, though in varying quantities, for the duration of the experiment from June to November in both years. Continuous sporulation at low levels is thought to be a survival mechanism, increasing the likelihood of a spore finding a suitable substrate, in contrast to releasing a high number of spores over a limited period like many mushrooms (Rockett and Kramer, 1974).

In the current study, sporulation tapered off toward summer, very slightly during 2010 and profoundly during 2011. When examining spore counts and their correlation to climatic conditions, it was found that there was a positive correlation between spore counts and rainfall during the trapping period, with a stronger correlation during 2011. When comparing the climatic conditions of 2010 to those of 2011, 2011 had an observably steeper decline in total rain between June and November, with considerably more rain falling during the first twelve weeks of the trial. Rainfall might have an effect on the occurrence and intensity of sporulation, though sporulation levels weren't found to be higher after rainfall events. This is in contrast with sporulation levels recorded for *Eutypa lata*, *Phomopsis* and *Botryosphaeriaceae* spp. during similar trials (Van Niekerk *et al.*, 2011). Average temperature, with a weak negative correlation to spore counts, likewise has an effect on

sporulation, with sporulation decreasing as average temperature increases. Nevertheless, spores were available throughout the collection time, which makes inoculum available for potential infections during pruning and suckering in the field. Fischer (2008) found that *Fomitiporia mediterranea* was able to colonise young vines via pruning wounds and was present on wounds and wood directly adjacent to wounds.

The diversity of *Inonotus* and related species occurring on diseased grapevine in the Western Cape is remarkable not only when compared to the Hymenochaetales found on *Vitis vinifera* in other grapevine growing regions, but also when compared to the spectrum of known *Inonotus s.l.* and *Fomitiporella* species occurring in Africa itself. Ryvar den and Johansen (1980) and Ryvar den (1999) listed a total of six species, namely *F. caryophylli*, *Inonotus pegleri* Ryvar den, *Inonotus ochroporus* (Van Der Byl) Pegler, *Inonotus patouillardii* (Pat. & R. Heim) Pegler, *Inonotus microsporus* Ryvar den, *Inonotus afromontanus* Ryvar den and *Inonotus palmicola* Ryvar den as occurring in the south of the Sahara. The species listed were described from East and Southern Africa (specifically Zimbabwe). No further descriptions of *Inonotus s.l.* or related species have been made from Africa, despite several recent publications dealing with Hymenochaetales in various parts of Sub-Saharan Africa (Decock *et al.*, 2005; Roberts and Ryvar den, 2006; Amalfi *et al.*, 2010; Yombiyeni *et al.* 2011). Ryvar den (1998) suggested that monomitic fruit bodies may make *Inonotus* better adapted to temperate-boreal climates, which may explain the lack of diversity in African *Inonotus* species. If that is the case, the Western Cape's Mediterranean climate may make it more suitable for *Inonotus s.l.* species than other parts of South Africa or Africa. The Western Cape also has a highly diverse natural ecosystem with a large number of introduced hosts, both agricultural and ornamental. This makes it difficult to speculate on the geographic origin or original hosts of the Hymenochaetales taxa occurring in this area.

Inonotus setuloso-croceus is a species that is not frequently documented and has not been sequenced prior to this publication. It has only been recorded from hardwoods in Japan and Australia (Buchanan and Ryvar den, 1993). Finding it in vegetative form on grapevine and as fruit bodies on *Salix* in South Africa suggests that grapevine may not be an ideal host for fruit body formation. Fruit bodies were also found specifically in large cavities, like woodpecker holes, on the trunks of *Salix* trees, another factor that may impact upon optimal conditions for fruit body formation which may not be present in grapevine. It is tempting to speculate that the species may have been introduced along with plant species introduced from Australasia, such as *Eucalyptus*, a known host according to Ryvar den (2005). This may be an indication of further hosts to examine in the future.

Despite the lack of further fruit bodies representing the missing *Inonotus*, *Inocutis* and *Fomitiporella* species on grapevine, the discovery of *Inonotus setuloso-croceus* on *Salix* is

an indication that the missing fruit bodies may be found on hosts other than grapevine. It seems likely that while conditions in *Vitis vinifera* are suitable for infection and vegetative growth, conditions may not be optimal for fruit body formation. This is one of several possible reasons why fruit bodies representing the remaining taxa remain undetected. Other possibilities include the difficulty of finding fruit bodies under the grapevine cordon and bark and the removal of infected vines before inspection for fruit bodies. Nevertheless, based on the discovery of *Inonotus setulosus-croceus* on *Salix*, it would seem that examining potential alternative hosts is the most promising possibility for finding the remaining taxa.

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Table 1. South African *Inonotus* s./ isolates included in this study.

Taxon	STE-U	Location	GenBank accession number
<i>Fomitiporella viticola</i>	7038	Stellenbosch	JQ038891
<i>Fomitiporella viticola</i>	7039	Stellenbosch	JQ038892
<i>Fomitiporella viticola</i>	7141	Riebeeck Kasteel	KP279295
<i>Fomitiporella viticola</i>	7148	De Rust	KP279296
<i>Fomitiporella viticola</i>	*7799	Stellenbosch	KP279298
<i>Fomitiporella viticola</i>	*7798	Vredendal	KP279299
	2	7147 Oudtshoorn	JQ038893
	2	7154 Calitzdorp	JQ038894
	2	7155 Calitzdorp	KP279297
	3	7109 Constantia	KP279300
	3	7136 Grabouw	JQ038896
	3	7174 Ashton	JQ038895
	3	7178 Montagu	KP279301
	4	7042 Stellenbosch	JQ038897
	4	7043 Stellenbosch	JQ038898
<i>Inonotus setulosus-croceus</i>	*7801	Somerset West	KP279294
<i>Inonotus setulosus-croceus</i>	*7802	Stellenbosch	XXXXXX
<i>Inonotus setulosus-croceus</i>	7090	Stellenbosch	JQ038904
<i>Inonotus setulosus-croceus</i>	7106	Constantia	JQ038903
<i>Inonotus setulosus-croceus</i>	7165	Franschoek	KP279292
<i>Inonotus setulosus-croceus</i>	7173	Somerset West	KP279293
	5	7126 Darling	JQ038900
	5	7129 Darling	JQ038899
	5	7143 Tulbagh	KP279302
	5	7153 Ladismith	KP279303
	6	7133 Malmesbury	JQ038901
	6	7134 Malmesbury	JQ038902
	8	7138 Botrivier	JQ038905
	8	7139 Botrivier	JQ038906

All isolates from *Vitis vinifera* except STE-U7801 and STE-U7802, which were taken from *Salix* sp.

All isolates are from White *et al.* 2010 except STE-U7798, 7799, 7801 and 7801,

* Fruit body available

Table 2. Morphological characters of *Fomitiporella* species.

Name	Distribution	Basidiospores	Hyphal system	Setae	Fruit body	Pores	Reference
<i>Fomitiporella caryophilli</i>	Australia	subglobose	dimitic	none	perennial	10-12/mm	Cunningham, 1965
	Java	2.5-5 x 2-2.5µm			pileate - effuse-reflexed		Ryvarden, 1980
	E. Africa	smooth, yellow tinted			solitary, woody		
<i>Fomitiporella cavicola</i>	Portugal, France, UK Czech Republic, Bulgaria	broadly ellipsoid	dimitic	none	perennial	5-6/mm	Melo and Cardoso, 2007;
		4.5-5.5 x 3.5-4.5µm smooth reddish brown			thin, resupinate		Kotlaba and Pouzar, 1997; Ainsworth, 1999; Fischer, 2001
<i>Fomitiporella floridana</i>	USA	globose, smooth	unknown	unknown	broadly effused	5-6/mm	Murrill, 1907
<i>Fomitiporella johnsoniana</i>	USA	subglobose	dimitic	frequent variable shape	resupinate - effuse-reflexed	7-9/mm	Murrill, 1907
	East Africa	3 - 4µm brownish			inseperable		Ryvarden, 1980
<i>Fomitiporella langloisiana</i>	USA	subglobose 3,5 - 4,5µm ferruginous	unknown	unknown	effused	6/mm	Murrill, 1907
<i>Fomitiporella viticola</i>	South Africa	ovoid-ellipsoid 4.5-5 x 3.5-4 smooth, yellowish-brown	dimitic	none	perennial resupinate - effuse-reflexed	3-5/mm	This study

Table 3. Analysis of variance between spore counts for both vineyard sites, P2 (Pinotage) and NVB (Table grapes) during the 2010 season. Sources of variance are variance between sites and variance within sites.

Source of Variation	df	Type I SS	Mean Square	F	P-value
Between Sites	1	176.0869565	176.0869565	1.16197	0.28693
Within Sites	44	6667.826087	151.541502		

Table 4. Analysis of variance between spore counts for both vineyard sites, P2 (Pinotage) and NVB (Table grapes) during the 2011 season. Sources of variance are variance between sites and variance within sites.

Source of Variation	df	Type I SS	Mean Square	F	P-value
Between Groups	1	712.1956522	712.1956522	2.37676	0.13032
Within Groups	44	13184.6087	299.6501976		

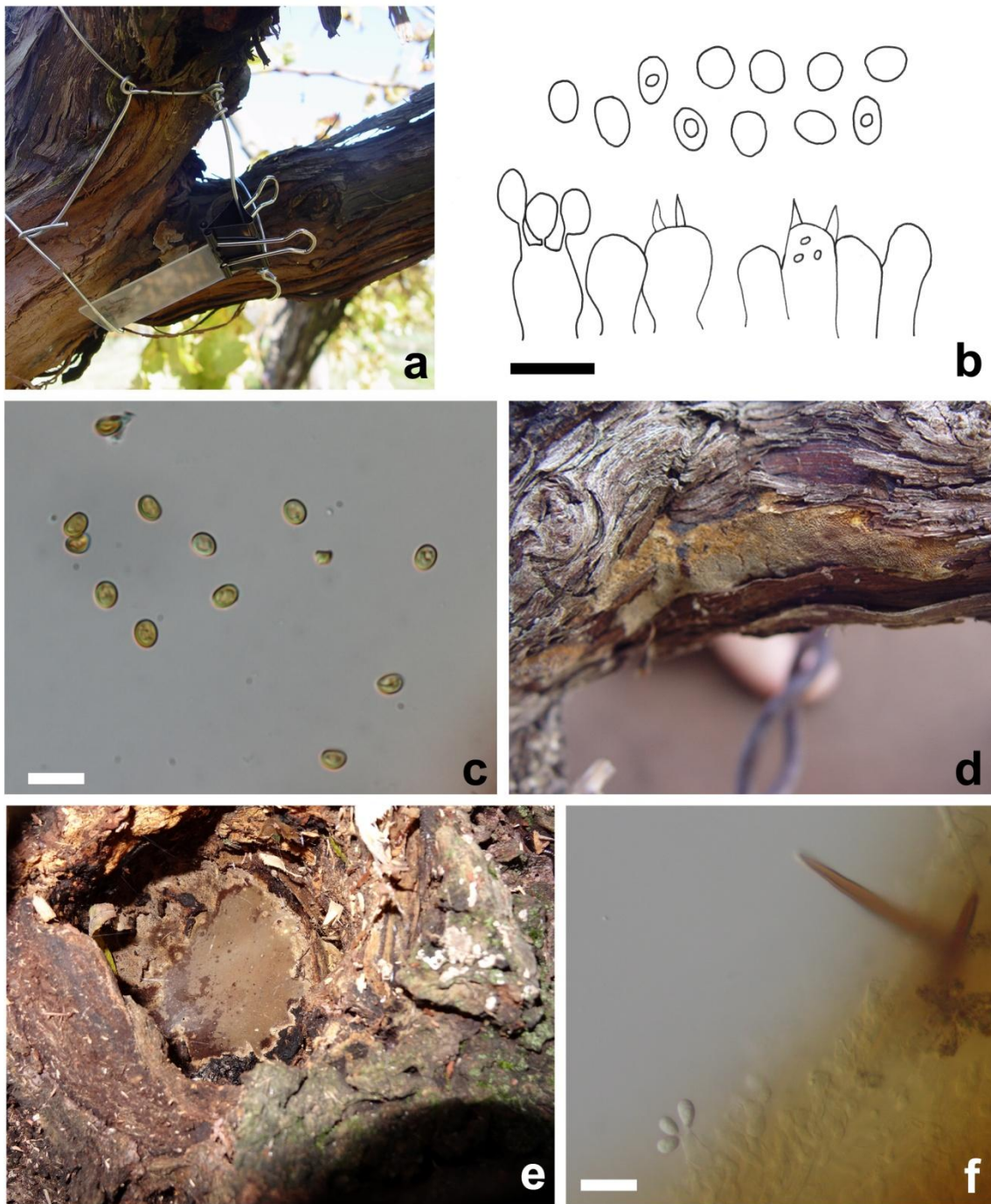


Figure 1. a. Spore trap attached to fruit body of *Fomitiporella viticola*. b. Basidia and basidiospores of *Fomitiporella viticola*. c. Pigmented basidiospores of *Fomitiporella viticola*. d. Fruit body of *Fomitiporella viticola* on *Vitis*. e. Fruit body of *Inonotus setulosus-croceus* on *Salix*. f. Basidium with basidiospores and pigmented setae of *Inonotus setulosus-croceus* from *Salix*. Scale bars indicate 10 μm.

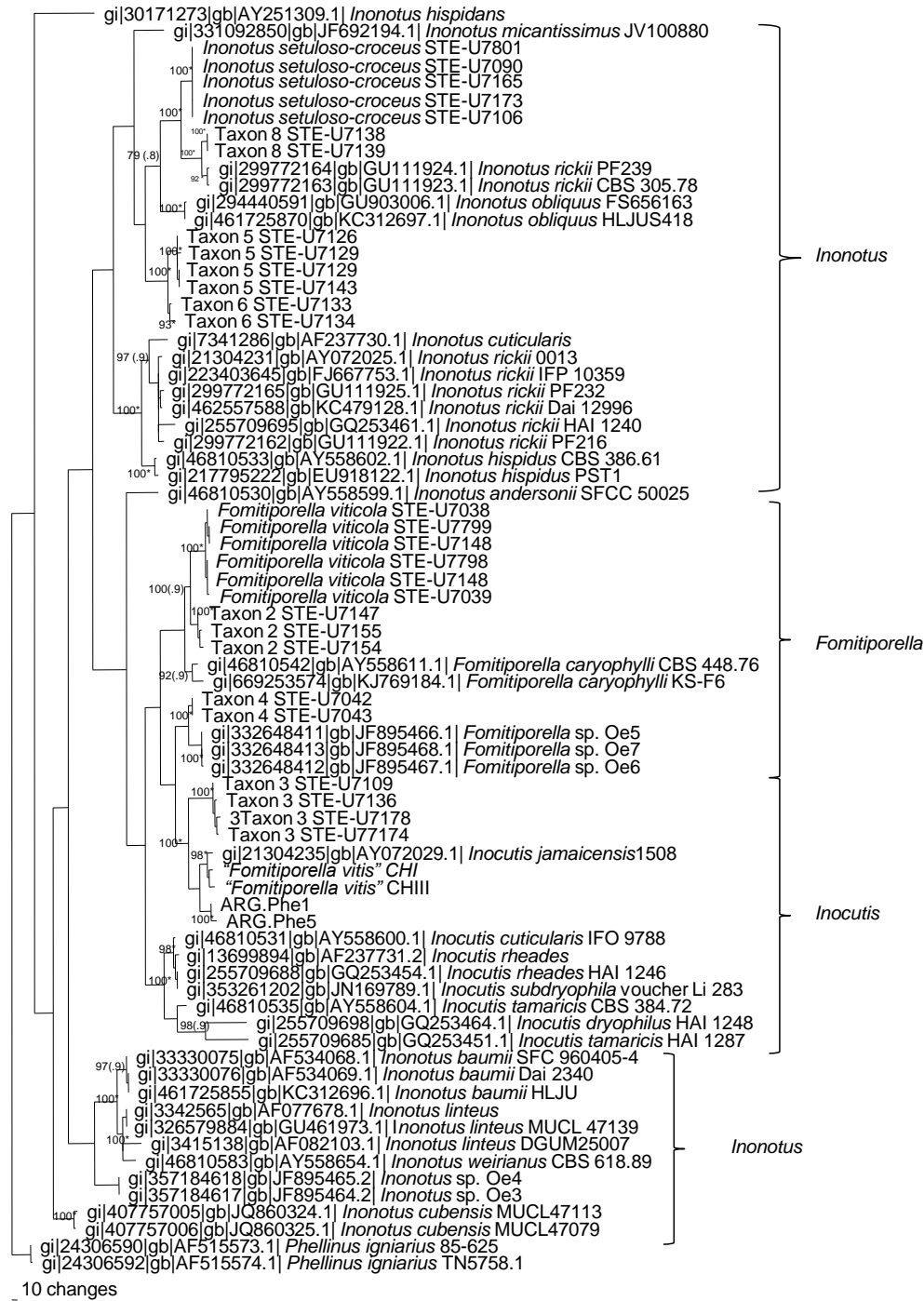


Figure 2. An ITS phylogeny of South African *Inonotus*, *Inocutis* and *Fomitiporella* spp. Bootstrap values and probability values are indicated. Probability values of 1.0 are indicated by an asterisk.

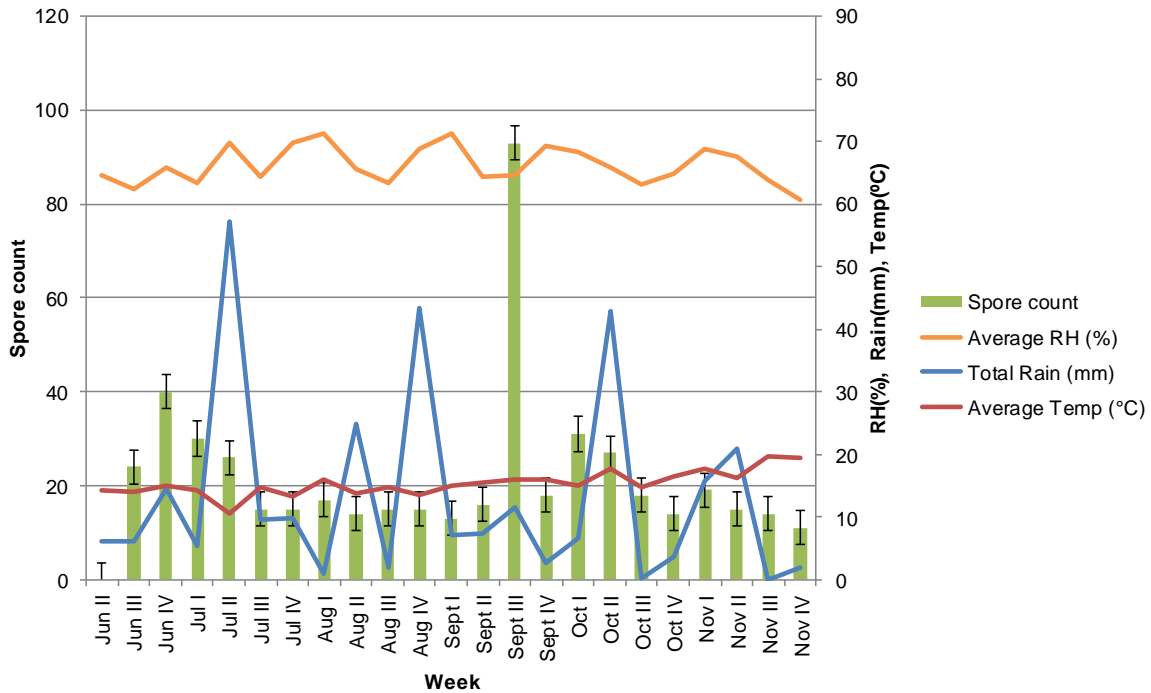


Figure 3. Spore count for all spore traps, average relative humidity, total rainfall and average temperature during every week of the sampling period in 2010.

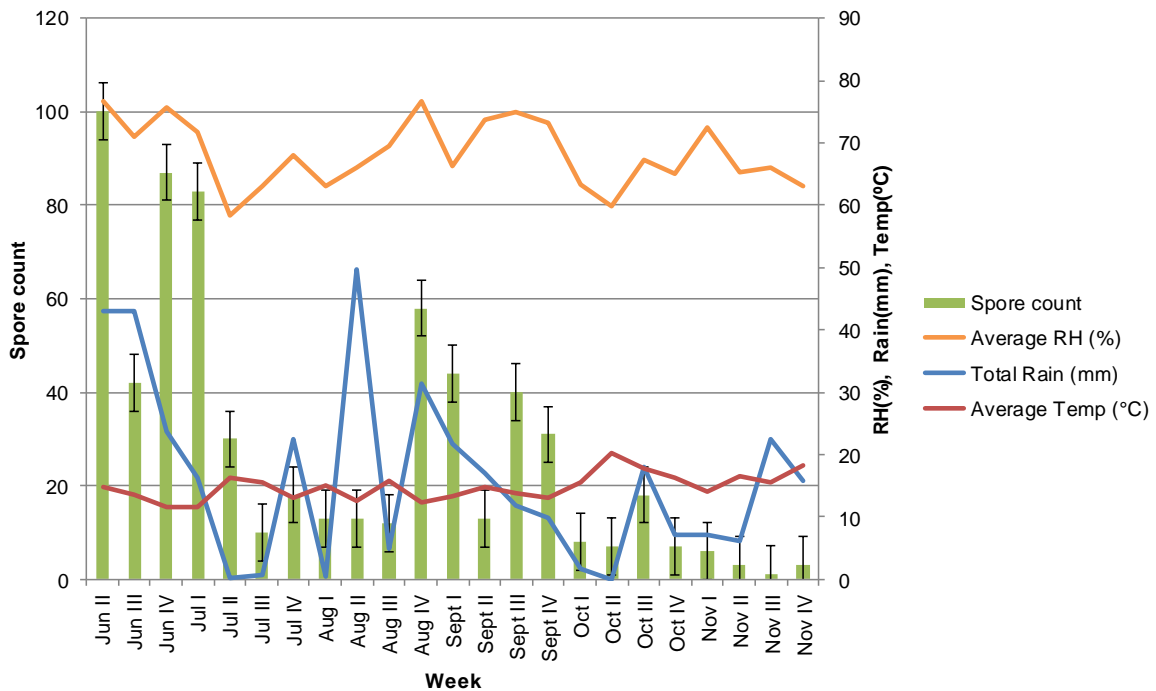


Figure 4. Spore count for all spore traps, average relative humidity, total rainfall and average temperature during every week of the sampling period in 2011.

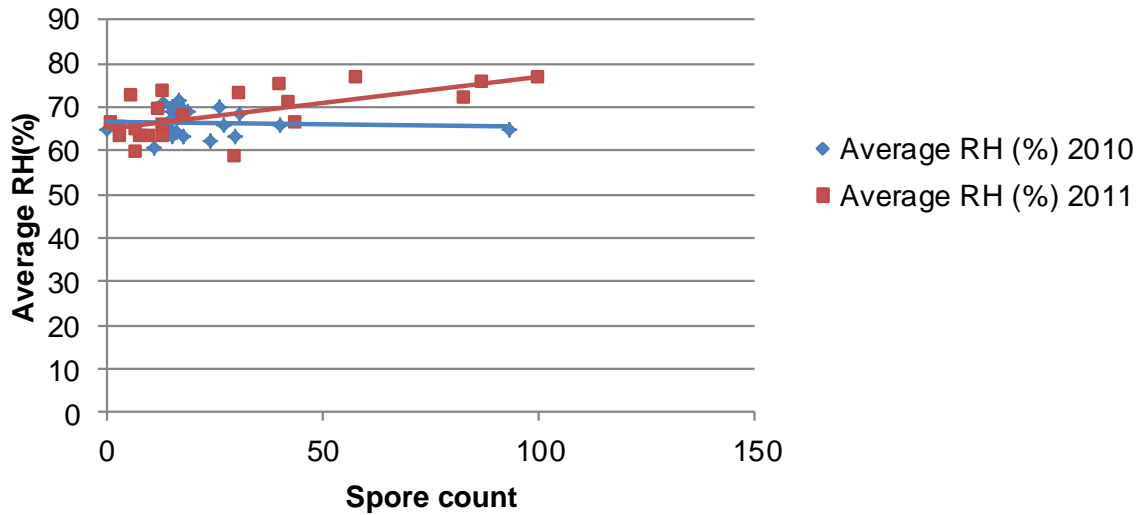


Figure 5. Average relative humidity in relation to spore count during 2010 and 2011. The correlation coefficient (r^2) for spore count to average relative humidity (r^2) was -0.1084 during 2010 and 0.5398 during 2011.

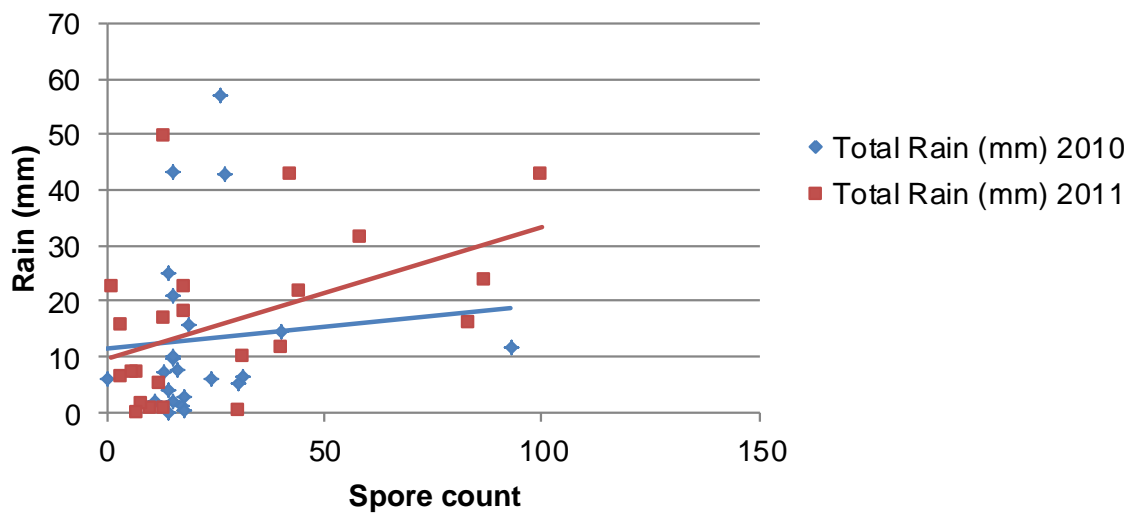


Figure 6. Total rainfall in relation to spore count during 2010 and 2011. The correlation coefficient (r^2) for spore count to total rainfall was 0.0613 during 2010 and 0.394 during 2011.

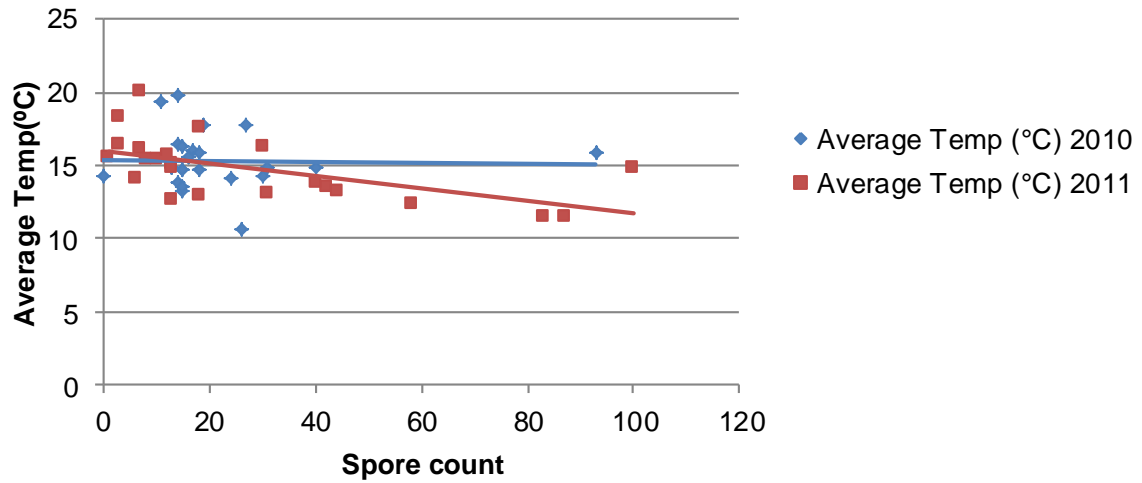


Figure 7. Average temperature in relation to spore count during 2010 and 2011. The correlation coefficient (r^2) for spore count to average temperature was -0.0413 during 2010 and -0.464 during 2011.

CHAPTER 5

Pathogenicity of South African Hymenochaetales taxa isolated from esca-infected grapevines

ABSTRACT

Little is known about the pathogenicity and aetiology of Hymenochaetales taxa associated with esca in South Africa. Ten taxa were subjected to basic enzyme assays to determine which ligninolytic enzymes were secreted by each taxon. In addition, a field trial was undertaken to determine the pathogenicity of ten South African Hymenochaetales taxa associated with esca in grapevine. Twenty-seven fungal isolates and two negative controls were inoculated into wounds made on mature grapevines of the cultivars Shiraz and Mourvèdre. Inoculated vines were evaluated after 24 months. The results of the enzyme assays indicated a difference in enzyme secretion among taxa and also between isolates of the same taxa. All isolates secreted cellulase and laccase, but there was a difference in isolates' ability to secrete manganese peroxidase and lignin peroxidase. . The results of the pathogenicity trial showed that all of the isolates used were capable of causing the characteristic white rot symptom in the wood. There were clear differences in susceptibility to white rot between the two cultivars tested, namely Shiraz and Mourvèdre. The cultivars also differed in which taxa proved to be more virulent. On Shiraz, Taxon 6 (an *Inonotus* sp.), *Phellinus resupinatus* and *Inonotus setuloso-croceus* were significantly virulent. On Mourvèdre, Taxon 3 (an *Inocutis* sp.) was significantly virulent.

INTRODUCTION

Field trials proving pathogenicity, the potential ability to cause disease or abnormalities in a host (Bos and Parlevliet, 1995), involving white rot are very rarely undertaken on grapevine or any other host. The result is that the aetiology of the organisms that cause one of the defining features of mature esca is not understood very well. Differences in virulence, the severity of disease manifestation in infected individuals (Thomas and Elkington, 2004), between the species that cause white rot are not fully understood either. In literature, there have been four trials of varying sizes and complexity on mature vines (Chiarappa, 1997; Sparapano *et al.*, 2000; Sparapano *et al.*, 2001; Gatica *et al.*, 2004) and two on young vines (Larignon and Dubos, 1997; Diaz *et al.*, 2013). Additionally, one of the trials tested the rotting ability of *Fomitiporia mediterranea* M. Fisch. on wooden blocks (Larignon and Dubos, 1997).

Chiarappa (1997) successfully performed inoculations of *Phellinus igniarius* on 7-year-old commercial vines and established *P. igniarius* (L.) Quél. as the main causal organism of the spongy decay symptom of the disease known as black measles in California. Larignon and Dubos (1997) inoculated *P. punctatus* P. Karst. (*Fomitiporia mediterranea*) on Cabernet Sauvignon cane segments, which were rooted for two months and grown in the glasshouse and the field for four months and a year, respectively, and wooden blocks taken from healthy Cabernet Sauvignon vines which were incubated for a year. The young vine inoculations of *F. mediterranea* showed brown vascular streaking, but the researchers were unable to re-isolate the basidiomycete from the inoculated plants. The wood blocks inoculated with *F. mediterranea* showed soft white rot after twelve months.

Sparapano *et al.* (2000) observed white rot symptoms two years after inoculating *Fomitiporia mediterranea* on 13-year-old Sangiovese vines and, during inoculations on six- and nine-year-old Italia and Matilde vines, could detect the first signs of white rot after six months. Sparapano *et al.* (2001) included *Fomitiporia mediterranea* in a cross-inoculation trial with *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. and L. Mugnai and *Phaeomoniella chlamydospora* W. Gams, Crous, M.J. Wingf. and L. Mugnai on mature grapevines and found that *F. punctata* was able to cause limited, localised white rot within three years after inoculation. In Argentina, researchers performed a limited experiment with an undescribed *Phellinus* sp. associated with the Argentine grapevine trunk disease, “hoja de malvón” (Gatica *et al.*, 2004). This species was later identified as *Inocutis jamaicensis* (Murrill) A.M. Gottlieb, J.E. Wright & Moncalvo (Lupo *et al.*, 2006). Five 13 year old plants were inoculated with the *Phellinus* sp. and showed internal and external symptoms of the disease within 6 years after inoculation. Diaz *et al.* (2013) attempted inoculation of a Chilean *Inocutis* sp. on axenic plantlets incubated for 28 days, rooted 2 year old grapevines incubated for 15 months, grapevine shoots incubated for 60 days and detached grapevine shoots incubated for 14 days as part of a larger pathogenicity trial of several pathogens associated with esca in Chile. In the Chilean experiment, the *Inocutis* sp. caused brown vascular discolouration in all the inoculations; however, the incubation time of all the Chilean trials was considerable shorter than in the case of Sparapano *et al.* (2000) and Gatica *et al.* (2004).

White rot in wood is caused by the degradation of primarily lignin, but also cellulose, within the wood cell-walls. Lignin and cellulose degradation are affected by extracellular enzymes released by wood rotting fungi, which break up the complex components of the cell wall (Manion, 1981). Lignin is a complex compound to degrade, and only white rot basidiomycetes have been found to do it efficiently (Songulashvili, 2006). Three enzymes have been found to be essential for lignin degradation, namely a copper containing

phenoloxidase, laccase and two heme-containing peroxidases, lignin peroxidase (LiP) and manganese-dependent lignin peroxidase (MnP) (Overton *et al.*, 2006; Songulashvili, 2006). According to Morgenstern *et al.* (2010), it is unlikely that ligninolytic processes would be possible without production of either lignin peroxidase or manganese peroxidase. Past trials involving enzymatic assays and basidiomycetes involved with esca have shown that *Fomes igniarius* produces laccase and peroxidases and *Fomitiporia punctata* (*F. mediterranea*) produces laccase and peroxidase (Chiarappa, 1959; Mugnai *et al.*, 1999). Having an indication of the types of enzymes secreted by the South African Hymenochaetales may provide some insight into the aetiology of these organisms, if not their pathogenicity.

South African vineyards are widely affected by trunk diseases, including esca. White *et al.* (2011a) characterised ten novel basidiomycete taxa belonging to the Order Hymenochaetales which were associated with white rot symptoms on vines affected by esca (White *et al.* 2011b). The South African Hymenochaetales taxa associated with the esca disease complex represent several distinct genera. Four taxa could be identified based on morphological characteristics, namely *Fomitiporella viticola*, *Fomitiporia capensis*, *Phellinus resupinatus*, and *Inonotus setulosocroceus* (Chapter 2, 3, 4; Cloete *et al.* 2014). The other taxa represent species from the genera *Fomitiporella* (Taxon 2), *Inocutis* (Taxon 3 and 4) and *Inonotus* (Taxon 5, 6 and 8), but these have not been described due to the lack of both fruit bodies for description and suitable reference sequences on GenBank. Given the diversity in species and genera associated with esca in South Africa, there is an expectation of variation in pathogenicity between different taxa. The main objectives of this study were i) to determine the ability of these taxa to induce white rot in mature vines by inoculating mature asymptomatic vines in the field with all ten taxa and ii) to conduct basic *in vitro* enzymatic studies to determine which ligninolytic and cellulose-degrading enzymes were secreted by these taxa.

MATERIALS AND METHODS

Fungal isolates

Twenty-seven isolates representing ten South African Hymenochaetales taxa were selected from White *et al.* (2011a) for pathogenicity testing (Table 1). The cultures are maintained in the culture collection of the Department of Plant Pathology at Stellenbosch University (STE-U). Two to three isolates (two in cases where only two isolates of a certain taxon was available) of every taxon originating from different cultivars and locations were selected for inoculation (Table 1). An isolate of *Acremonium strictum* was selected as negative control. An uninoculated control consisting of uncolonised toothpicks and uncolonised Potato

Dextrose Agar (PDA, Biolab, Merck, Gauteng, SA) was also used. Three weeks prior to inoculation, isolates were plated out on unamended Potato Dextrose Agar, and on triple-sterilised wooden toothpicks cut into 1 cm segments.

For the *in vitro* enzyme assays, the same isolates that were used in the pathogenicity study in addition to some reference isolates representing species associated with esca from other countries, as well as strains representing *Phellinus igniarius* and *Inonotus hispidus* were used (Table 2). Isolates were grown out on PDA plates two weeks before enzyme assays were carried out.

Enzyme assays

Enzyme assays for lignin peroxidase, manganese peroxidase, cellulase and laccase were performed on the 34 isolates indicated in Table 2. Plugs of 4mm diameter were cut from the margins of 2 week old colonies representing all isolates and plated out in triplicate on the respective media. Assays were repeated. For the manganese peroxidase, lignin peroxidase and laccase activity assays, negative reactions were scored as 0. Uncertain reactions, where one to five plates had a positive reaction, were scored as 0/1. Positive reactions, where all plates had a positive reaction, were scored as 1.

Manganese peroxidase

Mycelial plugs of 4mm diameter were plated onto a medium containing manganese sulphate at either 80 mg/l or 100 mg/l (Overton *et al.*, 2006). Plates were incubated at 25°C for 20 days with 12 hour light-dark cycles. The presence of manganese peroxidase was indicated by a rust-coloured discolouration in the medium.

Lignin peroxidase

Mycelial plugs of 4mm diameter were plated onto a medium made up of 5% maltose, 1.4% agar and 0.03% anisidine (Sigma-Aldrich, Gauteng, SA) according to Conesa *et al.* (2000) and incubated for 14 days at 30°C. The production of peroxidase was indicated by a purple halo forming around colonies after the plates were flooded with a solution of 50 mM Na-tartrate buffer at pH 3, 50 µM H₂O₂ and 2 mM 3,4-Dimethoxybenxyl alcohol (Sigma-Aldrich, Gauteng, SA).

Cellulase

Mycelial plugs of 4mm diameter were plated onto a medium containing 0.5% carboxymethyl-cellulose with 0.3% NaNO₃, 0.1% KH₂PO₄, 0.1 % yeast extract and 0.05% MgSO₄ (St. Leger *et al.*,1997) and incubated at 25°C for 7 days. After incubation, staining and destaining was done with Congo Red (1mg/ml) and 1 M NaCl, respectively. The isolates secreting cellulase formed a light halo caused by the cellulose degradation. This halo zone and the colony diameter were measured and expressed as a ratio of halo to colony diameter.

Laccase

Plugs of 4mm diameter were plated onto a 1.5% malt extract bacto agar medium containing 1% tannic acid (Merck) with an adjusted pH of 4.5. The presence of laccase was indicated by the medium turning brown (Rigling, 1995).

Pathogenicity trial

Site selection and plant material

Two vineyards in the Stellenbosch region of the Western Cape, South Africa, were selected for inoculation. Both vineyards were 10 years old, one Shiraz and the other Mourvèdre. The vineyards were approximately 16 km apart. Prior to inoculation, vineyards were inspected for external symptoms of esca and dieback and were spot-tested for internal wood discolouration by cutting open randomly chosen, healthy-looking cordons. Suitable sites for inoculation were marked in advance. Inoculation sites were selected on asymptomatic vines and on well-developed cordons of equal diameter, as far away as possible from existing pruning wounds and spurs.

Experimental design

The trial was laid out in a randomised block design. Experimental units consisted of one wound per vine and fungal treatments consisted of a single pathogen isolate. The negative controls consisted of uncolonised PDA and a non-pathogen control (*Acremonium strictum* STE-U 6296). A total of 29 treatments were applied, representing multiple isolates of all ten basidiomycete taxa, a negative and an uninoculated control. Each treatment was replicated ten times.

Inoculations

During spring in September and October 2010, the two test vineyards were inoculated with selected fungal isolates (Fig. 1). The inoculations were performed according to the

experimental design. The inoculation method was similar to the method detailed in Sparapano *et al.* (2000), but adapted to be less harsh to vines and to minimise the risk of contamination. Each inoculation site was manually cleared of excess bark and sprayed with 70% ethanol solution. A 4mm drill bit was used to drill 10 mm wounds into the wood. The drill bit was sterilised with 70% ethanol between inoculations. A colonised toothpick and a 1cm² piece of colonised growth medium were inserted into each inoculation wound. Wounds were sealed with petroleum jelly (Vaseline, Unilever, SA) and covered with several layers of Parafilm (Bemis Flexible Packaging, Neenah, WI, USA). The inoculated vines were inspected at regular intervals for foliar symptoms.

Retrieval and sample processing

In October and November 2012, respectively, inoculations on the Mourvèdre and Shiraz blocks were retrieved. A 30 cm piece of cordon around each wound site was removed and immediately taken to the laboratory. Cordon pieces were stripped of excess bark and split lengthwise through the inoculation site with a bandsaw (Toolmate, DT group, Denmark). All internal discolouration lengths and wound sites were measured and photographed. Any wood rot found to occur was measured lengthwise. Samples were triple sterilised in 70% ethanol (30s), undiluted bleach (NaOCl)(2min) and 70% ethanol (30s) and left to dry in the laminar flow cabinet. Isolations from internal symptoms were made at five positions, the first at the proximal end of the symptom, the second in the middle of the symptom, the third at the wound site, the fourth in the middle of the symptom on the distal side of the wound and the fifth on the distal end of the symptom. Five wood pieces were extracted from every isolation point. Isolated wood pieces were placed on PDA plates amended with chloramphenicol and incubated on the lab bench at 23 – 25 °C. Emerging basidiomycete colonies were sub-cultured and kept for identification.

Molecular identification of isolated colonies

Bester *et al.* (2014) designed primers in order to identify basidiomycete fungal isolates taken from trial samples. Isolates of which the identity could not be confirmed by this protocol were cloned and sequenced according to the protocol detailed in Cloete *et al.* (2014).

Statistical processing

Pathogenicity, defined in this study as the ability of the fungal agent to cause white rot as a primary rot-inducing agent, was calculated by the measurement of white rot occurring on inoculated vines compared to the negative and uninoculated controls to ascertain which isolates and taxa could be classified as pathogenic compared to controls. Lesion lengths,

defined as any dark discolouration surrounding the point of inoculation, were also measured in order to ascertain whether there was a significant difference in internal discolouration formed between controls and treated wounds. The data were subjected to analysis of variance (ANOVA) and the means were compared by Fischer's least significant difference (LSD) with $P = 0.05$. Analysis was performed using SAS 9.2 (SAS Institute Inc, Cary, North Carolina, USA). The incidence rates were calculated according to the absence or presence of white rot at the inoculation site and calculated as a percentage of the total number of vines inoculated with that particular isolate. The re-isolation percentages were calculated as a percentage of the inoculated basidiomycete recovered from isolation.

RESULTS

Enzyme assays

Manganese peroxidase

All isolates representing Taxon 2 and the single isolate of Taxon 8 (STE-U7139) were able to produce manganese peroxidase (MnP) (Table 2). *Fomitiporella viticola*, Taxon 3, Taxon 6 and *Inonotus setulosocroceus* had variation between isolates with some displaying positive, some negative and some uncertain results. Taxon 4 and a single isolate of Taxon 5 (STE-U7126) could not produce MnP. *Fomitiporella vitis*, *Phellinus alni*, *Fomitiporia polymorpha*, and *Fomitiporia mediterranea* had positive results for MnP activity. *Fomitiporia australiensis* had mixed results between the two isolates tested. *Inocutis jamaicensis*, *Phellinus igniarius* and *Inonotus hispidus* had uncertain results.

Lignin peroxidase

All reference isolates could produce lignin peroxidase (LiP), with the exception of *Phellinus alni*, *P. igniarius* and *Inonotus hispidus*. All isolates representing *Fomitiporella viticola*, Taxon 2, Taxon 6, *Inonotus setuloso-croceus*, *F. capensis* and *P. resupinatus* could produce LiP, as could two of the Taxon 5 isolates. One of the three Taxon 5 (STE-U 7126) isolates and the Taxon 8 isolate (STE-U 7139) had uncertain results. None of the Taxon 3 or Taxon 4 isolates were able to produce LiP. Results are indicated in Table 2.

Laccase

All isolates were able to produce laccase (Table 2).

Cellulase

All isolates tested were able to produce cellulase (Fig. 2). Two isolates of *Fomitiporella viticola* (STE-U7141, STE-U7148), all the isolates of Taxon 3 and the *Phellinus alni* reference isolate produced a halo: colony size ratio of more than two. Only four isolates, one representing Taxon 5 (STE-U7126), one *Inonotus setulosocroceus* (STE-U7090), one *F. capensis* (STE-U7096) and the reference isolate for *F. mediterranea* produced ratios smaller than 1.25.

Pathogenicity trials

None of the inoculated vines produced foliar symptoms associated with esca or any external symptoms associated with esca or other trunk diseases during the 2 year incubation period. When cut open, inoculated samples displayed an ellipse-shaped interior discolouration with the broadest area around the wound site (Fig. 3). Discoloured tissue was dark brown to black and hard. In some samples, light to dark yellow rotted tissue could be observed (Fig. 3). Rotten tissue was soft, spongy and moist to the touch. The symptoms types were evaluated separately as brown discoloured lesions and white rot.

The lesion lengths of the dark discoloured tissue lesions on all inoculated plants did not differ significantly from the control lesions in Shiraz (Table 3), and there were no significant differences between taxa ($P=0.6340$) or between isolates within taxa ($P=0.3978$). In Mourvèdre, there was a significant difference between taxa ($P<0.0001$), though not between isolates within taxa ($P=0.7951$) (Table 4) and only Taxon 3 was able to form lesions which differed significantly from the control (Table 5).

The results of the pathogenicity trial showed that all of the isolates were capable of causing the characteristic white rot symptom in the wood to some extent. None of the control plants had white rot. The extent of white rot observed on Shiraz (0.2 – 5.8 mm) was significantly less than that observed in Mourvèdre (1.4 – 40.9 mm). In Shiraz, there was a difference between taxa ($P=0.0083$) (Table 6) with Taxon 6 producing larger rot lengths than the other taxa. There was a significant difference between isolates within a taxon ($P=0.03$), but most isolates did not differ significantly from control (Table 7). Single isolates of Taxon 6 (STE-U 7133), *I. setulosocroceus* (STE-U 7090) and *P. resupinatus* (STE-U 7055) proved significantly virulent.

In Mourvèdre, there was variation between taxa with regard to rot lengths ($P<0.0001$) (Table 8) with Taxon 3 and Taxon 2 proving significantly virulent (Table 9). There was no significant difference between isolates within taxa in Mourvèdre ($P=0.1838$) (Table 9).

During reisolation from Shiraz, 26.89 % of isolates were recovered from inoculated vines. In Mourvèdre, 65.17 % of isolates were recovered from inoculated vines (Table 10). A

white rot incidence rate of 19.6 % was calculated for Shiraz. Mourvèdre's white rot incidence rate was 48.6%.

DISCUSSION

Based on the results of the basic enzyme assays, variation in virulence between isolates and taxa may manifest in the array of ligninolytic enzymes secreted by the various taxa. The reference isolates, chosen for their documented ability to cause white rot on various hosts, also displayed variation in the types of enzymes secreted. Although all isolates were able to produce laccase and cellulase, there was variation between taxa in terms of their ability to produce manganese peroxidase and lignin peroxidase, two critical enzymes in lignin degradation (Overton *et al.*, 2006; Morgenstern *et al.*, 2010). All the South African taxa could produce either lignin peroxidase or manganese peroxidase to a certain extent, except for Taxon 4, an *Inocutis* sp., which could produce neither. Only two South African taxa could not produce any lignin peroxidase, namely the two putative *Inocutis* species (Taxon 3 and Taxon 4). *Inocutis jamaicensis*, the reference isolate, produced both manganese peroxidase and lignin peroxidase. Among the reference isolates, the two *Phellinus* species, *P. alni* and *P. igniarius* and *Inonotus hispidus* could not produce lignin peroxidase, but could produce manganese peroxidase to varying degrees. Unlike *P. alni* and *P. igniarius*, all isolates of the the South African *Phellinus* sp., *P. resupinatus*, produced lignin peroxidase. Based on Morgenstern *et al.* (2010)'s assertion that lignin degradation is not efficiently achieved by laccases alone, and that peroxidases are necessary for the process to occur, there is an expectation that Taxon 4, an *Inocutis* species, would not be able to cause extensive white rot within a short period of time. More detailed investigation into the enzymes secreted by novel Hymenochaetales species is needed.

The occurrence of any white rot in inoculated samples showed that all of the South African Hymenochaetales taxa are pathogenic and have potential to cause white rot symptoms on mature commercial vines within two years. Due to the relatively short incubation time the extent of the rot development was not always significantly different from the controls. In comparison, Gatica *et al.* (2004) and Chiarappa (1997) left inoculated plants in the field for six and eight years, respectively. Sparapano *et al.* (2000) concluded that *Fomitiporia mediterranea* could be considered a primary pathogen after observing white rot symptoms within two years. Several valuable observations may be gleaned from the data in this current study, as these trials are rarely undertaken on such a scale.

The taxa that showed a statistical difference between lengths of white rot and the controls, differed between Shiraz and Mourvèdre. On Shiraz, Taxon 6, an *Inonotus* sp., *P. resupinatus* and *I. setulosocroceus* could be considered more virulent. This difference is

mostly due to some variation between isolates, with some isolates from all the previously mentioned taxa being significantly more virulent than other isolates of the same taxa. Taxon 3, an *Inocutis* sp., was the only significantly virulent taxon on Mourvèdre. There was no significant difference between isolates within taxa in the Mourvèdre block.

These differences in virulent taxa between cultivars could be ascribed to, among other factors, differences in enzyme profiles between taxa as discussed in the previous section, fungal suitability to colonisation of the substrate and various physiological differences between the two cultivars.

When comparing the extent of white rot symptoms measured during the trial, there is a clear difference between the two cultivars tested in the trial. Cultivar differences in sensitivity to grapevine trunk diseases have been subject to several studies (Peros and Berger, 1994; Sosnowski *et al.*, 2007), though no studies regarding sensitivity to rot have been published. Mourvèdre is a cultivar known to be particularly susceptible to esca while not being particularly sensitive to other trunk diseases (McGourty, 2003). Presumably, cultivar differences in sensitivity may be due to plant defenses, such as the formation of polyphenols which inhibit peroxidases and phenoloxidas (Del Rio *et al.*, 2004). Cultivar differences may also be due to physical factors such as differences in wood density between cultivars, a phenomenon that is easily observable in the field, though not documented in detail. Physiological factors also play an important role in plant resistance against trunk disease. During a study on pruning wound protection, Rolshausen *et al.* (2010) demonstrated that a cultivar with a documented susceptibility to trunk disease, Cabernet Sauvignon, had lower lignin content than a tolerant cultivar, Merlot, making it easier for pathogens to physically penetrate the grapevine wood. It stands to reason that factors such as lignin content will influence, not only the ability of pathogens to penetrate wood, but also the rapidity of development of symptoms such as white rot.

Grapevine wound response is a complex process consisting of many factors. Among these factors is the creation of physical barriers to prevent colonisation and spread by pathogens. Cell walls are fortified with additional lignin and pectin, tyloses and gums are formed and enzyme-inhibiting phenolic compounds accumulate around the infected zone to slow down the spread of infection (Del Rio *et al.*, 2001; Edwards *et al.*, 2007; Mutawila *et al.*, 2011). In Sparapano *et al.* (2000), a discolouration similar to the one found in the current trial was formed on either side of inoculations; however, they recorded a significant difference between inoculated and uninoculated vines. During the current trial, the length of internal discolouration surrounding all inoculations was measured. There was little to no variation between taxa in terms of lesions formed. On Shiraz, the most striking result was the fact that the negative and uninoculated controls didn't form lesions statistically different from most of

the fungal taxa inoculated. On Mourvèdre, Taxon 3 was the only fungal inoculation to form lesions that were statistically longer than those formed by the rest of the treatments and the negative and uninoculated controls didn't differ significantly from the other fungal taxa treatments. This may indicate that Mourvèdre has a less robust response to wounding than Shiraz and may explain the difference in the extent of white rot found in both cultivars. If fungal isolates recovered after a two year period can be interpreted as a possible reflection of the host's ability to prevent colonisation, the percentage of isolates recovered from Shiraz (26,89%) compared to those recovered from Mourvèdre (65,17%) could be seen as an indication of the efficacy of the cultivar Shiraz's short term defences.

During reisolation, inoculated cultures could be recovered from all points of isolation. Multiple isolations were made from five points along the length of discoloured internal tissue. In Shiraz and Mourvèdre, inoculated basidiomycetes could be recovered from all points on the lesion.

As in the case of Bos and Parlevliet (1995), pathogenicity of white rot-causing organisms should be defined as the ability of a species to form white rot. This trial demonstrated that all South African Hymenochaetales taxa have the potential to be primary inducers of white rot on grapevine to varying degrees, given enough time and the right circumstances. Taxa, and isolates within the same taxa, vary in their ability to produce enzymes, as well as their ability to produce rot in the host. There were dramatic differences between the two cultivars tested in terms of their susceptibility to white rot, which will play a role in their overall susceptibility to esca in the long run.

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Table 1. Hymenochaetales isolates from White *et al.* (2011a) used in a pathogenicity study conducted on 10 year old vines between October 2010 and October 2012 in Stellenbosch.

Taxon	Isolate (STE-U number)	Origin	Cultivar isolated from
<i>Fomitiporella viticola</i>	7038	Stellenbosch	Sauvignon blanc
	7141	De Rust	Chenin blanc
	7148	Riebeeck Kasteel	Chenin blanc
Taxon 2 (<i>Fomitiporella</i> sp.)	7147	Oudtshoorn	Hanepoot
	7154	Oudtshoorn	Hanepoot
	7155	Calitzdorp	Pinotage
Taxon 3 (<i>Inocutis</i> sp.)	7109	Constantia	Sauvignon blanc
	7136	Grabouw	Sauvignon blanc
	7174	Ashton	Sauvignon blanc
Taxon 4 (<i>Fomitiporella</i> sp.)	7042	Stellenbosch	Chenin blanc
	7043	Stellenbosch	Chenin blanc
Taxon 5 (<i>Inonotus</i> sp.)	7126	Darling	Chenin blanc
	7143	Tulbagh	Chenin blanc
	7153	Ladismith	Chenin blanc
Taxon 6 (<i>Inonotus</i> sp.)	7133	Malmesbury	Pinotage
	7134	Malmesbury	Pinotage
<i>Inonotus setulosus-croceus</i> .	7090	Stellenbosch	Ruby Cabernet
	7106	Constantia	Sauvignon blanc
	7165	Franschhoek	Chenin blanc
Taxon 8 (<i>Inonotus</i> sp.)	7138	Botrivier	Sauvignon blanc
	7139	Botrivier	Sauvignon blanc
<i>Fomitiporia capensis</i>	7096	Franschhoek	Chenin blanc
	7135	Grabouw	Chardonnay
	7168	Hermanus	Chardonnay
<i>Phellinus resupinatus</i>	7055	Marken	Prime seedless
	7098	Kanon Eiland	Sultana
	7105	Marchand	Sultana
<i>Acremonium strictum</i>	6296		

Table 2. The results of assays testing for manganese peroxidase, lignin peroxidase and laccase activity on Hymenochaetales isolates from White *et al.* (2011a) as well as reference isolates. The study, consisting of three plates per isolate, was repeated and a total of six plates were evaluated per isolate.

Taxon	Isolate (MF or STE-U number) ^a	Manganese peroxidase ^b		Lignin peroxidase ^c	Laccase ^d
		80 ppm	100ppm		
<i>Fomitiporia mediterranea</i> 45/23	MF1	1	1	1	1
<i>Fomitiporia australiensis</i> 22485	MF2	1	1	1	1
<i>Fomitiporia australiensis</i> 22486	MF3	0/1	0/1	1	1
<i>Phellinus alni</i> TW 162	MF4	1	1	0	1
cf. <i>Fomitiporella vitis</i> , "Chile.I"	MF5	1	1	1	1
<i>Fomitiporia polymorpha</i> 91-42/2	MF6	1	1	1	1
<i>Inocutis jamaicensis</i> "ARG 10"	MF7	0/1	0/1	1	1
<i>Phellinus igniarius</i> 83-1022	MF8	0/1	0/1	0	1
<i>Inonotus hispidus</i>	MF9	1	0/1	0	1
<i>Fomitiporella viticola</i>	7038	0/1	1	1	1
	7141	0/1	1	1	1
	7148	1	1	1	1
Taxon 2 (<i>Fomitiporella</i> sp.)	7147	1	1	1	1
	7154	1	1	1	1
	7155	1	1	1	1
Taxon 3 (<i>Inocutis</i> sp.)	7109	0	0/1	0	1
	7136	0/1	0/1	0	1
	7174	1	0/1	0	1
Taxon 4 (<i>Fomitiporella</i> sp.)	7042	0	0	0	1
	7043	0	0	0	1
Taxon 5 (<i>Inonotus</i> sp.)	7126	0	0	0/1	1
	7143	0/1	0/1	1	1
	7153	0/1	1	1	1
Taxon 6 (<i>Inonotus</i> sp.)	7133	0	0	1	1
	7134	1	1	1	1
<i>Inonotus setulosus-croceus</i>	7090	1	1	1	1
	7106	1	1	1	1
	7165	0/1	0/1	1	1
Taxon 8 (<i>Inonotus</i> sp.)	7139	1	1	0/1	1
<i>Fomitiporia capensis</i>	7096	0/1	1	1	1
	7135	1	1	1	1
	7168	1	1	1	1
<i>Phellinus resupinatus</i>	7055	1	1	1	1
	7098	0/1	0/1	1	1
	7105	0/1	0/1	1	1

^aMFreference isolates from the personal collection of Michael Fischer.

^b Manganese peroxidase activity defined by 1=all plates discoloured, 1/0=one to five discoloured, 0=no plates discoloured.

^c Lignin peroxidase activity defined by 1=all plates formed halo, 1/0= one to five plates formed halo, 0=no plates formed halo.

^d Laccase activity defined by 1=all plates turned brown, 1/0=one to five plates turned brown, 0=no plates turned brown.

Table 3. Analysis of variance for the lengths of brown discoloured lesions on Shiraz inoculated with representative Hymenochaetales isolates. Sources of variance are variance between taxa and variance between isolates within taxa.

Source	Degrees of Freedom	Type I SS	Mean Square	F-value	P-value
Taxa	11	1601.83504	145.621367	0.81	0.634
Isolates	17	3244.63066	190.860627	1.06	0.3978

Table 4. Analysis of variance for the lengths of brown discoloured lesions on Mourvèdre inoculated with representative Hymenochaetales isolates. Sources of variance are variance between taxa and variance between isolates within taxa.

Source	Degrees of Freedom	Type I SS	Mean Square	F-value	P-value
Taxa	11	38932.9857	3539.36234	9.05	<.0001
Isolate	17	4698.16321	276.36254	0.71	0.7951

Table 5. Mean lengths of brown discoloured lesions (mm) produced in the cordons of Mourvèdre vines inoculated with representative isolates of Hymenochaetales taxa.

Mean	N	Taxon
65.668 ^a	30	Taxon 3
37.511 ^b	10	Negative control
36.575 ^{bc}	20	Taxon 4
33.552 ^{bc}	30	<i>I. setulosus-croceus</i>
32.646 ^{bc}	30	<i>F. viticola</i>
31.549 ^{bc}	30	Taxon 5
29.346 ^{bcd}	30	<i>F. capensis</i>
28.682 ^{bcd}	18	Taxon 8
28.146 ^{bcd}	30	Taxon 2
26.315 ^{bcd}	30	<i>P. resupinatus</i>
24.465 ^{cd}	20	Taxon 6
18.303 ^d	10	Uninoculated control
LSD (P=0.05) 12.202		

^{a-d} Values within a column followed by the same letter are not significantly different.

Table 6. Analysis of variance for the lengths of white rot on Shiraz inoculated with representative Hymenochaetales isolates. Sources of variance are variance between taxa and variance between isolates within taxa.

Source	Degrees of Freedom	Type I SS	Mean Square	F-value	P-value
Taxa	11	733.714	66.701	2.38	0.0083
Isolates	17	853.732	50.219	1.79	0.03

Table 7. Least squares mean white rot lengths (mm) between different Hymenochaetales taxa inoculated into Shiraz vines.

LSMean	Taxon	Isolate
10.075 ^a	Taxon 6	STE-U7133
7.958 ^{ab}	<i>Inonotus setulosus-croceus</i>	STE-U7090
6.523 ^{abc}	<i>Phellinus resupinatus</i>	STE-U7055
3.77 ^{bcd}	Taxon 3	STE-U7109
3.472 ^{bcd}	<i>Fomitiporella viticola</i>	STE-U7148
3.011 ^{dc}	Taxon 8	STE-U7139
2.714 ^{dc}	Taxon 5	STE-U7143
2.482 ^{dc}	Taxon 5	STE-U7126
2.447 ^{dc}	<i>Phellinus resupinatus</i>	STE-U7105
2.16 ^{dc}	Taxon 5	STE-U7153
2.129 ^{dc}	<i>Phellinus resupinatus</i>	STE-U7098
2.016 ^{dc}	Taxon 6	STE-U7134
1.549 ^d	Taxon 3	STE-U327
1.449 ^d	<i>Inonotus setulosus-croceus</i>	STE-U7090
1.435 ^d	<i>Fomitiporella viticola</i>	STE-U7141
1.43 ^d	Taxon 8	STE-U7138
0.909 ^d	<i>Fomitiporia capensis</i>	STE-U7135
0.774 ^d	Taxon 3	STE-U7136
0.746 ^d	<i>Inonotus setulosus-croceus</i>	STE-U7165
0.701 ^d	<i>Fomitiporella viticola</i>	STE-U7038
0.692 ^d	Taxon 2	STE-U7155
0.606 ^d	Taxon 4	STE-U7042
0 ^d	Uninoculated control	Uninoculated control
0 ^d	<i>Fomitiporia capensis</i>	STE-U7168
0 ^d	<i>Fomitiporia capensis</i>	STE-U7096
0 ^d	Taxon 2	STE-U7154
0 ^d	Taxon 2	STE-U7147
0 ^d	Taxon 4	STE-U7043
0 ^d	Negative control	Negative control

^{a-d} Values within a column followed by the same letter are not significantly different.

Table 8. Analysis of variance for the lengths of white rot on Mourvèdre inoculated with representative Hymenochaetales isolates. Sources of variance are variance between taxa and variance between isolates within taxa.

Source	Degrees of Freedom	Type I SS	Mean Square	F-value	P-value
Taxa	11	33578.647	3052.604	9.86	>.0001
Isolates	17	6915.493	406.794	1.31	0.1838

Table 9. Mean white rot lengths (mm) produced in the cordons of Mourvèdre vines inoculated with representative isolates of Hymenochaetales taxa.

Mean	N	Taxon
40.869 ^a	30	Taxon 3
12.009 ^b	30	Taxon 2
11.069 ^{bc}	30	<i>Fomitiporella viticola</i>
10.138 ^{bc}	29	Taxon 5
7.278 ^{bc}	29	<i>P. resupinatus</i>
7.171 ^{bc}	29	<i>I. setuloso-croceus</i>
6.216 ^{bc}	20	Taxon 4
5.397 ^{bc}	26	<i>F. capensis</i>
3.571 ^{bc}	19	Taxon 6
1.428 ^{bc}	18	Taxon 8
0 ^c	7	Uninoculated control
0 ^c	8	Negative control
LSD ($P=0.05$) 11.563		

^{a-d} Values within a column followed by the same letter are not significantly different.

Table 10. Incidence of Hymenochaetales isolates recovered from inoculated Shiraz and Mourvèdre vines.

Taxon	Isolate	Shiraz^a	Mourvèdre^a
<i>Fomitiporella viticola</i>	7038	2	9
	7141	5	9
	7148	1	8
Taxon 2	7147	2	10
	7154	6	10
	7155	4	8
Taxon 3	7109	2	8
	7136	0	9
	7174	2	7
Taxon 4	7042	1	3
	7043	2	6
Taxon 5	7126	4	8
	7143	4	6
	7153	7	7
Taxon 6	7133	6	5
	7134	5	7
<i>Inonotus setulosus-croceus</i>	7090	5	4
	7106	4	8
	7165	2	6
Taxon 8	7138	0	0
	7139	2	4
<i>Fomitiporia capensis</i>	7135	0	6
	7168	0	6
	7096	0	2
<i>Phellinus resupinatus</i>	7055	5	5
	7105	4	9
	7098	3	10
Total isolates recovered^b		78	180

^a Isolates recovered out of ten inoculated vines.

^b Total isolates recovered are out of 270 inoculated vines.



Figure 1. Inoculation of vines in the field. (a, b.) A pre-selected site is cleared of excess bark and surface sterilised. (c.) A hole is drilled in the cordon with a sterilised drill bit. d. A colonised toothpick is inserted in the hole. (e,f.) The hole is covered with petroleum jelly and wrapped in Parafilm.

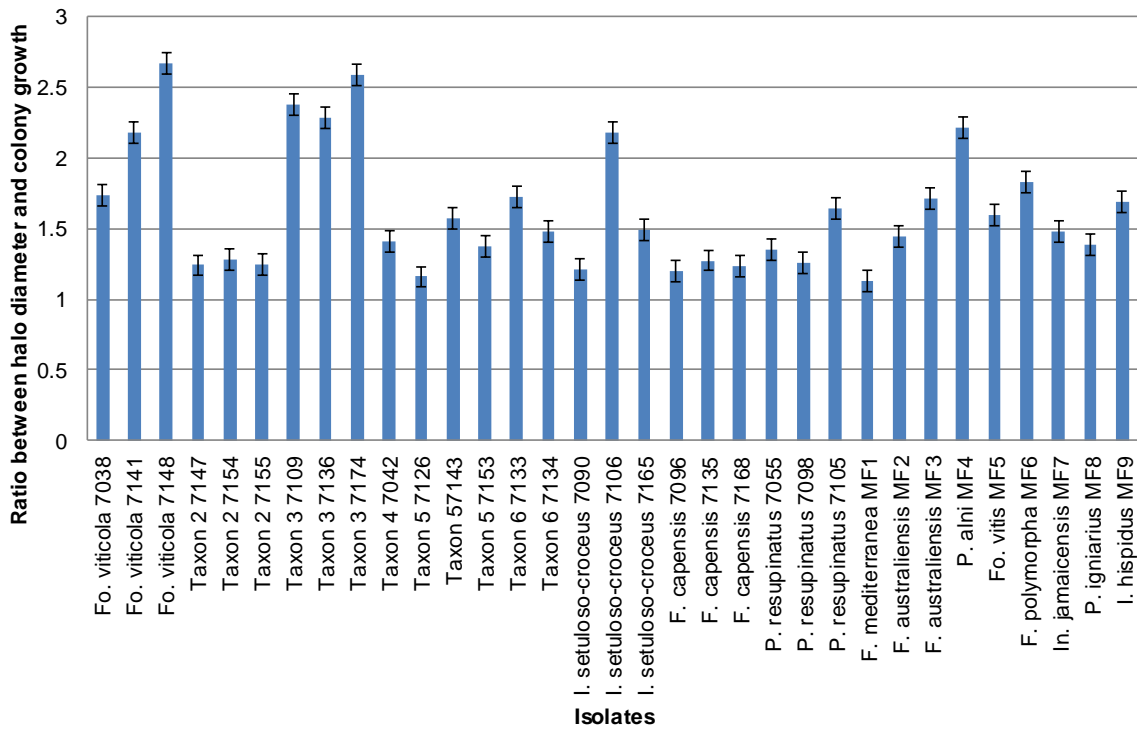


Figure 2. Ratio of halo diameter to colony growth obtained during the cellulase assays on the reference isolates and Hymenochaetales isolates from White *et al.* (2011a). Bars represent the standard error of the mean between repeats within experiment.

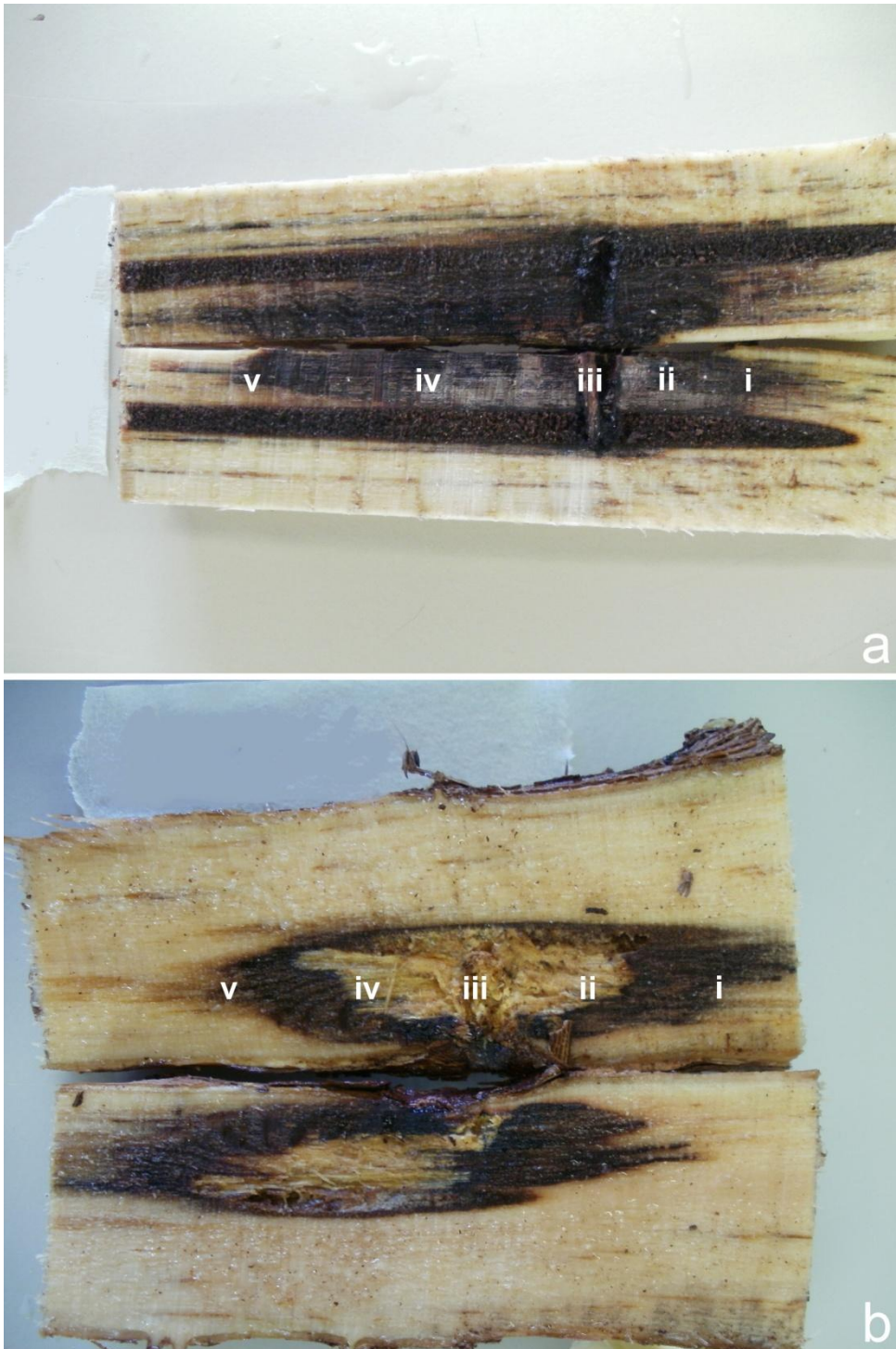


Figure 3. Isolation sites on inoculated grapevine samples. (a.) A discoloured lesion without rot on Shiraz. i. Isolation site on the proximal end of the lesion. ii. Isolation site in the middle of the lesion. iii. Isolation at wound site. iv. Isolation site in the middle of the lesion. v. Isolation site on the distal end of the lesion. b. A discoloured lesion with white rot on Mourvèdre, i-v the same as on Shiraz

CHAPTER 6

Concluding remarks and prospective work

INTRODUCTION

The aims of this study were to characterise the basidiomycetes involved in esca in South African vineyards and to investigate their epidemiology and pathogenicity. This knowledge forms a crucial part of understanding the dynamics of this disease complex under South African conditions. From ten Hymenochaetales taxa associated with the white rot symptom in South Africa, three novel species, *Fomitiporia capensis* M.Fisch. *et al.*, *Phellinus resupinatus* M.Fisch *et al.* and *Fomitiporella viticola* M. Fisch. *et al.* were described. Another species, *Inonotus setuloso-croceus* (Ciel. & Rodw.) P.K. Buchanan & Ryvardeen, was identified from grapevine after the recovery of two fruit bodies from *Salix* L. A spore trapping study on *Fomitiporella viticola* was done over two years, from June until the end of November in 2010 and 2011. An enzyme assay was conducted to examine the types of enzymes secreted by the South African Hymenochaetales taxa and some reference isolates. Finally, the pathogenicity of all ten taxa was investigated in a two year field trial (between September 2010 and November 2012) on mature vines.

NOVEL HYMENOGHAETALES TAXA ON GRAPEVINE IN SOUTH AFRICA

During a previous study on esca in South Africa, White *et al.* (2011) found ten different taxa belonging to the genera *Fomitiporella* Murr., *Fomitiporia* Murr., *Inocutis* Fiasson and Niemelä, *Inonotus* P. Karst. and *Phellinus* Qué. associated with the white rot symptom that forms part of mature esca on grapevines. The taxa were characterised based on ITS sequences from mycelial cultures which were obtained through isolations made from diseased vines. In order for a species to be formally described, a fruit body in reasonable condition must be available. During this study, only three species could be identified initially from fruit bodies found on grapevine. *Fomitiporella viticola* and *Fomitiporia capensis* are the most commonly isolated basidiomycetes on diseased grapevines in South Africa. These species accounted for 44% and 27%, respectively, of isolates in this study. A number of fruit bodies representing these species were found all over the Western Cape. *Phellinus resupinatus* was also relatively easily found in the Northern Cape and Limpopo province. No fruit bodies of other taxa were found on grapevine. This was despite many research trips to areas where the initial isolations were made, as well as other areas heavily affected by esca.

The discrepancy between the amount of white rot found in vineyards and the amount of fruit bodies found is well documented in Italy (Cortesi *et al.*, 2000), Germany (Fischer, 2006), Argentina (Gatica *et al.*, 2004) and Australia (Edwards *et al.*, 2001; Fischer *et al.*, 2005). According to Fischer (2006), a ratio of more or less 100:1 can be expected for vegetative mycelium to fruit bodies in Germany. Fischer (2006) gives the following three possible reasons for the discrepancy between the occurrence of white rot and the occurrence of fruit bodies. Badly rotted grapevines are often removed from the vineyard in accordance with good viticultural practices, possibly before fruit bodies have the opportunity to form. Fruit bodies are difficult to spot and may simply be missed in surveys. Finally, fruit bodies may occur primarily on hosts other than *Vitis vinifera* L. The spread of *Fomitiporia mediterranea* is due to basidiospores from within outcrossing populations (Fischer, 2002; Jamaux-Despreaux and Peros, 2003). This points to the last two possibilities, as fruit bodies must be present in some form in order for basidiospores to be available as an inoculum source.

During this study, grapevines of different ages and on different trellising systems were searched during various seasons. The research team made use of compact mirrors to examine the nooks and crannies underneath cordons and tore off bark on the trunks and cordons to examine the wood underneath. Vineyards from which the novel taxa were isolated from white rot symptoms were surveyed thoroughly for a number of years. It is possible that fruit bodies are present, but still undetected. Since the detection of fruit bodies is difficult, spore trapping could aid in the identification of vineyards with fruiting structures. The total DNA could be isolated from slide washings and taxon specific primers could be used to test for the presence of the spore DNA on the slide (Bester *et al.*, 2014).

HOST RANGE

Fischer (2006) states that white rotting or lignicolous basidiomycetes occupy a wider host range within their centre of distribution, and that lignicolous basidiomycetes are often quite cosmopolitan. The occurrence of *Fomitiporia mediterranea* on *Actinidia* in Greece and Italy (Elena and Paplomatis, 2002; Di Marco *et al.*, 2004), *Citrus* in Greece (Elena *et al.*, 2006) and *Inocutis jamaicensis* on *Eucalyptus* are examples of how esca-related lignicolous basidiomycetes are no exception. The diversity of native and introduced flora in the Western Cape is such that there are countless opportunities for examining potential alternative hosts for the occurrence of fruit bodies still unaccounted for on grapevine. The fruit bodies representing Taxon 7, morphologically identified as *Inonotus setuloso-croceus*, were found in woodpecker holes on *Salix* soon after starting the search for fruit bodies on alternative hosts. Since then, *Fomitiporia capensis* has been found on *Quercus* and *Psidium*, and

Fomitiporella viticola on *Psidium* in the Western Cape (unpublished data). The alternative host hypothesis would seem to be the most promising avenue to find and describe the remaining six taxa.

SPORE TRAPPING AND EPIDEMIOLOGY

Esca, along with the other trunk diseases, is a contributor to the lack of longevity in vineyards worldwide. Little to no information is available regarding the epidemiology of the lignicolous Hymenochaetales that cause the white rot symptom associated with esca. *Fomitiporia mediterranea*, the most well-studied of the esca-related Hymenochaetales, infects via basidiospores that are released continuously for 190 to 250 days a year (Fischer, 2002; Jamaux-Despreaux and Peros, 2003; Fischer, 2009). The long period of sporulation means a high likelihood that spores will find a surface to infect on grapevines or other hosts.

While no direct correlation between sporulation events in *Fomitiporella viticola* and climate factors could be established, spores were available in low levels throughout the trapping period lasting approximately 161 days. Van Niekerk *et al.* (2010) found spore levels and spore events of *Eutypa lata*, *Phomopsis* and Botrosphaeriaceae to be dependent on rain, humidity, wind speed and temperature. Spores were available throughout the trapping period (June to September), suggesting a risk to exposed pruning wounds during this time. Although Van Niekerk *et al.* (2010) didn't trap any *Phaeoconiella chlamydospora* and *Phaeacremonium* spp. in South Africa, Eskalen and Gubler (2001) and Eskalen *et al.* (2004) trapped *Phaeoconiella chlamydospora* and *Phaeoacremonium inflatipes* throughout the year in California. Pruning wounds are generally thought to be the primary portal of entry for grapevine trunk pathogens; although recent research has shown that sucker wounds are also susceptible (Lecomte and Bailey, 2011; Makatini *et al.*, 2014). Under South African conditions, pruning wounds are susceptible to infection for more than three weeks (Van Niekerk *et al.*, 2011) and sucker wounds were susceptible during the four weeks it was assessed (Makatini *et al.*, 2014). The availability of inoculum for all the trunk disease pathogens, including *Fomitiporella viticola*, makes the likelihood of infection during pruning and suckering higher, assuming that pruning wounds are the primary infection portal for the Hymenochaetales taxa.

Aside from basidiospores being the primary infective agent, little is known regarding the infection pathway of the Hymenochaetales on grapevine. Fischer (2009) detected *Fomitiporia mediterranea* on the surface of pruning wounds and adjacent wood of young grapevines using species-specific primers. Hymenochaetales have never been isolated from from eight-month old pruning wounds during several trials in the grapevine trunk disease

group at Stellenbosch (pers. comm. Francois Halleen). Be that as it may, the Hymenochaetales are fairly slow-growing compared to very common pathogens such as the Botryosphaeriaceae, and material should be checked using the primers developed by Bester *et al.* (2014) in order to make further comparisons to Fischer (2009). Further research on this topic should be undertaken, especially with regard to conditions conducive to spore germination and wound susceptibility.

DISEASE MANAGEMENT

Disease management of esca and other trunk pathogens is mainly reliant on pruning wound protection as a way to prevent infection. Wound protection makes use of physical barriers, fungicides or biological agents to prevent infection at the wound site for a period of time. Various chemical agents, mostly benzimidazoles including benomyl, carbendazim and thiophanate-methyl have been shown to be effective against *E. lata* (Sosnowski *et al.*, 2008; Rolshausen *et al.*, 2010), though benomyl has been phased out in most grapevine growing countries and the use of carbendazim is under revision in almost all grapevine growing countries. Carbendazim and thiophanate-methyl are also effective at preventing infection by *Phaemoniella chlamydospora*, *Phaeoacremonium* spp. and *Inocutis* spp. (Diaz and Latorre, 2013). Certain demethylation inhibitors, flusilazole and tebuconazole have also been effective against trunk disease pathogens (Sosnowski *et al.*, 2008; Diaz and Latorre, 2013). Biological control agents including various *Trichoderma* spp. have been tested quite extensively on grapevine to prevent the colonisation of wounds by trunk pathogens. *Trichoderma* spp. were found to reduce pathogen occurrence in wounds by between 10 and 66%, though there was a huge variation in efficacy between cultivars due to interactions between grapevine wounds and the biological control agent (Mutawila *et al.*, 2011). *Trichoderma* species have been tested as a wound treatment against rot fungi. After testing *Trichoderma* species as a protective agent against *Inonotus hispidus* and several *Ganoderma* species on six different tree species, Schubert *et al.* (2008) recorded a significant preventative effect compared to controls.

Viticultural practices such as the removal of infected wood material from the vineyard, a practice known as sanitation, play an important part in the reduction of inoculum pressure in the management of trunk diseases such as esca and *Eutypa dieback* (Di Marco *et al.*, 2000; Sosnowski *et al.*, 2011). The efficacy of sanitation as a strategy to manage lignicolous Hymenochaetales could be reduced by the availability of fruit bodies on a wide range of alternative hosts, making wound protection the more attractive management strategy.

With the exception of carbendazim and thiophanate-methyl and the *Trichoderma*

species in Schubert *et al.* (2008), none of these control agents have been tested on Hymenochaetales. Testing the efficacy of a range of control methods, chemical and biological, against the South African taxa presents more opportunities for future research.

PATHOGENICITY STUDIES AND WHITE ROT FORMATION

Pathogenicity field trials involving lignicolous Hymenochaetales are very rarely undertaken on grapevine or any other host and the aetiology of these organisms is not understood very well. During this study, all ten Hymenochaetales taxa were inoculated on mature, field-grown grapevines. The results showed a vast difference in cultivar susceptibility, as well as differences in virulent species between cultivars. The occurrence of varying degrees of white rot after a relatively short infection period for all inoculated isolates was notable. Sparapano *et al.* (2000) had similar results two years after inoculating *Fomitiporia mediterranea* on 13 year old Sangiovese vines. During inoculations of *Fomitiporia mediterranea* on six and nine year old Italia and Matilde vines in the same study, the first signs of white rot could be detected after as little as six months. The results of that study were taken as an indication that *Fomitiporia mediterranea* could act as a primary pathogen on grapevine. More work needs to be done in order to determine the mechanism and timing of symptom development, as well as studying the variation between isolates and taxa.

One explanation for the variation in symptom development between isolates, taxa and grapevine cultivar, involves the profile of enzymes secreted by the fungi. During the preliminary enzyme assays, there was considerable variation in the type of enzymes secreted. According to Overton *et al.* (2006) and Morgenstern *et al.* (2010), laccase and a form of heme-containing peroxidase, manganese peroxidase or lignin peroxidase, are essential for lignin degradation, a crucial process in the development of white rot. All taxa produced laccase and, with the exception of Taxon 4, all taxa could produce either manganese peroxidase or lignin peroxidase. Further purification and description of these enzymes could provide more answers regarding the inter- and intraspecific variation in virulence and also shed some light on the broader infection process of these fungi.

CONCLUSION

South African vineyards are plagued by an unprecedented diversity of basidiomycetes of the order Hymenochaetales. Out of ten taxa initially identified, four could be described. These were *Fomitiporella viticola*, *Fomitiporia capensis*, *Inonotus setulosus-croceus* and *Phellinus resupinatus*. The publication of *Fomitiporella viticola* and *Phellinus resupinatus* will allow the

formal use of these names. The other six taxa, including two *Fomitiporella* spp., one *Inocutis* sp. and three *Inonotus* spp., remain at large in the field. The fruit bodies will most likely be found on hosts other than grapevine. The South African Hymenochaetales taxa are capable of forming white rot on vines given enough time, and, along with the other grapevine trunk pathogens, represent a clear threat to producers concerned about prolonging the longevity of their vines. Future work needs to include further studies on the host range, infection pathways and methods of control, including pruning wound protection.

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