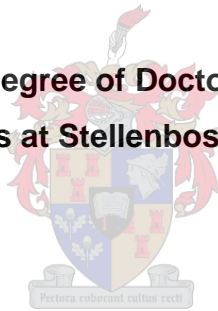


**IDENTIFICATION AND CHARACTERISATION OF DIATRYPACEAE SPECIES  
ASSOCIATED WITH DECLINING GRAPEVINES AND ALTERNATIVE HOSTS IN  
SOUTH AFRICA**

**PROVIDENCE MOYO**

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



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## SUMMARY

Grapevine trunk diseases have devastating impacts on the sustainability of viticulture, worldwide. *Eutypa dieback*, in particular, has caused large economic losses and premature mortality of vines. This disease has, for many years, been associated with the Diatrypaceae fungus, *Eutypa (E.) lata*. Several species of Diatrypaceae were, however, recently discovered to be associated with *Eutypa dieback*-affected grapevines in different grape growing areas including Australia, Chile, Spain and United States of America. No extensive study has been conducted to identify and characterise the species of Diatrypaceae in South Africa.

Surveys were conducted in vineyards located in different grape growing regions of the Western Cape and Diatrypaceae fungi were isolated from grapevines with dying spurs or wood with wedge-shaped necrosis in cross section, as well as from perithecia on dead wood. Isolates were studied using phylogenetic analyses of combined DNA sequences of the internal transcribed spacer regions (ITS1 and ITS2) and 5.8S rRNA gene as well as partial  $\beta$ -tubulin gene. Morphological characteristics of perithecia were also studied. Morphological and phylogenetic analyses revealed the presence of seven Diatrypaceae species to occur on grapevine in South Africa, namely *Cryptovalsa (C.) ampelina*, *C. rabenhorstii*, *E. consobrina*, *E. lata*, *Eutypella (Eu.) citricola*, *Eu. microtheca* and *E. cremea*, which was described as a new species. The most common species isolated from dying spurs, in order of abundance, were *C. ampelina* (46.4% of total number of isolates), *Eu. citricola* (26.8%), *E. lata* (20.1%), *E. cremea* (4.3%), *Eu. microtheca* (1.2%), *E. consobrina* (0.6%) and *C. rabenhorstii* (0.6%). On the other hand, from wedge-shaped necrosis, *E. lata* represented the most frequent species (89.2% of all isolates obtained) followed by *Eu. citricola* (8.5%), *E. cremea* (1.4%) and *C. ampelina* (0.9%). Five species namely, *E. lata*, *C. ampelina*, *E. cremea*, *Eu. citricola* and *Eu. microtheca* were found to produce perithecia on dead grapevine wood. These results suggest that *Eutypa dieback* in South Africa can be associated with several Diatrypaceae species.

Different fruit and ornamental trees occurring near vineyards were investigated to determine whether they are colonised by Diatrypaceae species, which are associated with *Eutypa dieback* of grapevine. Isolates of Diatrypaceae were collected from these trees showing symptoms of dieback, cankers and perithecia. Isolates were analysed by morphological and phylogenetic analyses as described above. Fourteen species namely, *C. ampelina*, *E. consobrina*, *E. lata*, *Eu. citricola*, *Eu. microtheca*, *E. cremea*, *Cryptosphaeria (Cr.) multicontinentalis*, *Cr. ligniota*, *Diatrypella* sp., *Eu. leprosa*, *Eu. australiensis* and three undescribed *Eutypella* species were identified from 29 different fruit and ornamental trees, occurring in close proximity to vineyards. The five most prevalent species were *E. lata*, *C. ampelina*, *E. cremea*, *Eu. citricola* and *Eu. microtheca*, which were also the most prevalent on grapevine. These findings suggest that cross infections are possibly occurring between

grapevine and other woody hosts growing near vineyards in South Africa. These five species were also the only Diatrypaceae species isolated from stone fruit trees. Pathogenicity of these five Diatrypaceae species on stone fruit trees (apricot and plum) was also determined. In these pathogenicity studies, all five species were pathogenic on both apricot and plum, producing brown-red discolouration, typical of *Eutypa* dieback of apricot.

Finally, pathogenicity of Diatrypaceae species identified from grapevine and other woody hosts in South Africa was evaluated on grapevine, under field conditions. Artificial inoculations of these fungal species were conducted on fresh pruning wounds and lignified shoots of Cabernet Sauvignon as well as green shoots of Cabernet Sauvignon and Sauvignon blanc. After 10 months, all the species caused disease symptoms (brown discolouration) on pruning wounds and lignified shoots of Cabernet Sauvignon. Disease symptoms were also observed on green shoots of both cultivars. Pathogenicity results revealed that several species including *C. ampelina*, *Eu. microtheca*, *Eu. leprosa*, and *Eu. citricola* were equally virulent as the well-known pathogen, *E. lata*. Quantitative real-time PCR (qPCR) assays were also developed for the detection and quantification of *E. lata* and *C. ampelina* in grapevine wood. The qPCR assays were specific and successfully quantified target taxa in artificially inoculated wood samples.

The present study provides knowledge on the identity of Diatrypaceae species associated with declining grapevines and other woody hosts occurring adjacent to vineyards in South Africa. This knowledge, together with qPCR assays can be useful in early diagnosis of infection caused by Diatrypaceae species in vineyards. Furthermore, pathogenicity studies have shown that many Diatrypaceae species, including those obtained from other woody hosts, are pathogenic to grapevine. As such, this study forms the platform for further studies aimed at managing Diatrypaceae species causing disease on grapevine in South Africa.

## OPSOMMING

Wingerdstamsiektes het 'n verwoestende impak op die volhoubaarheid van die wynbou industrie wêreldwyd. *Eutypa*-terugsterf, in besonder, veroorsaak groot ekonomiese verliese en vroegtydige terugsterf van wingerde. Hierdie siekte was vir baie jare slegs met die Diatrypaceae swam, *Eutypa (E.) lata*, geassosieer. Verskeie spesies van Diatrypaceae is onlangs ontdek wat verband hou met *Eutypa*-terugsterf geaffekteerde wingerde in verskillende wingerd produksie areas in Australië, Chili, Spanje en die Verenigde State van Amerika. Tot dusvêr is daar nog geen uitgebreide studie gedoen om die spesies van Diatrypaceae in Suid-Afrika te identifiseer en te karakteriseer nie.

Opnames is in wingerde gedoen wat in verskillende wingerdproduksiestreke van die Wes-Kaap geleë is, waartydens Diatrypaceae swamme geïsoleer is vanuit wingerdstokke met sterwende lote of hout met wigvormige nekrose in deursnit, asook van geslagtelike vrugstrukture (perithecia) op dooie hout. Isolate is ondersoek met behulp van filogenetiese analise van gekombineerde DNS volgordes van die interne getranskribeerde spasiëer streke (ITS1 en ITS2), die 5.8S rRNS gene en gedeeltelike  $\beta$ -tubulin gene. Die morfologiese kenmerke van perithecia was ook ondersoek. Die morfologiese en filogenetiese analise het die voorkoms van sewe Diatrypaceae spesies op wingerd in Suid-Afrika bevestig, waaronder *Cryptovalsa (C.) ampelina*, *C. rabenhorstii*, *E. consobrina*, *E. lata*, *Eutypella (Eu.) citricola*, *Eu. microtheca* en *E. cremea*, wat beskryf is as 'n nuwe spesie. Die mees algemene spesie wat geïsoleer is vanuit sterwende lote, in volgorde van voorkoms, was *C. ampelina* (46,4% van die totale aantal isolate), *Eu. citricola* (26,8%), *E. lata* (20,1%), *E. cremea* (4,3%), *Eu. microtheca* (1,2%), *E. consobrina* (0,6%) en *C. rabenhorstii* (0,6%). Daarteen was *E. lata* die mees algemene spesie wat geïsoleer was van wigvormige nekrose (89,2% van alle isolate verkry), gevolg deur *Eu. citricola* (8,5%), *E. cremea* (1,4%) en *C. ampelina* (0,9%). Daar is bevind dat vyf spesies, waaronder *E. lata*, *C. ampelina*, *E. cremea*, *Eu. citricola* en *Eu. microtheca* perithecia op dooie wingerdhout kan produseer. Hierdie resultate dui daarop dat *Eutypa*-terugsterf in Suid-Afrika geassosieer kan word met verskeie Diatrypaceae spesies.

Verskillende vrugte en ornamentele bome wat naby aan wingerde voorkom is ondersoek om vas te stel of dit deur Diatrypaceae spesies gekoloniseer word wat verband hou met *Eutypa*-terugsterf van wingerd. Isolate van Diatrypaceae is ingesamel vanaf hierdie bome wat simptome van terugsterf of kankers of perithecia toon. Die isolate is daarna ontleed deur morfologiese en filogenetiese analise soos wat dit hierbo beskryf is. Veertien spesies, waaronder *C. ampelina*, *E. consobrina*, *E. lata*, *Eu. citricola*, *Eu. microtheca*, *E. cremea*, *Cryptosphaeria (Cr.) multicontinentalis*, *Cr. ligniota*, *Diatrypella* sp., *Eu. leprosa*, *Eu. australiensis* en drie onbeskryfde *Eutypella* spesies, is geïdentifiseer vanaf 29 verskillende vrugte en ornamentele bome wat naby aan wingerde geleë is. Die vyf mees algemene spesie

was *E. lata*, *C. ampelina*, *E. cremea*, *Eu. citricola* en *Eu. microtheca*, wat ook die mees algemene spesie op wingerd was. Hierdie bevindinge dui daarop dat wedersuidse infeksies in Suid-Afrika moontlik plaasvind tussen wingerd en ander houtagtige gashere in die nabyheid daarvan. Hierdie vyf spesies was ook die enigste Diatrypaceae spesies wat geïsoleer was vanuit steenvrugtebome. Die patogenisiteit van hierdie vyf Diatrypaceae spesies is ook bepaal op steenvrugtebome (appelkoos en pruim). Hierdie studie het bevind dat al vyf spesies patogenies is op beide appelkoos en pruim en veroorsaak bruin-rooi verkleuring, wat 'n tipiese simptome van Eutypa-terugsterf op appelkoos is.

Ten slotte is die patogenisiteit van Diatrypaceae spesies, wat geïsoleer is vanaf wingerd en ander houtagtige gashere in Suid-Afrika, geëvalueer op wingerd onder veldkondisies. Hierdie swam spesies is kunsmatig geïnokuleer op vars snoeiwonde en gelygnifiseerde lote van Cabernet Sauvignon, asook groen lote van Cabernet Sauvignon en Sauvignon Blanc. Al die spesies het siekte simptome (bruin verkleuring) op snoei wonde en gelygnifiseerde lote van Cabernet Sauvignon veroorsaak na 10 maande. Siektesimptome is ook waargeneem op groen lote van beide kultivars. Die patogenisiteitsresultate het daarop gedui dat verskeie spesies, insluitend *C. ampelina*, *Eu. microtheca*, *Eu. leprosa* en *Eu. citricola* ewe virulent is as die welbekende patoog, *E. lata*. Kwantitatiewe intyd PKR (kPKR) toetse is ook ontwikkel vir die diagnose en kwantifisering van *E. lata* en *C. ampelina* in wingerdhout. Die kPKR analises was spesifiek en kon die teiken taksa suksesvol kwantifiseer in geïnokuleerde houtmonsters.

Die huidige studie verskaf kennis oor die identiteit van Diatrypaceae spesies in Suid-Afrika, wat verband hou met die terugsterf van wingerde en ander houtagtige gashere in die nabyheid daaraan. Hierdie kennis, tesame met kPKR analises, kan 'n nuttig hulpmiddel wees in die vroeë diagnose van Diatrypaceae spesies in wingerde. Die patogenisiteitstudies het verder getoon dat baie Diatrypaceae spesies, insluitend dié van ander houtagtige gashere, patogenies is op wingerd. Hierdie studie vorm dus die platform vir verdere navorsing wat gemik is op die bestuur van Diatrypaceae spesies wat siekte op wingerd in Suid-Afrika veroorsaak.

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## CHAPTER 1

### **Biology, detection and the role of Diatrypaceae fungi in Eutypa dieback of grapevine**

#### **1.1 INTRODUCTION**

Grapevine production in many regions of the world is constrained by several factors and grapevine dieback and trunk diseases are considered one of the major constraints that lead to poor performance of vines. Among these diseases, *Eutypa* dieback is known to cause large economic losses and premature mortality of vines. A survey conducted on Cabernet Sauvignon vineyards within the Stellenbosch wine region, in South Africa, revealed that more than 32% of vines were infected with *Eutypa* dieback (Halleen *et al.*, 2001) and losses incurred as a result of the disease for the 2000/2001 farming season in the Stellenbosch region in South Africa amounted to approximately R1.7 million (Van Niekerk *et al.*, 2003). In Australia, the disease has been attributed to yield losses of at least 860 and 740 kg/ha in Shiraz and Cabernet Sauvignon vineyards, respectively (Wicks and Davies, 1999) while in California, economic losses of up to US\$ 260 million per year have been reported (Siebert, 2001).

For many years, *Eutypa lata* (Pers.) Tul. & C. Tul (Ascomycota, Diatrypaceae) has been considered to be the sole cause of *Eutypa* dieback on grapevines and hence its biology, epidemiology and management have been extensively studied (Carter and Price, 1974; Moller and Kasimatis, 1978; Trese *et al.*, 1980; Petzoldt *et al.*, 1981; Sosnowski *et al.*, 2011). Recent studies have, however, reported the existence of several Diatrypaceae fungi occurring either alone or in combination with *Eutypa* (*E.*) *lata* in dieback and canker-affected vines (Trouillas and Gubler, 2004; Pitt *et al.*, 2010; Díaz *et al.*, 2011; Trouillas *et al.*, 2010, 2011; Luque *et al.*, 2012; Rolshausen *et al.*, 2014). These findings raised questions as to whether these newly discovered Diatrypaceae fungi are a threat to the sustainability of the grapevine industry and have led researchers to speculate on their role in *Eutypa* dieback and implications in the efforts to manage the disease.

Management of *Eutypa* dieback has been focused on the control of *E. lata* and involves cultural practices such as surgical removal of infected wood and reworking vines (Sosnowski *et al.*, 2011) as well as delayed pruning (Petzoldt *et al.*, 1981; Kasimatis and Vilas, 1985) which coincides with periods of low inoculum pressure (Ramos *et al.*, 1975; Petzoldt *et al.*, 1983). Chemical protection of pruning wounds has been found to be effective in reducing incidence of *E. lata*, however, pruning wounds remain susceptible to infection for several weeks and chemicals do not persist on the wound until it is completely healed (Munkvold and Marois, 1995; Chapuis *et al.*, 1998; Kotze *et al.*, 2011; Van Niekerk *et al.*, 2011). Protection of

wounds with biological agents has been used as an alternative approach to chemical control and has been found to be effective against *E. lata* (Carter and Price, 1974; Ferreira *et al.*, 1991; Munkvold and Marois, 1993b; John *et al.*, 2004). Biological agents have different modes of action which may aid in the delay of the development of resistance to chemicals by pathogens and they persist on pruning wounds thus offering long term protection. Despite the extensive research carried out on Eutypa dieback and different control methods showing great potential in reducing the impact of the disease in vineyards, no eradication solution has been found and the disease still remains an important limiting factor in grapevine production worldwide.

Eutypa dieback develops slowly and usually symptoms are observed 3-8 years post infection (Carter, 1988) and by then, the disease is well developed such that control becomes difficult and hence diagnostic methods which allow monitoring the latent presence of pathogens in plant tissue are warranted. The occurrence of additional Diatrypaceae fungi on Eutypa dieback-affected grapevines further complicates the management of the disease. Traditionally, diagnosis of Eutypa dieback is done through visual inspection of foliar symptoms and plating out infected woody tissue on media and identification of the asexual morph in culture (Lardner *et al.*, 2005). However, species belonging to the Diatrypaceae are difficult to differentiate solely based on morphological characteristics of the asexual morph (Glawe and Rogers, 1984) and hence, rapid, sensitive and specific diagnostic tools are necessary to correctly identify the pathogens. Correct and rapid identification is a fundamental step in executing effective management strategies. In this chapter, the biology, molecular detection, control strategies and possible role of Diatrypaceae species in the development of Eutypa dieback will be discussed.

## **1.2 Biology of Diatrypaceae**

### *1.2.1 Taxonomy*

The family Diatrypaceae is an ascomycetous group which was established in 1869 (Nitschke) and belongs to the order Xylariales. According to the Dictionary of Fungi (Kirk *et al.*, 2008), the Xylariales consists of nine families and more than 229 genera. Members of this order are considered to be endophytic or saprophytic although a number of species are known to be serious plant pathogens (Glawe and Rogers, 1984). The Xylariales are characterised by features such as presence of stromata, perithecial ascomata which can be superficial or embedded in stroma, cylindrical asci which is usually octosporous and pigmented ascospores (Kirk *et al.*, 2008). The family Diatrypaceae is considered as a sister family to Xylariaceae (Glawe and Rogers, 1984). Morphological characteristics used to define the limits of the family Diatrypaceae include perithecia embedded in a stroma which is usually well developed. The

family is also characterised by having long-necked perithecia with sulcate or non-sulcate ostioles and clavate to spindle shaped asci with an apical apparatus consisting of a refractive apical invagination that terminates in a minute apical ring. The long-stalked asci often have a truncate apex and contain allantoid ascospores (Glawe and Rogers, 1984).

Genera within the Diatrypaceae are traditionally recognised on the basis of stromatic characters as proposed by Rappaz (1987) and the family consists of 13 accepted genera (Kirk *et al.*, 2008). Main characters used to distinguish genera within the Diatrypaceae include the degree of stromatal development (well or poorly developed), disposition of perithecia, type of host tissue (bark and/or wood) in which stromata occur and number of ascospores in an ascus (Glawe and Rogers, 1984; Rappaz, 1987). Genera in the Diatrypaceae include *Eutypa* Tul. & C. Tul., *Eutypella* (Nitschke) Sacc., *Echinomyces* Rappaz, *Cryptovalsa* (Ces. & De Not.), *Cryptosphaeria* Ces. & De Not., *Diatrype* Fr. and *Diatrypella* (Ces. & De Not.) De Not. Features used in differentiating species include colour of stromatal surface, ornamentation of perithecial ostioles, configuration of perithecial necks and ascus size (Glawe and Rogers, 1984).

Traditionally, classification of the Diatrypaceae has mainly relied on morphology of the sexual morphs. The conidial states are indistinguishable and are not always produced in culture and hence, cannot be used to distinguish taxa either at genus or species level (Glawe and Rogers, 1984; Pildain *et al.*, 2005; Carmaran *et al.*, 2006). The separation of genera and subsequent identification of taxa within the Diatrypaceae using characters of the sexual morphs is also challenging because much overlap of taxonomic characters exists among genera of the family (Glawe and Rogers, 1984). Development of stromatic characters of the same species may vary as a result of the host or tissue (either bark or wood) they occupy and environment (Carmaran *et al.*, 2006) thus, confident generic or species boundaries cannot be made. Complications in morphological identification of the Diatrypaceae have led many researchers to make use of DNA sequence data from a few gene loci to identify species within this family (Lecomte *et al.*, 2000; Rolshausen *et al.*, 2004; Luque *et al.*, 2006; Catal *et al.*, 2007). Furthermore, studies have also attempted phylogenetic classification to investigate the reliability of morphological characters in identifying Diatrypaceae species. Phylogenetic studies of Diatrypaceae species based on sequences of the internal transcribed spacer regions (ITS), ITS1 and ITS2, (including the 5.8S rRNA gene) was conducted by Acero *et al.* (2004) while Carmaran *et al.* (2006) determined phylogenetic relationships among Diatrypaceae species with octosporous asci. Results of these two studies demonstrated the difficulties and unreliability associated with the use of morphological characteristics in recognising different species within this family.

The production of secondary metabolites has proven useful in distinguishing species in other groups of fungi including Pleosporaceae and Nectriaceae (Mule *et al.*, 1997; Andersen *et al.*, 2001). Apart from studies which focused on metabolites produced by *E. lata* and a few

additional Diatrypaceae species including *Cryptovalsa (C.) ampelina* (Nitschke) Fuckel, *Eutypa lata* var. *aceri* Rappaz, *Eutypa petrakii* var. *petrakii* Rappaz and *Eutypa laevata* (Nitschke) Sacc. (Lardner *et al.*, 2006; Rolshausen *et al.*, 2006), production of secondary metabolites has not been extensively investigated within species in the Diatrypaceae. Species within the Xylariaceae, a sister family of Diatrypaceae, were shown to produce secondary metabolites and this trait was found to be constant and deemed reliable to be used as an additional tool to distinguish between morphologically similar species (Whalley and Edwards, 1995). This approach might therefore, need to be investigated within the Diatrypaceae and possibly be used as an additional taxonomic character for species delimitation in the group. A combination of morphological characters, biochemical and phylogenetic analyses might allow for confident identifications to be made in the Diatrypaceae.

### 1.2.2 Ecology

Species belonging to the Diatrypaceae have been characterised from a wide range of plant hosts and many have been considered to occur as saprophytes or weak parasites, but some species are known to cause extensive damage to natural ecosystems and economically important crops. For example, *Cryptosphaeria (Cr.) populina* (Pers.) Sacc. and *Eutypella (Eu.) parasitica* R.W. Davidson & R.C. Lorenz are pathogens of *Populus tremuloides* Michx. (Hinds, 1981) and *Acer pseudoplatanus* L. (Jurc *et al.*, 2006), respectively. *Eutypa lata* is one of the economically important pathogens among the Diatrypaceae. This fungus causes severe dieback and canker diseases in agricultural crops such as grapevine (*Vitis vinifera* L.) (Moller and Kasimatis, 1978), apricot (*Prunus armeniaca* L.) and European plum (*Prunus domestica* L.) (Carter, 1957). On grapevines, the only problematic Diatrypaceae species has, for many years, been *E. lata*. However, several species within this family have recently been reported to be associated with Eutypa dieback symptoms on grapevines (Pitt *et al.*, 2010; Trouillas *et al.*, 2010, 2011; Luque *et al.*, 2012) and it is thought that the shift in habit from saprophytic to pathogenic maybe a result of the introduction of new hosts (Trouillas and Gubler, 2010).

### 1.2.3 Host range and distribution

Members of Diatrypaceae have been reported to occur on several different hosts which are related or unrelated, worldwide. A few species among the Diatrypaceae are known to be host specific. For example, *Diatrypella betulina* (Peck) Sacc. is restricted to *Betula* spp., *Cr. populina* seems to be exclusive to *Populus* spp. (Glawe & Rogers, 1984) and *Eutypa maura* (Fr.) Sacc. has only been found on *Acer pseudoplatanus* L. (Rappaz, 1987). Other taxa such as *E. lata*, *Eutypa leptoplaca* (Mont.) Rappaz and *C. ampelina* have, however, been reported from numerous host genera (Trouillas *et al.*, 2011). Diatrypaceae species have been isolated from plant families which include Betulaceae, Fabaceae, Fagaceae, Juglandaceae, Moraceae, Oleaceae, Platanaceae, Rosaceae, Rutaceae, Salicaceae, Sapindaceae and

Ulmaceae, (Carter, 1957; Vasilyeva and Stephenson, 2005; Damm *et al.*, 2009; Trouillas *et al.*, 2011; Chacón *et al.*, 2013; Mehrabi *et al.*, 2015). Genera of Diatrypaceae differ on the host tissue in which their stromata occur. Thus, some genera are associated with the bark while some occur in decorticated wood, although some have no apparent preference as they may occur on both host tissues (Glawe and Rogers, 1984).

A number of diatrypaceous fungi are global in their distribution but others seem to be restricted. *Eutypa lata*, *E. leptoplaca* and *C. ampelina* are examples of taxa which are widely distributed and *Diatrypella betulina*, in contrast, is only known from North America (Glawe and Rogers, 1984). Many of the diatrypaceous fungi are only reported to be restricted in their geographical range probably based on the presence of their sexual morphs, but the possibility exists that many can be occurring outside their recognised distribution as endophytes. Several Diatrypaceae species have been described and reported, on grapevine and other woody hosts, from different geographical locations including the eastern United States as well as Arkansas and Texas (Vasilyeva and Stephenson, 2004, 2005, 2006, 2009), California (Trouillas and Gubler, 2004; Trouillas *et al.*, 2010), Australia (Pitt *et al.*, 2010; Trouillas *et al.*, 2011), South Africa (Mostert *et al.*, 2004; Safodien *et al.*, 2005; Damm *et al.*, 2009; Moyo *et al.*, 2016), Chile (Díaz *et al.*, 2011), Spain (Luque *et al.*, 2012), Argentina (Carmaran *et al.*, 2009), Panama (Chacón *et al.*, 2013), Mexico (Paolinelli-Alfonso *et al.*, 2015), Iran (Mehrabi *et al.*, 2015, 2016) and Brazil (Almeida *et al.*, 2016). Diatrypaceae species have been reported from different plant hosts and in the light of emerging new species isolated from grapevine and several hosts within the vicinity of vineyards (Trouillas *et al.*, 2010, 2011), it is possible that many species of Diatrypaceae remain to be discovered.

### **1.3 Detection and identification from symptomatic tissues**

Diagnosis of Eutypa dieback has mainly been by visual observations of foliar symptoms characteristic of the disease. These foliar symptoms often do not appear until several years after infection has occurred (Carter, 1988) and by then, the pathogens would be well established and have spread in most parts of the vine. Furthermore, Eutypa dieback symptoms vary from year to year (Sosnowski *et al.*, 2007) which makes diagnosis on the basis of foliar symptoms inaccurate. Following visual observations in the field, identification of causal organisms involves plating pieces of infected tissue onto growth medium (usually potato dextrose agar) and microscopic examination of cultural growth and morphology. This approach is time consuming, requires taxonomic expertise and hampered by the fact that the causal organisms are slow growing and therefore can be overgrown by fast growing fungi which are usually isolated from symptomatic tissues and thereby result in misdiagnosis (Lardner *et al.*, 2005). In addition, sexual morphs of Diatrypaceae species are not produced

in culture, the hyphae lack diagnostic features and there is inconsistency of conidial production (Glawe and Rogers, 1984; Carter, 1991) and thus absolute identifications cannot be made based on cultures.

The need for diagnostic methods that allow early assessment of plant infection and the difficulties associated with traditional detection and identification methods of Diatrypaceae species have led to the increased focus on techniques that are less time consuming, more sensitive and specific. Techniques that have been used to detect and evaluate the identity, classification and evolutionary relationships between Diatrypaceae fungi include serology (Francki and Carter, 1970; Price, 1973; Gendloff *et al.*, 1983; Octave *et al.*, 2009), fatty acid analysis (Ferreira and Augustyn, 1989), biochemical data (Mahoney *et al.*, 2003; Lardner *et al.*, 2006; Rolshausen *et al.*, 2006) and molecular approaches. Molecular methods have shown potential to be more sensitive, specific and are used more widely in the detection and identification of Diatrypaceae species than fatty acid analysis, biochemical analysis and serological techniques.

Molecular techniques have now become standard approaches in many studies and have paved the way to reliable detection, identification, classification and determination of evolutionary relationships of many fungal groups. Most molecular detection methods are based on polymerase chain reaction (PCR) amplification of DNA regions of interest and choosing a suitable target DNA region, which has little intraspecific sequence variation and sufficient interspecific variation, is a fundamental step in developing a good PCR-based detection method (Cooke *et al.*, 2007). DNA-based techniques in combination with morphological traits have proved to be of great value for characterisation of species as well as for inferring phylogenetic relationships in fungi.

Molecular identification of diatrypaceous species implicated in *Eutypa dieback* has been achieved using phylogenetic analysis, random fragment length polymorphism (RFLP) patterns and conventional PCR based on specific primers. DNA-based markers have been designed to detect and identify Diatrypaceae fungi within infected wood. PCR markers available are specific to *E. lata* (Lemcote *et al.*, 2000; Lardner *et al.*, 2005; Catal *et al.*, 2007), *C. ampelina* (Luque *et al.*, 2006) and nested PCR-assays which are able to detect low levels of DNA in infected plants have also been developed for *E. lata* and *Eutypella vitis* (Schwein.: Fr.) Ellis & Everh. (Catal *et al.*, 2007). Polymerase chain reaction-RFLP markers from the ITS region were used to distinguish *E. lata* from other diatrypaceous species (Rolshausen *et al.*, 2004). DNA phylogenies based on ITS,  $\beta$ -tubulin and RNA polymerase II subunit II genes have been used to determine new diatrypaceous species and evolutionary relationships among species and have proved to be useful (Acero *et al.*, 2004; Trouillas *et al.*, 2011; Rolshausen *et al.*, 2014). Although conventional PCR procedures offer speedy diagnosis in comparison to traditional methods, these procedures have their drawbacks which include the need for post-PCR sample



handling (sequencing for identification of the PCR product) as well as unreliability in quantifying target DNA in samples (Schena *et al.*, 2004; Ward *et al.*, 2004).

Real-time quantitative PCR (also known as qPCR) is an advanced PCR technique which employs the use of non-specific DNA binding dyes (e.g. SYBR Green) and fluorogenic probes which are specific to target DNA (e.g. TaqMan, Molecular beacons and Scorpion PCR) to monitor amplification of amplicons during thermocycling (Schena *et al.*, 2004; Ward *et al.*, 2004; Cooke *et al.*, 2007). This method is becoming the choice tool for identification of plant pathogens because it rapidly provides reliable results, can detect and quantify pathogen DNA in samples simultaneously and no sample handling is required after PCR which saves time, labour and also avoids the risk of contamination (Ward *et al.*, 2004; Cooke *et al.*, 2007). In addition, high specificity and sensitivity are easily achieved by qPCR as compared to classical PCR methods (Gachon *et al.*, 2004).

The use of qPCR to detect and quantify DNA of a number of grapevine trunk pathogens has previously been reported (Overton *et al.*, 2004; Aroca *et al.*, 2008; Martín *et al.*, 2012; Pouzoulet *et al.*, 2013) and several studies have reported on PCR-based identification and detection of Diatrypaceae species on grapevine (Lemcote *et al.*, 2000; Rolshausen *et al.*, 2004; Lardner *et al.*, 2005; Luque *et al.*, 2006; Catal *et al.*, 2007). Although qPCR has been used to study expression profiles of grapevine genes expressed in response to infection by *E. lata* (Camps *et al.*, 2014), the use of qPCR for detection and quantification of different species of Diatrypaceae on grapevine has never been reported previously. Despite no reports on the use of qPCR on the detection of Diatrypaceae species on grapevine or any economically important crop, several important applications for this tool are noteworthy. For instance, the tool can be used to determine the rate or extent of colonisation of grapevine tissue by pathogens as well as to detect the presence of pathogens in symptomless grapevine tissues.

#### **1.4 Eutypa dieback of grapevines and the role of Diatrypaceae species in disease development**

Eutypa dieback is problematic in all areas of the world where grapevines are cultivated. This disease develops slowly and as a result, its symptoms do not usually appear until grapevines are over 8 years old (Carter, 1988). Symptoms of the disease are commonly observed after bud break in early spring. Generally, vines affected with Eutypa dieback exhibit symptoms such as poorly developed clusters; a dark and wedge-shaped necrosis of wood; dieback of spurs, canes and portions of the cordon as well as tattering of leaves; stunted shoots with shortened internodes, cupped leaves and yellowing leaves as well as cankers which usually start at the pruning wound and visible when the bark is removed (Moller and Kasimatis, 1978; Mauro *et al.*, 1988). These symptoms were collectively known as 'dead arm disease' and

initially associated with *Phomopsis (P.) viticola* (Sacc.) Sacc. (Coleman, 1928). Several studies around the world, however, found an association of *E. lata* with such symptoms in grapevines (Dye and Carter, 1976; Kouyeas *et al.*, 1976; Moller *et al.*, 1977; Moller and Kasimatis, 1978; 1981) and this resulted in the role of *P. viticola* in this disease being reconsidered.

*Eutypa lata* is an important plant pathogen with a wide geographical distribution and infects a large number of agricultural crops (Carter, 1991). This pathogen was first reported as a vascular pathogen of apricots (Carter, 1957) and only considered as a saprophyte on grapevines by Carter (1960) but was later associated with vascular diseases of grapevines (Moller *et al.*, 1974; Moller and Kasimatis, 1975). The proof that *E. lata* was responsible for dieback of grapevines was provided by Moller and Kasimatis (1978) who completed Koch's postulates. Since then Eutypa dieback has been attributed to *E. lata*. The fungus infects pruning wounds and colonise the vascular system, produces cell wall degrading enzymes and phytotoxins (Tey-Rulh *et al.*, 1991; Rudelle *et al.*, 2005, Rolshausen *et al.*, 2008) which then result in soft rot of the vascular system and foliar symptoms, respectively (English and Davis, 1978; Moller and Kasimatis, 1978; Carter, 1991; Munkvold and Marois, 1995). As a result of the destruction that *E. lata* caused in vines and the notion that this fungus was the principal causative agent of the disease, its biology has been studied extensively by plant pathologists around the world.

As with any infectious disease, a chain of events must happen usually one after another for the disease to develop and spread. These series of events include the ability of the pathogen to adhere, penetrate, colonise, grow, reproduce, disperse and survive within host tissue. The disease cycle of Eutypa dieback takes a long time to become complete. Infection is initiated when ascospores are released into the air, usually in winter and early spring, from mature perithecia embedded in a stroma which is formed on dead wood of the host (Moller and Carter, 1965). Rainfall events of at least 1-2 mm or an equivalent in overhead irrigation or snowmelt is required for the ascospores to be released (Ramos *et al.*, 1975; Pearson, 1980; Trese *et al.*, 1980; Van Niekerk *et al.*, 2010) after which they land and adhere onto fresh pruning wounds where they are washed into exposed vessels and germinate. Once the stroma is wet, ascospore release continues for as long as the perithecia remain wet. The hyphae of *E. lata* penetrate the cells either directly or via pits (English and Davis, 1978). In response to invasion, the cells surrounding infected vessels produce phytoalexins and form gums and tyloses in an effort to limit the spread of the pathogen (Rudelle *et al.*, 2005). The pathogen colonises and grows within vessels by producing enzymes which leads to wood degradation and soft rot (English and Davis, 1978). The fungus also produces secondary metabolites, especially acetylenic compounds including eutypine, eulatachromene and eulatinol, which have been shown to be phytotoxic (Mahoney *et al.*, 2003; Lardner *et al.*, 2006). Although

ascospore germination occurs quickly, proliferation of the mycelium occurs slowly such that symptoms of the disease are not apparent until about 3-8 years after infection (Carter, 1988). If the infected parts of the host are not removed, the affected arm or trunk eventually dies which allows the fungus to produce stromata which bears perithecia in which ascospores are produced.

The number of diatrypaceous fungi isolated from grapevines showing Eutypa dieback symptoms has, however, increased in the recent years. At least 17 additional diatrypaceous fungi have recently been isolated from necrotic grapevine wood tissue and fruiting bodies on surfaces of grapevine wood worldwide. These species are: *Eutypa leptoplaca* (Mont.) Rappaz, *Eutypa* sp., *E. laevata*, *Cryptosphaeria pullmanensis* Glawe, *C. ampelina*, *Cryptovalsa rabenhorstii* (Nitschke) Sacc., *Diatrype* sp., *Diatrype oregonensis* (Wehm.) Rappaz, *Diatrype stigma* (Hoffm.), *Diatrype whitmanensis* J.D. Rogers & Glawe, *Diatrypella vulgaris* Trouillas, W.M. Pitt & Gubler, *Diatrypella verrucaeformis* (Ehrh.) Nitschke, *Eu. vitis*, *Eutypella leprosa* (Pers. ex Fr.) Berl., *Eutypella citricola* Speg., *Eutypella microtheca* Trouillas, W.M. Pitt & Gubler and *Eutypella scoparia* (Schwein.: Fr.) Ellis & Everh. (Mostert *et al.*, 2004; Trouillas and Gubler, 2004; Rolshausen *et al.*, 2006; Catal *et al.*, 2007; Trouillas *et al.*, 2010; Díaz *et al.*, 2011; Trouillas *et al.*, 2011; Rolshausen *et al.*, 2014; Paolinelli-Alfonso *et al.*, 2015). Studies have found that the same species collected from grapevine also occurred on other hosts often found adjacent to vineyards (Trouillas *et al.*, 2010, 2011) and isolates of species found on other hosts were pathogenic to grapevine which shows that inoculum from neighbouring hosts plays an important role in disease development (Trouillas and Gubler, 2010).

The identification of several Diatrypaceae species on grapevine exhibiting Eutypa dieback has cast doubt on *E. lata* being the sole causal agent of the disease. Pathogenicity of the additional Diatrypaceae fungi on grapevines has been investigated in Australia (Pitt *et al.*, 2013), California (Trouillas and Gubler, 2010; Rolshausen *et al.*, 2014), South Africa (Mostert *et al.*, 2004), Chile (Díaz *et al.*, 2011) and Spain (Luque *et al.*, 2006) and results have shown these species to be pathogenic on grapevines with some producing lesions and vascular discolourations similar to those produced by *E. lata* (Trouillas and Gubler, 2010, Pitt *et al.*, 2013). Surveys showed that some of the newly reported species namely *C. ampelina*, *Eutypella* spp., *Diatrypella* spp. and *Eutypa* spp. were even more widespread and abundant than *E. lata* (Pitt *et al.*, 2010; Rolshausen *et al.*, 2014). These reports suggest that the Eutypa dieback symptoms, originally thought to be caused primarily by *E. lata*, could also be caused by other diatrypaceous fungi harboured by grapevines or even by interactions of the species. Whether these additional species are capable of producing perennial grapevine cankers, foliar symptoms as well as secondary metabolites similar to those produced by *E. lata*, however, still needs to be determined.

Although little is known about the biodegradation potential of the majority of diatrypaceous fungi, a few species have been shown to have the ability to produce cellulolytic enzymes (Pildain *et al.*, 2005; Rolshausen *et al.*, 2008), moderately degrade lignin and hence, can be considered to be soft rot fungi (Worrall *et al.*, 1997). The biology of these additional diatrypaceous species needs to be studied and their role in Eutypa dieback fully established. Nevertheless, studies have found that several of these species produce stromata on old grapevine wood (Trouillas and Gubler, 2004; Mostert *et al.*, 2004; Pitt *et al.*, 2010; Trouillas *et al.*, 2010, 2011). These findings suggest that the species are capable of completing their life cycle on grapevine and probably invade the host via pruning wounds, colonise and grow within the vascular tissues before killing the wood and forming stromata with perithecia (Trouillas and Gubler, 2004).

### **1.5 Control strategies for Eutypa dieback on grapevines**

Eutypa dieback has been widely studied, however, no eradicated measures have been found and with the disease still being a major limiting factor in most grapevine producing areas, several approaches have been developed to reduce the impact of the disease. Prevention of the introduction of causal pathogens into the vineyard is the most reliable control strategy. Eutypa dieback pathogens infect vines through wounds, and pruning wounds made during winter are the primary entry point (Moller and Kasimatis, 1980). To reduce the incidence of Eutypa dieback, pruning wounds are protected by application of fungicides, wound sealants and biological control agents as well as manipulating the time of pruning. Although studies have shown that several diatrypaceous species, other than *E. lata*, may be involved in the development of the disease, most control strategies available have been developed mainly to control *E. lata* and these studies only focused on the pruning wounds of one-year-old canes. Pruning wound protection on wounds, other than the one-year-old canes, such as those created during the removal of old wood and retaining fruiting spurs for the current season crop has not been studied. Chemical control strategies are often preferred to limit disease losses, but these do not provide long term protection to pruning wounds while biological and cultural control strategies are partially effective, but they also have their shortfalls. Effective control can be achieved by combining these various strategies in an integrated management to prevent or reduce pathogen infections and disease.

#### **1.5.1 Chemical control**

Efforts to manage Eutypa dieback via chemical means involve the application of fungicides on pruning wounds after pruning. Chemical products such as benomyl, carbendazim, flusilazole have been tested *in vitro* and *in vivo* for their ability to reduce infection by *E. lata* and were reported to be effective against this grapevine dieback pathogen (Munkvold and Marois,

1993a, Sosnowski *et al.*, 2008; Halleen *et al.*, 2010). However, reports of ineffective control of chemicals such as imazilil sulphate in vineyards exist (Sosnowski *et al.*, 2008) and negative effects of some chemicals have also been reported. For example, although effective control of *E. lata* was achieved by use of boron, this product was found to cause bud failure at the first internode below the treated wound in California (Rolshausen and Gubler, 2005). The use of mixtures of physical barriers, such as acrylic paints and fungicides have also been shown to reduce infection of 1-year old canes against *E. lata* (Sosnowski *et al.*, 2008). Acrylic paint acts as barrier to prevent spores from entering the exposed xylem vessels while the fungicide prevents the germination of spores which enter the xylem vessels through cracks developing as a result of the contracting paint as it dries or it prevents colonisation of the wound by spores which could have landed before the mixture was applied on the wound. However, the effectiveness of such mixtures in the long run when applied to larger wounds, for instance those made on cordons and trunks, is unknown (Sosnowski *et al.*, 2008). There have been no reports of chemical management of other diatrypaceous fungi, other than *E. lata*, in the field but fungicides including carbendazim, flusilazole, pyrimethanil and tebuconazole have been tested *in vitro* for their ability to inhibit mycelial growth of a few diatrypaceous fungi found to be pathogenic to grapevines and all were found to be effective except for pyrimethanil (Gramaje *et al.*, 2012).

Although fungicides application provides immediate protection to pruning wounds, the efficacy of fungicides decreases with time (Munkvold and Marois, 1993b; Kotze *et al.*, 2011) and thus, they do not provide long term protection of grapevine pruning wounds since these remain susceptible to trunk pathogens for more than 4 weeks (Van Niekerk *et al.*, 2011). Furthermore, if it rains immediately after application of fungicides, the chemical residues may be washed off from pruning wounds (Munkvold and Marois, 1993a), rendering the fungicides ineffective.

### 1.5.2 Biological control

Biological control of plant diseases makes use of microorganisms with the ability to suppress plant pathogens and its importance cannot be contested especially in response to increased environmental awareness and deregistration of effective fungicides. Biological agents are capable of colonising grapevine wounds and persisting in the wood subsequently offering long term protection. Evidence of such protection is reported by Carter and Price (1974) and Hunt *et al.* (2001) who were able to isolate *Fusarium lateritium* Nees: Fr. and *Trichoderma* species from pruning wounds 15 weeks and 8 months after inoculation, respectively. Other organisms that have been shown to inhibit infection by *E. lata* include *Cladosporium herbarum* (Pers.: Fr.) Link (Munkvold and Marois, 1993b), *Bacillus subtilis* (Ehrenberg) Cohn. (Ferreira *et al.*, 1991) and *Erwinia herbicola* (Löhnis) Dye (Schmidt *et al.*, 2001). *Trichoderma* (*T.*) species, in

different formulations, have shown great potential to protect grapevine pruning wounds from *E. lata* and other trunk disease pathogens. Studies by John *et al.* (2005) and Kotze *et al.* (2011) found a spore suspension of *T. harzanium* and *Trichoderma*-based products, Vinevax and Eco77, to greatly reduce *E. lata* infection of fresh pruning wounds in the field, respectively. For efficient control, biological agents need approximately 1-2 weeks to colonise the wounds and improve their competitiveness before being challenged with pathogens. This, however, creates a window of susceptibility to infection by pathogens (Carter and Price, 1975) and varying environmental conditions in different geographical regions may also influence their performance (Stabb *et al.*, 1994; Bull *et al.*, 1997).

### 1.5.3 Cultural practices

Complete control of Eutypa dieback is impossible once the disease is established in a vine and cultural practices are warranted in reducing pathogen inoculum in vineyards. A number of cultural practices to reduce the impacts of the disease in vineyards have been documented. The most reliable method to control the disease is through surgical removal of diseased wood and reworking the vine (Sosnowski *et al.*, 2011). The disease, however, develops slowly and symptoms are usually visible between 3 and 8 years after infection (Carter, 1988) and by this time the pathogen would have colonised the woody tissue and therefore, removal of infected wood becomes a costly procedure. Recommendations have been made to encourage farmers to prune grapevines late in winter when temperatures have risen because wounds heal faster and colonisation of wounds by pathogen antagonists is greater at higher temperatures compared to low temperatures (Munkvold and Marois, 1995). Spore trapping results of a trial conducted in the Western Cape Province of South Africa, however, have shown that ascospores of *E. lata* are available in vineyards from winter up until spring (Van Niekerk *et al.*, 2010). An additional study conducted in the Stellenbosch wine region of South Africa, also highlighted that pruning grapevines late in winter could result in higher wound infection by trunk disease pathogens (Mutawila *et al.*, 2016). A common result reported by different studies is that spores of *E. lata* are often released during or after rainfall events (Ramos *et al.*, 1975; Pearson, 1980; Trese *et al.*, 1980; Carter, 1991; Van Niekerk *et al.*, 2010) and hence, pruning during wet weather is not advised.

## 1.6 Conclusion

From the above review, it is evident that grapevines exhibiting Eutypa dieback symptoms are colonised by several Diatrypaceae species and other woody hosts could act as inoculum reservoirs to adjacent vineyards. In South Africa, only four Diatrypaceae species namely *E. lata*, *C. ampelina*, an undescribed *Eutypa* sp. and a *Eutypella* species (Mostert *et al.*, 2004; Safodien *et al.*, 2005) have been reported on grapevine. No extensive surveys for perithecia

of Diatrypaceae species have been carried out in South Africa and in recent years, problems of spur dieback have been reported in vineyards in the Western Cape Province of South Africa. The extent of the diversity of Diatrypaceae in South African vineyards is largely unknown and therefore, how the diversity of this group of fungi compares with those reported in other studies around the world will be of great interest. Results of pathogenicity tests of *C. ampelina* showed that this species was a weak pathogen of grapevine (Mostert *et al.*, 2004). The pathogenicity tests were, however, conducted on one-year-old rooted grapevines under glasshouse conditions and thus, the performance and that of potentially new Diatrypaceae species under field conditions is unknown in South Africa and thus needs to be investigated. Differentiation of Diatrypaceae species based on conidial characteristics is difficult because these are indistinguishable (Glawe and Rogers, 1984) and the presence of more than one species of Diatrypaceae in infected grapevine tissue warrants a precise and accurate tool to rapidly detect and identify the respective fungi. The biology and epidemiology of Diatrypaceae species in South Africa need to be understood which is important in the development and optimisation of new management strategies against Eutypa dieback of grapevines.

### **1.7 Aim of the study**

The overall aim of the study was to identify and characterise the different species of Diatrypaceae that may be involved in Eutypa dieback in South Africa. More specifically, the objectives were to:

- Identify and characterise Diatrypaceae species associated with spur dieback and wedge-shaped necrosis characteristic of Eutypa dieback, in declining grapevines.
- Identify Diatrypaceae species occurring on other woody hosts often found in close proximity to vineyards as well as determine pathogenicity of Diatrypaceae species on stone fruits which, besides grapevines, are one of the most economically important fruit crops in South Africa.
- Conduct pathogenicity tests of Diatrypaceae species on field grown grapevines and evaluate qPCR protocols for the detection and quantification of specific Diatrypaceae species on grapevine wood.

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## CHAPTER 2

### Diversity of Diatrypaceae species associated with dieback of grapevines in South Africa, with the description of *Eutypa cremea* sp. nov

#### 2.1 ABSTRACT

Species in the Diatrypaceae are well-known saprobes and pathogens of woody hosts. Recent studies in Australia, California and Spain have revealed an extensive diversity of Diatrypaceae species on grapevines affected by *Eutypa* dieback. However, little is known regarding the diversity of these species in South African vineyards. The aim of this study was therefore, to identify and characterise Diatrypaceae species associated with dieback symptoms of grapevine in South Africa. Isolates were collected from dying spurs of vineyards aged four- to eight-years-old, grapevine wood showing wedge-shaped necrosis, in cross section, as well as from perithecia on dead wood. The collected isolates were identified by phylogenetic analyses based on the internal transcribed spacer regions (ITS1 and ITS2) and 5.8S rRNA gene as well as partial  $\beta$ -tubulin gene. Isolates from perithecia were characterised based on their morphology as well as sequence data based on ITS and  $\beta$ -tubulin genes. Seven Diatrypaceae species were identified on grapevine, namely *Cryptovalsa* (*C.*) *ampelina*, *C. rabenhorstii*, *Eutypa* (*E.*) *consobrina*, *E. lata*, *Eutypella* (*Eu.*) *citricola*, *Eu. microtheca* and *Eutypa cremea* sp. nov. The dying spurs yielded the highest diversity of species when compared with the wedge-shaped necrosis and/or perithecia. *Cryptovalsa ampelina* was the most dominant species in the dying spurs followed by *Eu. citricola* whereas *E. lata* was the dominant species isolated from the wedge-shaped necrosis and perithecia. These results suggest that *C. ampelina* is a significant cause of spur dieback and *E. lata* is still an important grapevine canker pathogen in South Africa. However, in some cases, more than one species was isolated from a single symptom which suggests that synergistic interactions may be occurring leading to decline of grapevines. *Cryptovalsa rabenhorstii*, *E. consobrina*, *Eu. citricola*, *Eu. microtheca* and *E. cremea* are reported for the first time on grapevine in South Africa.



## 2.2 INTRODUCTION

An increase in the number of Diatrypaceae species isolated from grapevines affected by *Eutypa dieback* has recently been reported from different grape-growing countries worldwide (Trouillas and Gubler, 2004; Pitt *et al.*, 2010; Trouillas *et al.*, 2010, 2011; Díaz *et al.*, 2011; Luque *et al.*, 2012; Rolshausen *et al.*, 2014; Paolinelli-Alfonso *et al.*, 2015). *Eutypa dieback*-affected grapevines exhibit symptoms such as weak and stunted shoots with shortened internodes. The leaves are small, chlorotic, usually cupped and may become tattered and scorched later in the season. Clusters also become poorly developed. The formation of cankers, which are visible when the bark is removed and usually start at the pruning wound, is an important diagnostic symptom of *Eutypa dieback*-affected grapevines. A cross section of the canker reveals a dark and wedge-shaped necrosis of wood tissues (Moller and Kasimatis, 1978; Mauro *et al.*, 1988).

The failure of spurs to bud in spring is usually the first sign of *Eutypa dieback* infection. Symptoms of this disease appear initially in one or two spurs. Spurs retained from canes of *Eutypa dieback*-diseased vines usually do not bud in spring (Moller and Kasimatis, 1975) and with time, the disease spreads to adjacent spurs until the affected part can no longer produce shoots and dies (Moller and Kasimatis, 1975). *Eutypa dieback* is known to develop slowly and its symptoms are generally not found on grapevines younger than 5 years (Moller *et al.*, 1980) and do not usually appear until grapevines are over 8-years-old (Carter, 1988).

For many decades, *Eutypa dieback* has been attributed to the Diatrypaceae species, *Eutypa (E.) lata* (Pers.) Tul. & C. Tul. (Moller and Kasimatis, 1978; Ferreira, 1987; Carter, 1988). Grapevines become infected when ascospores of *E. lata* enter fresh wounds, resulting from pruning or mechanical damage. Ascospores which are the primary form of inoculum of *E. lata*, are produced from mature perithecia occurring within stroma found on dead wood, during rainfall periods. The ascospores germinate in xylem vessels and the fungus subsequently colonises the wood (Moller and Carter, 1965; Moller and Kasimatis, 1978). Recent studies have, however, reported the existence of several Diatrypaceae fungi on grapevines with symptoms of *Eutypa dieback* (Schilder *et al.*, 2003; Trouillas and Gubler, 2004; Pitt *et al.*, 2010; Trouillas *et al.*, 2010, 2011; Luque *et al.*, 2012; Rolshausen *et al.*, 2014). Studies have even reported that several of the newly reported species occurred more abundantly and were more widely distributed geographically compared to *E. lata*, which was originally thought to be the most abundant and widely distributed species (Pitt *et al.*, 2010; Trouillas *et al.*, 2011; Rolshausen *et al.*, 2014). These findings raised questions as to whether these newly discovered Diatrypaceae fungi are a threat to the sustainability of the grapevine industry and have led researchers to speculate on their role in *Eutypa dieback* and implications in the efforts to manage the disease. Results of pathogenicity trials of several of these

Diatrypaceae taxa on grapevine have further led researchers to postulate that *E. lata* is not solely responsible for symptoms associated with Eutypa dieback, but several species of Diatrypaceae are also involved in the development of this disease (Jordan and Schilder, 2005; Trouillas and Gubler, 2010; Pitt *et al.*, 2013; Rolshausen *et al.*, 2014).

Similar to other grape-growing countries, Eutypa dieback is considered the most important trunk disease in South Africa because of the severe damage and losses incurred as a result of the disease (Van Niekerk *et al.*, 2003). Recently poor budburst and spur dieback in Sauvignon blanc and Cabernet Sauvignon vineyards, including those as young as 4-years-old, in the Western Cape Province of South Africa have been observed. Spurs of the affected vines either produce weak shoots which die soon after bud break or the spurs do not produce new shoots at all in spring. These observations are disturbing considering young vineyards are also affected. The question which arises though concerns the extent to which the Diatrypaceae are involved in the phenomenon.

Although studies in other countries have provided evidence of the occurrence and pathogenicity of several diatrypaceous fungi on grapevine, the information presently available about the occurrence and identity of the species of Diatrypaceae associated with dieback of grapevine in South Africa is still limited. In fact, only four Diatrypaceae species have been isolated from grapevine in South Africa namely *E. lata*, *Cryptovalsa (C.) ampelina* (Nitschke) Fuckel, an unidentified *Eutypa* sp. and a *Eutypella* species (Ferreira, 1987; Mostert *et al.*, 2004; Safodien *et al.*, 2005). In the light of many Diatrypaceae species being discovered on Eutypa dieback-affected grapevines in different countries, there is a possibility that a large diversity of these species remains to be discovered in South African vineyards.

The overall aim of this study was therefore to identify Diatrypaceae species associated with dieback of grapevines. The first objective of this study was to determine whether Diatrypaceae species are associated with dying spurs in Cabernet Sauvignon and Sauvignon blanc vineyards severely affected by poor budburst and spur dieback. The second objective of the study was to identify Diatrypaceae species associated with the wedge-shaped necrosis on numerous wine grape cultivars in the Western Cape Province of South Africa. The third objective was to carry out surveys for perithecia of species of Diatrypaceae on grapevine in South Africa.

## **2.3 MATERIALS AND METHODS**

### **2.3.1 Sampling and isolate collection**

Four- to eight-year-old Sauvignon blanc and Cabernet Sauvignon vineyards were sampled in 2013 and 2014 to determine whether Diatrypaceae species are associated with dying spurs. Sampling was carried out in different locations in the Western Cape Province to represent

areas with different environmental conditions, namely Stellenbosch, Grabouw, Constantia, Durbanville and Robertson. Sampling was conducted in 19 Sauvignon blanc and 17 Cabernet Sauvignon vineyards. Ten dying spurs were collected from each vineyard, individually placed in bags and taken to the laboratory for processing. In the laboratory, spurs were split open through the pruning wounds to reveal internal symptoms and were then surface sterilised by submerging into 70% ethanol for 30s, 2.5% sodium hypochlorite for 2 min and again in 70% ethanol for another 30s. Subsequently, wood pieces were taken from different necrotic areas as well as from the border between live and dead tissue at the pruning wound and plated onto potato dextrose agar (PDA-C, Biolab, Midrand, South Africa) plates containing chloromycetin (250 mg/L). Plates were incubated at 24°C, exposed to approximately 12 hours of day light and 12 hours of darkness, and inspected daily for fungal growth for 4 weeks. Isolates of Diatrypaceae species were transferred onto new PDA-C for further identification.

To identify Diatrypaceae species associated with wedge-shaped necrosis (V-shaped necrosis), vineyards were selected on the basis of the presence of dieback symptoms which include dead spurs, stunted shoots, shortened internodes, dwarfed leaves with tattered margins and cankers. Cordons and trunks of such vines were cut in cross section and wood showing the wedge-shaped necrosis was then taken to the laboratory for isolations. Samples were collected from ten cultivars namely Cabernet Sauvignon, Chardonnay, Chenin blanc, Merlot, Pinot noir, Pinotage, Sauvignon blanc, Semillon, Shiraz and Viogner. Sampling was done in different locations in the Western Cape Province including Stellenbosch, Sommerset West, Constantia, Durbanville, Grabouw, Robertson, Barrydale, Paarl and Hermanus. Isolations were carried out by cutting small wood pieces from the border between live and dead tissue, of the wedge-shaped necrosis and plating onto PDA-C as described above.

Dead grapevine wood with black, charcoal-like stroma with fruiting bodies resembling those of *E. lata*, were collected from different areas mentioned above. Depending on availability of diseased wood, one to ten samples per vineyard block were randomly collected from the different sampling areas. Isolations from collected samples were achieved according to Trouillas *et al.* (2010). The stromata were sliced open, under a Nikon SMZ 1500 stereo microscope, using a scalpel to reveal perithecial contents. A drop of sterile water was then added on the opened perithecia. Perithecial contents were then collected with a needle, mounted in water on a microscopic slide and examined under a light microscope (Nikon Y-TV55), for morphological identification. Thereafter, 50 µl of sterile water was mixed with the contents of the slides by pipetting. This mixture was then transferred onto water agar in Petri dishes. Single spores were transferred to PDA-C plates within 48 hours and the plates were incubated at 24°C for 15-21 days. Thereafter, fungal identification was carried out.

## 2.3.2 Identification of Diatrypaceae isolates

### 2.3.2.1 Morphological characterisation of sexual morphs

This technique was used to characterize isolates of species found to produce fruiting bodies on grapevine wood. Perithecial contents were collected as described above and mounted in 10% lactic acid, examined microscopically and images were captured with a Nikon DS-Ri2 camera on a Nikon Y-TV55 light microscope at 1000x magnification. Images of stromata and measurements of perithecia diameter were captured using the Nikon DS-Ri2 camera on a Nikon SMZ 1500 stereo microscope. Measurements were made for representative samples of each species (either two or three samples of each species), 5% and 95% confidence intervals were calculated from the measurements of 50 ascospores, 20 asci (length=spore bearing part and width=width at the widest point) and 10 perithecia (diameter). Characteristics such as morphology of perithecia, asci and ascospores; number of ascospores per ascus as well as colour of ascospores were noted for each species as described by Rappaz (1987), Glawe and Rogers (1984) and Trouillas *et al.* (2011). Herbarium specimens of *Sphaeria consobrina* Durieu & Mont. (MC 5626 and MC 5627) and *Sphaeria leptoplaca* Durieu & Mont. (MC 8635 and MC 8636) from the Museum National d'Histoire Naturelle in France were also studied. Furthermore, wood from other woody hosts occurring in close proximity to vineyards were also examined.

### 2.3.2.2 Morphological and cultural characterisation of asexual morphs

For characterization of the asexual states, one or two isolates were characterized per species (depending on sporulation of the isolate). Three replications of each isolate were inoculated on PDA-C and incubated at 24°C. Visual observations of the colony color were made, every three days, from day of inoculation until day 30 after inoculation. Measurements of conidia were carried out at 30 days after inoculation and due to inconsistent sporulation of isolates, conidia size for species was obtained from either one replication and averaging measurements of up to three replications, depending on sporulation of isolates. In addition, colony morphology and growth characteristics on PDA-C of the CBS 286.87 isolate, designated as *Eutypa leptoplaca* (Durieu & Mont.) Rappaz, obtained from the Centraalbureau voor Schimmelcultures (CBS), Netherlands was examined.

### 2.3.2.3 Molecular characterisation

#### 2.3.2.3.1 DNA extraction

Mycelia from actively growing pure cultures resembling Diatrypaceae species were scraped directly from PDA-C and DNA was extracted using CTAB extraction buffer (2% CTAB, 1 M Tris, pH 7.5; 5 M NaCl; 0.5 M EDTA, pH 8.0) according to the following protocol: Glass beads (0.5 g) and 600 µl of CTAB were added to the mycelium in 2 ml tubes which were then shaken

for 7 min at  $30 \text{ s}^{-1}$  frequency using a Mixer Mill type MM 301 (Retsch GmbH & Co. KG, Germany) and incubated at  $65^{\circ}\text{C}$  for 30 min. Thereafter,  $400 \mu\text{l}$  of chloroform: isoamylalcohol (24:1) was added and tubes were centrifuged at  $15\,800 \times g$  for 10 min. The supernatant was transferred to fresh tubes and  $250 \mu\text{l}$  of 7.5 M cold ammonium acetate solution and  $600 \mu\text{l}$  cold isopropanol were added. The tubes were inverted to mix the solutions and incubated for 15 min at  $-20^{\circ}\text{C}$  before centrifugation at  $15\,800 \times g$  for 15 min. The supernatant was discarded and 1 ml of cold 70% ethanol added to the pellet before centrifuging at  $15\,800 \times g$  for 5 min. The supernatant was discarded and the pellets were left to dry at room temperature overnight. Pellets were dissolved in  $100 \mu\text{l}$  double distilled water and stored at  $4^{\circ}\text{C}$ .

#### 2.3.2.3.2 PCR amplification and DNA sequencing

All isolates were first subjected to amplification using species-specific primers to detect *C. ampelina* and *E. lata*. The species-specific primer pair, Camp1/Camp2R designed by Luque *et al.* (2006) was used to detect *C. ampelina* while the primer pair EL1/EL4 (Catal *et al.*, 2007) was used for the detection of *E. lata*. A touch down PCR protocol was used for the amplification of *C. ampelina* and was carried out in a  $25 \mu\text{l}$  reaction containing the following end concentrations: 1X PCR buffer, 1 mM  $\text{MgCl}_2$ ,  $0.2 \mu\text{M}$  each deoxyribonucleotide triphosphates (dNTPs), 1 mM each primer and 0.65 U Taq polymerase. The touch down PCR protocol consisted of two phases: The first phase included an initial denaturing at  $94^{\circ}\text{C}$  for 5 min, followed by 20 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing at  $68^{\circ}\text{C}$  for 30 s, and extension at  $72^{\circ}\text{C}$  for 30 s. Phase 2 consisted of 10 cycles of  $94^{\circ}\text{C}$  for 30 s,  $66^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 30 s and a final extension step at  $72^{\circ}\text{C}$  for 5 min. The EL1/EL4 PCR reaction was performed in a total volume of  $25 \mu\text{l}$  consisting of 1X PCR buffer, 3.5 mM  $\text{MgCl}_2$ , 0.8 mM dNTPs,  $0.5 \mu\text{M}$  of each primer, 0.65 U Taq Polymerase (Bioline, USA) and  $1 \mu\text{l}$  DNA template. PCR amplification was conducted using the following thermal regime: initial denaturing for 2 min at  $94^{\circ}\text{C}$ , 35 cycles of denaturing at  $94^{\circ}\text{C}$  for 1 min, followed by annealing at  $62^{\circ}\text{C}$  for 30 s, elongation at  $72^{\circ}\text{C}$  for 1 min, final extension at  $72^{\circ}\text{C}$  for 5 min.

For isolates in which amplification could not be obtained using species-specific primers, the internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S ribosomal RNA gene were amplified using primers ITS1/ITS4 (White *et al.*, 1990). Twenty isolates of *C. ampelina* and 25 isolates of *E. lata*, randomly chosen, were also included in this PCR to verify the specificity of the species-specific primers. Amplification of the ITS region was performed in a  $25 \mu\text{l}$  reaction volume containing 1X PCR buffer, 2.5 mM  $\text{MgCl}_2$ ,  $1 \mu\text{g}/\mu\text{l}$  bovine serum albumen, 0.2 mM dNTPs,  $0.25 \mu\text{M}$  each primer and 0.65 U of Bioline Taq polymerase. The PCR reaction consisted of denaturation at  $94^{\circ}\text{C}$  for 3 min, followed by 35 cycles of 30 s at  $94^{\circ}\text{C}$ , 30 s at  $52^{\circ}\text{C}$ , and 60 s at  $72^{\circ}\text{C}$ , and a final extension step at  $72^{\circ}\text{C}$  for 7 min.

Identification of the isolates was based on subjecting the ITS sequence data to the megablast function of the National Center for Biotechnology Information's (NCBI) GenBank nucleotide database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Depending on the number of isolates identified by the BLAST search, up to eleven representative isolates of each species, including *E. lata* and *C. ampelina*, were selected for amplification of the  $\beta$ -tubulin gene. The partial  $\beta$ -tubulin gene was amplified using the primer set, Bt2a/Bt2b (Glass and Donaldson, 1995). The PCR for the  $\beta$ -tubulin gene consisted of 1X PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.5  $\mu$ M primers, 0.65 units of Taq DNA polymerase and 1  $\mu$ l of DNA template and made up to 25  $\mu$ l with sterile water. The following conditions were set for the thermal cycler: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 5 min.

A GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) was used for all PCR reactions and all PCR products were separated by electrophoresis on 1% agarose gels, stained with ethidium bromide and viewed under UV light. Estimates of amplicon sizes were made against a GeneRuler 100 bp molecular marker (Thermo Scientific, South Africa). The MSB Spin PCRapase kit (Invitek, Berlin, Germany) was used to purify PCR products. The amplicons of both ITS and  $\beta$ -tubulin were sequenced in both directions using an ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA) with the primers used in the initial PCR reactions. The products were then analysed on an ABI Prism 3130XL DNA sequencer (Perkin-Elmer, Norwalk, CN).

#### 2.3.2.3.3 Phylogenetic analysis

The phylogenetic analyses for the combined datasets (ITS and  $\beta$ -tubulin) were performed using Maximum likelihood (ML) and Bayesian analyses. The ITS and  $\beta$ -tubulin sequences of representative isolates obtained in this study were respectively aligned with sequences of Diatrypaceae taxa retrieved from GenBank (Table 1) as well as the outgroup *Hypoxyylon fragiforme*. Sequences were aligned with MAFFT v7.017 (Kato *et al.*, 2002) in Geneious v8.1 (<http://www.geneious.com>, Kearse *et al.*, 2012), using the default parameters. The alignments were run through Gblocks v0.91b, using default parameters (Castresana, 2000) to exclude poorly aligned positions from subsequent phylogenetic inferences.

Maximum likelihood analyses were performed using PhyML (Guindon and Gascuel, 2003) in Geneious v8.1 under the general time reversible (GTR) model. Both the proportion of invariable sites and gamma distribution parameter were estimated and bootstrap support values were calculated from 1000 replicates. Clades with bootstrap support  $\geq 70\%$  were considered significant and highly supported (Hillis and Bull, 1993). Bayesian analyses were done with Mr Bayes v3.2.2 (Huelsenbeck and Ronquist, 2001) using the Markov Chain Monte Carlo (MCMC) method. Four MCMC chains were run simultaneously and trees were saved

every 100th generation. The first 25% of generated trees were discarded as the burn-in phase of the analyses. Posterior probabilities were assigned after a 50% majority-rule. Clades with Bayesian posterior probability  $\geq 95\%$  were considered significant (Alfaro *et al.*, 2003).

## 2.4 RESULTS

### 2.4.1 Sampling and isolate collection

A total of 360 dying spurs (Fig. 1A) were collected in this study, with 190 collected from Sauvignon blanc and 170 from Cabernet Sauvignon vineyards. Isolations were performed from different symptoms observed inside dying spurs (Fig. 1B-F). Subsequently, 68 (36%) and 71 (42%) of the total number of spurs collected from Sauvignon blanc and Cabernet Sauvignon, yielded Diatrypaceae isolates, respectively. Two hundred and ninety-six samples showing wedge-shaped necrosis (Fig. 1G) were collected from vineyards and 72% of these samples yielded Diatrypaceae isolates. A total of 81 grapevine wood pieces with perithecia of Diatrypaceae species were collected from vineyards in the Western Cape Province.

### 2.4.2 Identification of Diatrypaceae isolates

Maximum likelihood and Bayesian analyses of the combined data set generated trees with similar topology, with regards to the clades/subclades representing species isolated in this study. The ML tree is presented in Fig. 2 with ML bootstrap support values and Bayesian posterior probability scores at the nodes. In this tree, isolates obtained in this study were distinguished as belonging to seven species, residing in three genera namely *Eutypa* Tul. & C. Tul., *Eutypella* (Nitschke) Sacc. and *Cryptovalsa* (Ces. & De Not.). Eight isolates clustered as a strongly supported group (100%/1.00, ML bootstrap support and Bayesian posterior probability scores) with *Eutypella* (*Eu.*) *citricola* Speg. isolates. Ten isolates grouped with strong support (99%/1.00) with *C. ampelina* isolates obtained from Australia and California. Five isolates clustered with *Eutypella microtheca* Trouillas, W.M. Pitt & Gubler (74%/0.94) isolates from Australia whereas one isolate grouped with strong support (100%/1.00) with *Cryptovalsa rabenhorstii* (Nitschke) Sacc. isolates obtained from Australia. Five isolates clustered with strong support (99%/1.00) with *E. lata* isolates from California and Australia. One isolate grouped with a *Eutypa consobrina* (Mont.) Rappaz isolate with 100%/1.00 support. Eleven isolates grouped with the French isolate CBS 286. 87 from *Arundo donax* L., designated as *E. leptoplaca*. This group represents a previously misidentified taxon which is closely related to *Eutypa tetragona* (Duby) Sacc. In view of the isolate CBS 286. 87 and the 11 isolates obtained in this study, being phylogenetically distinct from other *E. leptoplaca* isolates, we then propose a new species name for this group.

The dying spurs presented the highest diversity of Diatrypaceae compared to the wedge-shaped necrosis on arms and trunks of declining grapevines. From a total of 360 dying spurs processed, seven species namely *C. ampelina*, *C. rabenhorstii*, *Eutypa cremea* sp. nov, *E. consobrina*, *E. lata*, *Eu. citricola* and *Eu. microtheca* were isolated and identified from the two grapevine cultivars sampled from the five different areas within the Western Cape Province of South Africa. *Cryptovalsa ampelina* was the most frequently isolated species (46.4% of the total number of Diatrypaceae isolates) from dying spurs. The next most common species isolated, in order of decreasing abundance were *Eu. citricola* (26.8%), *E. lata* (20.1%) and *E. cremea* (4.3%), *Eu. microtheca* (1.2%), *E. consobrina* (0.6%) and *C. rabenhorstii* (0.6%). Isolations from wedge-shaped necrotic sectors of grapevine, representing 10 cultivars collected from eight locations in the Western Cape, yielded four species namely *C. ampelina*, *E. lata*, *E. cremea*, and *Eu. citricola*. *Eutypa lata* represented the most frequent species detected in the wedge-shaped necrosis (89.2% of all Diatrypaceae isolates obtained). *Eutypella citricola* was the second most frequent species (8.5%), followed by *E. cremea* (1.4%) and *C. ampelina* (0.9%). In both symptomatic types (dying spurs and wedge-shaped necrosis), several samples were infected by more than one species. More than one fungal species was isolated from a single symptom in 17% and 4% of the samples that yielded Diatrypaceae species, for dying spurs and wedge-shaped necrotic sectors, respectively. *Cryptovalsa ampelina* and *E. lata* were found in 83% and 100% of the samples yielding more than one fungal species, in the dying spurs and wedge-shaped necrosis samples, respectively (Table 2).

The survey of fruiting structures in vineyards, revealed five species to produce fruiting bodies on dead grapevine wood. *Eutypa lata* fruiting bodies were found on 51% of the grapevine wood samples collected. Perithecia of *C. ampelina* were found on 38% of the wood pieces whereas *E. cremea*, *Eu. citricola* and *Eu. microtheca* perithecia were found on 6%, 4% and 1% of the wood pieces, respectively.

#### 2.4.2.1 Taxonomy

Surveys resulted in five species being found to produce perithecia on dead wood of grapevine. Stromatal features of four of these species namely *C. ampelina*, *E. lata*, *Eu. citricola* and *Eu. microtheca* have already been described on grapevine and hence are not described in this chapter. Nevertheless, these taxa are described in the next chapter. Phylogenetic analyses strongly supported the separation of the French isolate CBS 286.87 designated as *E. leptoplaca* by Rappaz (1987) from other *E. leptoplaca* isolates (CBS 287.87 and CBS 288.87). Since isolates grouped with this isolate represent a strongly supported clade with no proper species name, the following species name is proposed to accurately circumscribe this taxon:



***Eutypa cremea*** Moyo, P., Halleen, L. Mostert **sp. nov.**

(Fig. 3)

MycoBank: MB XXX

*Etymology*: in reference to the colony colour which is mostly cream.

*Stromata*: charcoal-like appearance, on wood or bark, poorly developed. *Perithecia* scattered or in rows (Fig. 1A), circular to ovoid, (228–) 232–376 (–395)  $\mu\text{m}$  diam. *Ostioles* apparent on surface of substrate, emerging on surface separately, 3-4 sulcate. *Asci* clavate, with apical ring, octosporous, long stipitate, (23–) 26–58.5 (–62)  $\times$  (5–) 5–7.5  $\mu\text{m}$ . *Ascospores* yellowish in colour, allantoid, (5.5–) 6–8  $\times$  1–2  $\mu\text{m}$ .

*Cultural characteristics on PDA-C*: Colonies are cream and flat, sometimes with white aerial mycelium. *Conidia* hyaline, filiform, (19–) 22–33  $\times$  1–1.6– (–1.7)  $\mu\text{m}$ .

*Hosts*: *Vitis vinifera* (grapevine), *Cinnamomum camphora* (camphor tree), *Morus* sp. (Mulberry), *Quercus* sp. (oak), *Quercus suber* (cork oak), *Arundo donax* (giant cane).

*Type specimen examined*: SOUTH AFRICA: Western Cape Province, Durbanville, Hooggelegen farm, on dead wood of *Vitis vinifera*, August 2014; Holotype: P. Moyo, collection number PREM 61675, Ex-type culture number STEU 8082.

*Additional specimens examined*: Algeria: *Sphaeria leptoplaca*, isolate MC 8636 (lectotype) on *Fraxinus* sp.; *Sphaeria leptoplaca*, isolate MC 8635 on *Arundo mauritanica*; *Sphaeria consobrina*, isolate MC 5627 on *Arundo mauritanica*; *Sphaeria consobrina* MC 5626 (neotype) on *Arundo mauritanica*.

*Other specimens*: SOUTH AFRICA: Western Cape Province, Darling, on dead wood of *Vitis vinifera*, November 2013, P. Moyo, culture number STEU 8101; Darling, on dead wood of *Vitis vinifera*, June 2012, F. Halleen, culture number STEU 8084; Franschoek, Allee Bleue, dead wood of *Morus* sp., September 2014, P. Moyo, culture number STEU 8130; Durbanville, Morgenster farm, on dead wood of *Quercus* sp., November 2013, P. Moyo, culture number STEU 8144; Stellenbosch, dead wood of *Cinnamomum camphora*, February 2014, P. Moyo, culture number STEU 8142.

*Notes*: Variation in cultural growth within individual isolates of this species was observed. For example, an individual isolate could be seen as cream and very flat with no apparent aerial mycelium on one PDA-C plate and be cream with apparent white aerial mycelium on another plate, despite being plated the same time and the two PDA-C plates exposed to same incubation conditions. *Eutypa cremea* is similar to *E. leptoplaca* and *E. tetragona* (*E. tetragona* as described by Rappaz, 1987) in having 3-4 sulcate ostioles emerging separately, yellowish ascospores and spherical to ovoid perithecia but these differ in the length of their spore bearing part of their asci. *Eutypa cremea* has a higher extreme spore bearing part [(23–) 26–58.5 (–62)  $\mu\text{m}$ ] compared to *E. leptoplaca* (25-40  $\mu\text{m}$ ) and *E. tetragona* [(30-45 (-55)  $\mu\text{m}$ ] (Rappaz, 1987). Additionally, *E. tetragona* has larger perithecia diameter [300-400 (- 500)  $\mu\text{m}$ ]

versus *E. leptoplaca* (150-300 µm) (Rappaz, 1987) and *E. cremea* [(228–) 232–376 (–395) µm].

## 2.5 DISCUSSION

There has been an increase in the number of studies of the Diatrypaceae occurring on grapevine recently and the use of DNA sequences makes it easier to identify taxa to generic and species level. Phylogenetic analyses allowed the identification of seven Diatrypaceae species from declining grapevines in South Africa, namely *C. ampelina*, *C. rabenhorstii*, *E. lata*, *E. consobrina*, *E. cremea*, *Eu. citricola* and *Eu. microtheca*. Besides *E. lata* and *C. ampelina*, the other five taxa found in this study are reported for the first time on grapevine in South Africa.

Phylogenetic results strongly supported the separation of the French isolate CBS 286.87 designated as *E. leptoplaca* by Rappaz (1987) from other isolates of *E. leptoplaca*. Trouillas and Gubler (2004) hypothesised that the isolate CBS 286.87 could be *E. consobrina* after finding close resemblance of the type material of *E. leptoplaca* to the type material of *E. consobrina*. The close resemblance of the type material of these two species was also observed in this study. However, *E. consobrina* could be distinguished from *E. cremea* based on phylogenetic analyses and morphological characteristics. *Eutypa consobrina* could be differentiated from *E. cremea* based on the apricot colour of its colonies on PDA-C (see Chapter 3). Furthermore, perithecia of *E. consobrina* were also larger in size [(249–) 262–451 (–468) µm diam.] and bore bigger asci [(25–) 27–69 (–82) × 5–7 µm] and ascospores [6–10 (–11) × (1–) 1.5–3 µm] in comparison to *E. cremea* (Chapter 3).

The identity of Diatrypaceae species associated with wedge-shaped cankers and spur dieback on grapevine was investigated and results showed that the frequency of isolation of these species from the two symptom types differed considerably. Wedge-shaped cankers on grapevine have been, in the past, been associated with *E. lata* (Moller and Kasimatis, 1978) and indeed this species was found to be the most dominant species in this symptom type in the current study. The high incidence of isolation of this pathogen indicates that the wedge-shaped necrosis could be attributed to *E. lata* and that this species is still an important grapevine canker pathogen in South Africa. *Eutypa lata* produces toxins (Tey-Ruhl *et al.*, 1991) and wood degrading enzymes (English and Davis, 1978; Schmidt *et al.*, 1999), which have been implicated in the decline of grapevine. The presence of additional Diatrypaceae species in the same tissue with *E. lata* further compromises the immunity of the vine and possibly enhance the decline of the infected grapevine.

Isolations from dying spurs resulted in seven species of Diatrypaceae being identified, with *C. ampelina* being the most isolated species from Cabernet Sauvignon and Sauvignon

blanc. *Cryptovalsa ampelina* co-occurred with all the other six species found to infect spurs, however, the frequency in which this species co-occurred with other species was lower than that in which it occurred alone in both cultivars. The high frequency with which *C. ampelina* was isolated agrees with results of Luque *et al.* (2014) who found this species to be the most abundant species within the Diatrypaceae in naturally infected grapevine spurs in Spain.

*Eutypa lata* infects hosts through ascospores which germinate and colonise the vascular tissue of the trunk and cordons of grapevine (Moller and Kasimatis, 1978, 1980). Mycelium of this fungus is not usually detected in annual canes or the foliage of infected grapevines (Rudelle *et al.*, 2005) and development of foliar symptoms in *Eutypa* dieback-affected grapevines has been accredited to the phytotoxins produced by *E. lata* inside the wood (Tey Rulh *et al.*, 1991). While the dieback of spurs and low frequency of isolation of *E. lata* could be explained by the translocation of toxins of this fungus from mature wood to the spurs, the higher incidence of isolation of *C. ampelina* in both cultivars could be explained by the capability of *C. ampelina* to better colonise younger wood of spurs. This also makes *C. ampelina* an important causal agent of spur dieback in the Western Cape of South Africa. The isolation of a diversity of species of Diatrypaceae in vineyards as young as four-years-old, however, could also suggest that these species are involved in the long process of the development of dieback of grapevines, or they may work synergistically with *E. lata* in symptom development.

A high number of isolates and diversity of Diatrypaceae species was found in the Cabernet Sauvignon compared to the Sauvignon blanc vineyards despite a lower number of dying spurs examined in the Cabernet Sauvignon vineyards. Although not statistically proven, these results might suggest that Cabernet Sauvignon is more susceptible to these fungi than Sauvignon blanc. Péros and Berger (1994) reported that Cabernet Sauvignon has greater susceptibility to *E. lata* compared with Sauvignon blanc and this could potentially be true for the other Diatrypaceae species. Apart from species that were isolated fairly frequently from spurs, *C. rabenhorstii* and *E. consobrina* were each isolated only once which suggests that their presence on grapevine might be influenced by certain conditions, for example the occurrence of other woody hosts in the vicinity of vineyards. This theory is supported by other studies which found diatrypaceous species occurring on grapevine to also occur in other woody hosts surrounding vineyards and postulated that these hosts act as inoculum sources to vineyards (Trouillas *et al.*, 2010, 2011). Furthermore, the low frequency of isolation of these species in the present study makes it difficult to establish the role played by these species in grapevine decline.

While *E. lata* has been known to be the most important diatrypaceous pathogen in many grapevine-producing areas of the world, *C. ampelina*, *Eu. microtheca* and *Eu. citricola* also seem to be widespread geographically having been reported in countries including Spain

(Luque *et al.*, 2012), Australia (Trouillas *et al.*, 2011), California (Trouillas *et al.*, 2010), Iran (Mehrabi *et al.*, 2015), Mexico (Paolinelli-Alfonso *et al.*, 2015) and Brazil (Almeida *et al.*, 2016). Before this study, *C. rabenhorstii* has only been reported on grapevine in Australia (Trouillas *et al.*, 2011). *Eutypa cremea* was isolated from grapevine samples collected from different areas in the Western Cape Province including Bonnievale, Darling, Durbanville, Constantia, Hermanus, Robertson, Slanghoek and Stellenbosch. This shows that this species occurs on a wide geographical area in South Africa and therefore, grapevine appears to be a good host for *E. cremea*. Furthermore, besides its isolation from grapevine, *E. cremea* has been associated with *Arundo donax* L. from Algeria (Rappaz, 1987) and other woody hosts including *Cinnamomum camphora* (L.) J. Presl., *Morus* sp., *Quercus* sp. in this study, which indicates its capability to infect multiple hosts. Results from the current study extend the host range of *E. consobrina* from *Arundo mauritanica* Poir. to *Vitis* spp. and further increases the number of different Diatrypaceae species found to colonise grapevine.

Surveys of sexual fruiting bodies revealed that five Diatrypaceae species have the ability to complete their life cycle on grapevine tissue. However, perithecia of species such as *Eu. microtheca*, *Eu. citricola* and *E. cremea* were not as prevalent as those of *C. ampelina* and *E. lata* in South African vineyards. Nevertheless, finding stromata of these species on dead wood of grapevine suggests that they may be involved in the dieback and eventually the death of grapevines. The epidemiology and biology of the newly discovered Diatrypaceae species are currently unknown on grapevine. However, the isolation of these species from necrotic tissues below pruning wounds of naturally infected spurs and from wedge-shaped necrosis typical of *Eutypa* dieback in this study suggests that, similar to *E. lata*, these species infect grapevine *via* pruning wounds on spurs and cordons and develop inside vascular tissues.

Prevention of the introduction of causal pathogens into the vineyard is the most reliable control strategy against *Eutypa* dieback. Prevention of infection is achieved by strategies which include remedial surgery, the use of pruning wound protectants and avoiding pruning immediately after rainfall events (Sosnowski *et al.*, 2008). To date, none of the prevention strategies has been evaluated for the potential to control or prevent any infection that could be caused by the newly discovered Diatrypaceae species on grapevine, with the exception of fungicides known to reduce *E. lata* infection. Although the efficacy of fungicides to prevent infection by these species was only tested *in vitro* and fungicides are yet to be tested *in vivo*, results of a study by Gramaje *et al.* (2012) showed that fungicides such as fluazinam, carbendazim and tebuconazole were effective against mycelial growth of species including *C. ampelina*, *Eu. citricola* and *Eu. microtheca*. Further research on management strategies against *Eutypa* dieback of grapevine may need to incorporate and be effective against all the newly discovered Diatrypaceae species.

In conclusion, the current study represents the first survey aimed at matching Diatrypaceae species to symptom type on grapevine and results obtained give insight into the aetiology of symptoms associated with dieback in South Africa. The study showed that *C. ampelina* and *E. lata* are the predominant diatrypaceous species associated with dying spurs and wedge-shaped necrosis on cordons or trunks of grapevine in South Africa, respectively. The isolation of more than one species in some samples, however, suggests that synergistic interactions occur leading to the decline of grapevines. Furthermore, the isolation of *E. consobrina* and *C. rabenhorstii* from dying spurs with no stromata found suggests that inoculum of these fungi originates from other sources in particular, other woody hosts in the vicinity of vineyards. Vineyards are usually established in close proximity of natural ecosystems as well as fruit orchards in South Africa and this poses risks with regards to unlimited availability of Diatrypaceae species inoculum. For this reason, it is therefore important to investigate other woody hosts as possible reservoirs of inoculum of these species.

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**Table 1.** List of Diatrypaceae isolates collected in this study and reference strains from GenBank, used for phylogenetic analysis.

Species	Host	Origin	Strain number	Genbank number	
				ITS	$\beta$ -tubulin
<i>Cryptosphaeria ligniota</i>	<i>Populus tremula</i>	Switzerland	CBS273.87	KT425233	KT425168
<i>Cryptosphaeria ligniota</i>	<i>Populus tremuloides</i>	Colorado, USA	ATCC46315	KT425234	KT425169
<i>Cryptosphaeria multicontinentalis</i>	<i>Populus balsamifera</i> subsp. <i>trichocarpa</i>	California, USA	DSIERRA600	KT425184	KT425119
<i>Cryptosphaeria multicontinentalis</i>	<i>Populus balsamifera</i>	Australia	NSW03PO	KT425237	KT425172
<i>Cryptosphaeria multicontinentalis</i>	<i>Populus</i> sp.	Oregon, USA	OREG100	KT425188	KT425123
<i>Cryptosphaeria pullmanensis</i>	<i>Populus trichocarpa</i>	Washington, USA	ATCC52655	KT425235	KT425170
<i>Cryptosphaeria pullmanensis</i>	<i>Populus nigra</i> 'italica'	California, USA	DWIN100	GQ293930	GQ294015
<i>Cryptosphaeria subcutanea</i>	<i>Salix borealis</i>	Norway	CBS240.87	KT425232	KT425167
<i>Cryptosphaeria subcutanea</i>	<i>Salix myrsinifolia</i>	Norway	DSUB100A	KT425189	KT425124
<i>Cryptovalsa ampelina</i>	<i>Fraxinus latifolia</i>	California, USA	DMO100	GQ293908	GQ293978
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	New South Wales, Australia	HVVIT04	HQ692558	HQ692459
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Riebeeck-Wes, WC, South Africa	STEU 8119	KY111665	KY111624
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Durbanville, WC, South Africa	STEU 8120	KY111666	KY111625
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Robertson, WC, South Africa	STEU 8114	KY111660	KY111619
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Robertson, WC, South Africa	STEU 8115	KY111661	KY111620
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Robertson, WC, South Africa	STEU 8122	KY111668	KY111627
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Robertson, WC, South Africa	STEU 8113	KY111659	KY111618
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Paarl, WC, South Africa	STEU 8117	KY111663	KY111622
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8121	KY111667	KY111626
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8118	KY111664	KY111623
<i>Cryptovalsa ampelina</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8116	KY111662	KY111621
<i>Cryptosphaeria rabenhorstii</i>	<i>Vitis vinifera</i>	Western Australia	WA07CO	HQ692620	HQ692522
<i>Cryptosphaeria rabenhorstii</i>	<i>Vitis vinifera</i>	Western Australia	WA08CB	HQ692619	HQ692523
<i>Cryptosphaeria rabenhorstii</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8112	KY111628	KY111607
<i>Diatrype brunneospora</i>	<i>Acacia longifera</i> subsp. <i>sophorae</i>	Australia	CNP01	HM581946	HQ692478
<i>Diatrype oregonensis</i>	<i>Quercus kelloggii</i>	California, USA	DPL200	GQ293940	GQ293999
<i>Diatrype stigma</i>	<i>Quercus</i> sp.	Irish Republic	CBS211.87	AJ302438	–
<i>Diatrypella verruciformis</i>	<i>Quercus</i> sp.	California, USA	DCH500	GQ293926	GQ293991
<i>Diatrypella vulgaris</i>	<i>Citrus paradisi</i>	New South Wales, Australia	HVGRF03	HQ692590	HQ692505
<i>Diatrypella vulgaris</i>	<i>Vitis vinifera</i>	New South Wales, Australia	CG8	HQ692595	HQ692502
<i>Eutypa consobrina</i>	<i>Arundo donax</i>	Almeria, Spain	F091,961	AJ302447	–
<i>Eutypa consobrina</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8092	KY111651	KY111596

Table 1. (continued)

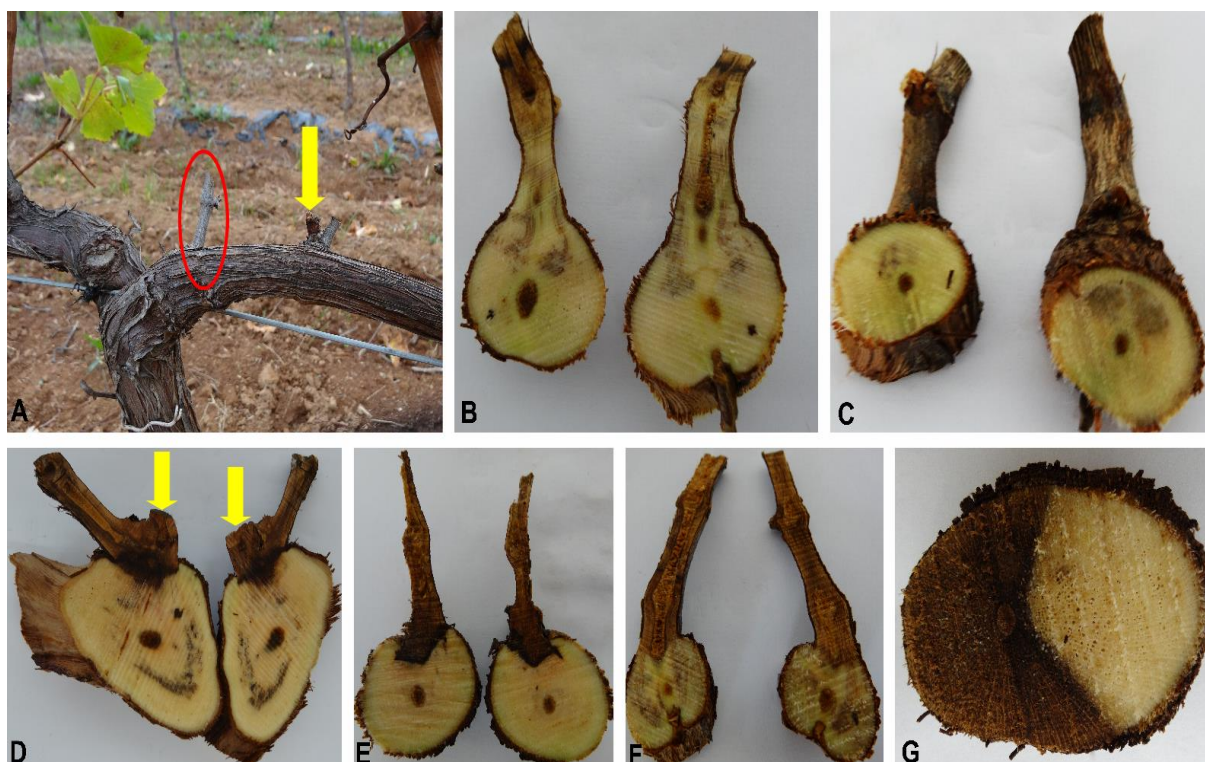
Species	Host	Origin	Strain number	Genbank number	
				ITS	β-tubulin
<i>Eutypa crustata</i>	<i>Ulmus</i> sp.	France	CBS210.87	AJ302448	DQ006968
<i>Eutypa laevata</i>	<i>Salix</i> sp.	Switzerland	CBS291.87	HM164737	HM164771
<i>Eutypa lata</i>	<i>Vitis vinifera</i>	California, USA	EAMS100	HM164730	HM164764
<i>Eutypa lata</i>	<i>Populus nigra</i> 'italica'	South Australia	SAPN01	HQ692616	HQ692500
<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8096	KY111654	KY111616
<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8097	KY111644	KY111617
<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Barrydale, WC, South Africa	STEU 8095	KY111643	KY111615
<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8093	KY111652	KY111613
<i>Eutypa lata</i>	<i>Vitis vinifera</i>	Sommerset West, WC, South Africa	STEU 8094	KY111653	KY111614
<i>Eutypa lata</i> var. <i>aceri</i>	<i>Acer campestre</i>	France	CBS217.87	HM164734	HM164768
<i>Eutypa lejoplaca</i>	<i>Acer pseudoplatanus</i>	Switzerland	020202-5	AY684221	AY684196
<i>Eutypa leptoplaca</i>	<i>Arundo donax</i>	France	CBS286.87	AY684225	AY684203
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8089	KY111648	KY111605
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8090	KY111649	KY111587
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8087	KY111646	KY111603
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8088	KY111647	KY111604
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Robertson, WC, South Africa	STEU 8086	KY111645	KY111602
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Hermanus, WC, South Africa	STEU 8091	KY111650	KY111606
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Durbanville, WC, South Africa	STEU 8082	KY111656	KY111598
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8083	KY111657	KY111599
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Bonnievale, WC, South Africa	STEU 8081	KY111655	KY111597
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Slanghoek, WC, South Africa	STEU 8085	KY111658	KY111601
<i>Eutypa cremea</i>	<i>Vitis vinifera</i>	Darling, WC, South Africa	STEU 8084	KY111642	KY111600
<i>Eutypa leptoplaca</i>	<i>Frangula alnus</i>	Switzerland	CBS287.87	AY684226	AY684204
<i>Eutypa leptoplaca</i>	<i>Cyssus hypoglauca</i>	Australia	CBS288.87	AY684227	AY684205
<i>Eutypa maura</i>	<i>Acer pseudoplatanus</i>	Switzerland	CBS219.87	AY684224	DQ006967
<i>Eutypa petrakii</i> var. <i>petrakii</i>	<i>Prunus spinosa</i>	Switzerland	CBS244.87	HM164735	HM164769
<i>Eutypa sparsa</i>	<i>Populus</i> sp.	Switzerland	3802-3a	AY684200	AY684219
<i>Eutypa tetragona</i>	<i>Laburnum alpinum</i>	Switzerland	190802-3	AJ684223	AY684202
<i>Eutypa tetragona</i>	<i>Sarothamnus scoparius</i>	France	CBS284.87	DQ006923	DQ006960
<i>Eutypella alsophila</i>	<i>Arthrocnemum fruticosum</i>	France	CBS250.87	AJ302467	–
<i>Eutypella australiensis</i>	<i>Acacia longifolia</i> subsp. <i>sophorae</i>	Australia	CNP03	HM581945	HQ692479

**Table 1.** (continued)

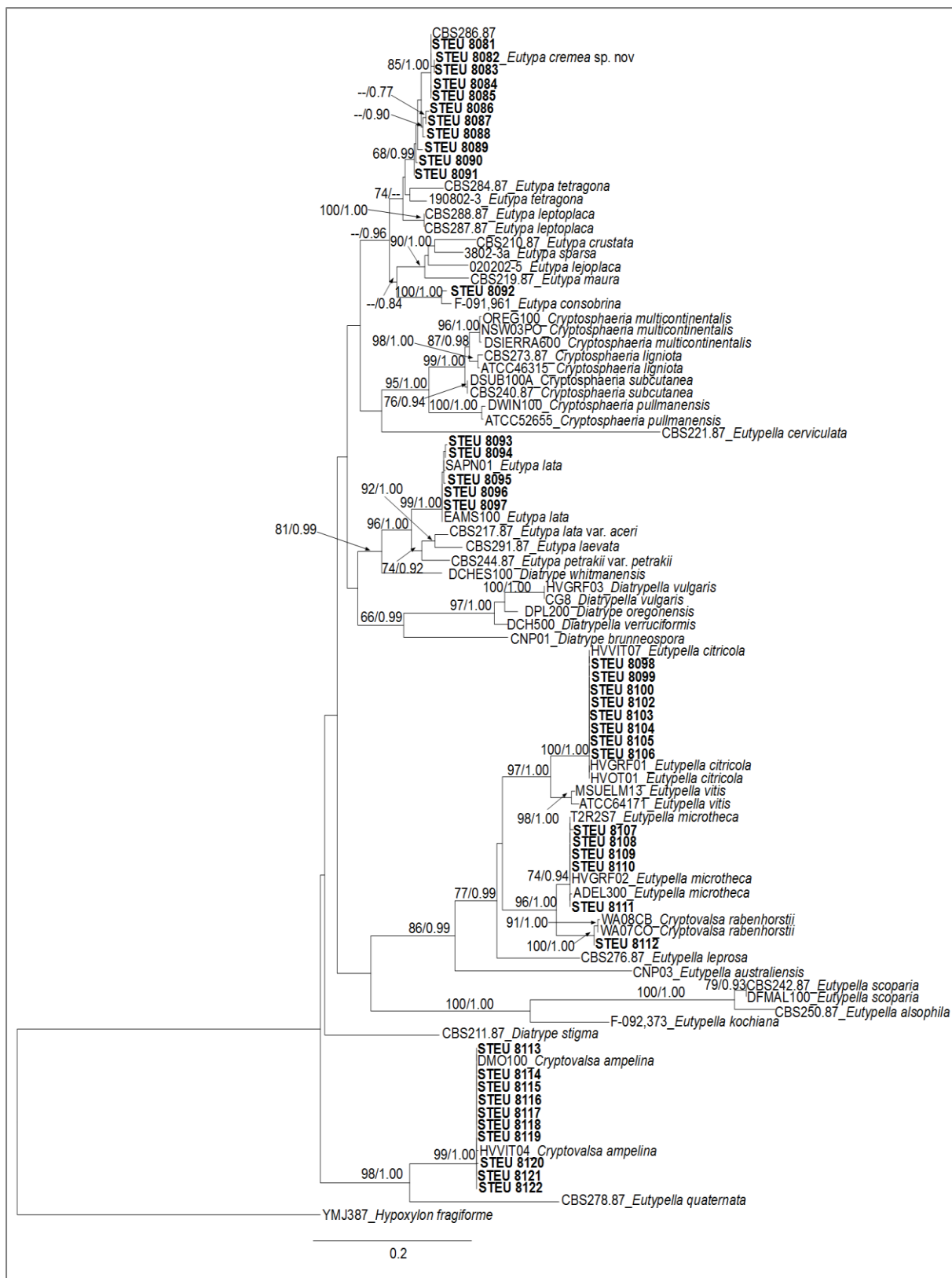
Species	Host	Origin	Strain number	Genbank number	
				ITS	$\beta$ -tubulin
<i>Eutypella cerviculata</i>	<i>Alnus glutinosa</i>	Switzerland	CBS221.87	AJ302468	–
<i>Eutypella citricola</i>	<i>Citrus paradisi</i>	New South Wales, Australia	HVGRF01	HQ692589	HQ692521
<i>Eutypella citricola</i>	<i>Citrus sinensis</i>	New South Wales, Australia	HVOT01	HQ692581	HQ692509
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	New South Wales, Australia	HVVIT07	HQ692579	HQ692512
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8106	KY111641	KY111595
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8099	KY111637	KY111589
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Worcester, WC, South Africa	STEU 8102	KY111635	KY111591
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8100	KY111638	KY111590
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8103	KY111639	KY111592
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Grabouw, WC, South Africa	STEU 8105	KY111640	KY111594
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8104	KY111636	KY111593
<i>Eutypella citricola</i>	<i>Vitis vinifera</i>	Constantia, WC, South Africa	STEU 8098	KY111634	KY111588
<i>Eutypella cryptovalsoidea</i>	<i>Ficus carica</i>	New South Wales, Australia	HVFIG02	HQ692573	HQ692524
<i>Eutypella kochiana</i>	<i>Atriplex halimus</i>	Almeria, Spain	F-092,373	AJ302462	–
<i>Eutypella leprosa</i>	<i>Tilia</i> sp.	Switzerland	CBS276.87	AJ302463	–
<i>Eutypella microtheca</i>	<i>Ulmus procera</i>	South Australia	ADEL300	HQ692560	HQ692528
<i>Eutypella microtheca</i>	<i>Citrus paradisi</i>	New South Wales, Australia	HVGRF02	HQ692569	HQ692533
<i>Eutypella microtheca</i>	<i>Vitis vinifera</i>	New South Wales, Australia	T2R2S7	HQ692566	HQ692532
<i>Eutypella microtheca</i>	<i>Vitis vinifera</i>	Worcester, WC, South Africa	STEU 8107	KY111629	KY111608
<i>Eutypella microtheca</i>	<i>Vitis vinifera</i>	Worcester, WC, South Africa	STEU 8110	KY111632	KY111611
<i>Eutypella microtheca</i>	<i>Vitis vinifera</i>	Stellenbosch, WC, South Africa	STEU 8109	KY111631	KY111610
<i>Eutypella microtheca</i>	<i>Vitis vinifera</i>	Robertson, WC, South Africa	STEU 8108	KY111630	KY111609
<i>Eutypella microtheca</i>	<i>Vitis vinifera</i>	Calitzdorp, WC, South Africa	STEU 8111	KY111633	KY111612
<i>Eutypella quartenata</i>	<i>Fagus sylvatica</i>	Switzerland	CBS278.87	AJ302469	–
<i>Eutypella scoparia</i>	<i>Robinia pseudoacacia</i>	France	CBS242.87	AJ302465	–
<i>Eutypella scoparia</i>	<i>Robinia pseudoacacia</i>	France	DFMAL100	GQ293962	GQ294029
<i>Eutypella vitis</i>	<i>Vitis labrusca</i>	Illinois, USA	ATCC64171	AJ302466	–
<i>Eutypella vitis</i>	<i>Vitis vinifera</i>	Michigan, United States	MSUELM13	DQ006943	DQ006999

**Table 2.** The diversity of Diatrypaceae fungi isolated from dying spurs of Cabernet Sauvignon (CS) and Sauvignon blanc (SB) vineyards as well as wedge-shaped necrosis on wood pieces collected from different vineyards in the Western Cape Province of South Africa.

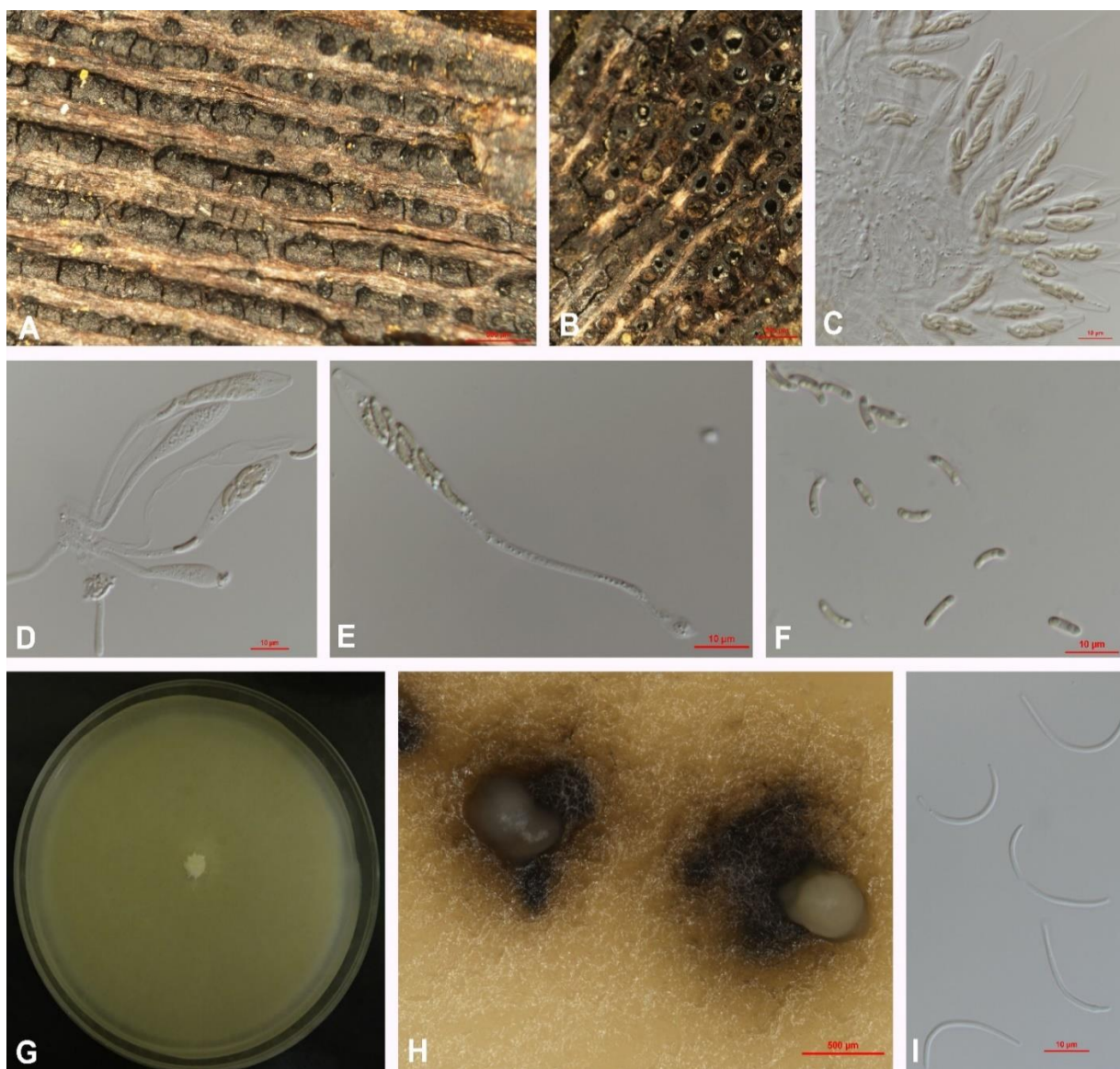
Species isolated	Number of samples		
	Dying spurs		Wedge-shaped necrosis
	SB	CS	
<i>Cryptovalsa ampelina</i>	27	29	1
<i>Eutypa lata</i>	17	7	181
<i>Eutypa cremea</i>	3	1	2
<i>Eutypella citricola</i>	13	17	11
<i>Eutypella microtheca</i>	–	1	–
<i>Cryptovalsa ampelina</i> + <i>Cryptovalsa rabenhorstii</i>	–	1	–
<i>Cryptovalsa ampelina</i> + <i>Eutypa lata</i>	5	–	1
<i>Cryptovalsa ampelina</i> + <i>Eutypa consobrina</i>	1	–	–
<i>Cryptovalsa ampelina</i> + <i>Eutypa cremea</i>	–	2	–
<i>Cryptovalsa ampelina</i> + <i>Eutypella citricola</i>	2	7	–
<i>Cryptovalsa ampelina</i> + <i>Eutypella microtheca</i>	–	1	–
<i>Cryptovalsa ampelina</i> + <i>Eutypa lata</i> + <i>Eutypella citricola</i>	–	1	–
<i>Eutypa lata</i> + <i>Eutypa cremea</i>	–	–	1
<i>Eutypa lata</i> + <i>Eutypella citricola</i>	–	3	7
<i>Eutypa cremea</i> + <i>Eutypella citricola</i>	–	1	–
<b>Total number of samples yielding Diatrypaceae isolates</b>	<b>68</b>	<b>71</b>	<b>204</b>
<b>Total number of samples collected</b>	<b>190</b>	<b>170</b>	<b>296</b>



**Figure 1.** Disease symptoms observed on dying spurs and cordons/trunks of naturally infected grapevines in the Western Cape Province of South Africa. **A.** Four-year-old grapevine showing a dying spur (circled in red) and poor bud burst (showed by yellow arrow) in the field. **B.** Brown streaks extending from pruning wound of dying spur. **C.** Brown sectorial necrosis seen from the reverse side of the dying spur presented in B. **D.** Brown/black streaks extending from dying spur which has a wound next next to it. **E.** Black line at the border of dead and live tissue. **F.** Brown streaks in a dying spur. **G.** Wedge-shaped necrosis.



**Figure 2.** Maximum likelihood tree derived from the combined analysis of ITS and  $\beta$ -tubulin sequence data. Maximum likelihood bootstrap support values and Bayesian posterior probability scores are given at the nodes. *Hypoxylon fragiforme* served as an outgroup. Isolates obtained from this study are in bold.



**Figure 3.** Morphology of *Eutypa cremea* from grapevine. **A.** Stromata showing perithecia in rows. **B.** Perithecial cavities. **C.** Asci attached to the hymenium. **D.** Mature and immature asci. **E.** Long stalked octosporous asci. **F.** Allantoid ascospores. **G.** Colony, on a 90 mm diam. PDA-C dish, after 15 days incubation at 24°C under 12 hours of intermittent lighting. **H.** Spore mass oozing from pycnidia after 30 days of incubation at 24°C. **I.** Filiform conidia.



## CHAPTER 3

### Diatrypaceae species associated with woody hosts adjacent to vineyards in South Africa

#### 3.1 ABSTRACT

Species in the Diatrypaceae occupy many woody hosts as saprophytes and pathogens. In this study, Diatrypaceae species were collected from woody hosts occurring near vineyards in South Africa. This study aimed to determine whether woody hosts are colonized by Diatrypaceae species, which are associated with *Eutypa* dieback of grapevine. Stone fruit trees were among woody hosts found to be colonised by Diatrypaceae species. South Africa is the leading producer of apricots in the Southern Hemisphere and the stone fruit industry contributes significantly to the economy of South Africa. Hence, it was also necessary to understand the role of Diatrypaceae species in dieback of apricot and plum by using pathogenicity tests. Wood of 39 different hosts showing symptoms of dieback, necrosis, cankers as well as fruiting bodies (perithecia) were sampled in different areas of the Western Cape and Limpopo Provinces of South Africa. Isolations were conducted to obtain isolates of Diatrypaceae fungi from symptomatic tissues. The fungi were identified based on phylogenetic analyses of the internal transcribed spacer regions (ITS1 and ITS2), 5.8S rRNA gene and partial  $\beta$ -tubulin gene as well as morphological characteristics of the sexual morphs. A total number of 289 isolates of Diatrypaceae were obtained and from these isolates, 14 species were identified. The five most frequently encountered species, in order of abundance, were *Eutypa* (*E.*) *lata*, *Eutypella* (*Eu.*) *citricola*, *E. cremea*, *Eu. microtheca* and *Cryptovalsa* (*C.*) *ampelina*. These five species were found to dominate, with regards to abundance, on grapevine in a previous study in South Africa. These results indicate the potential for infection routes between grapevine and other woody hosts and therefore, other woody hosts should be considered as potential inoculum sources of Diatrypaceae pathogens to vineyards. Furthermore, woody hosts serve as primary hosts for the survival of these species in the absence of vineyards. First reports in South Africa include *Cryptosphaeria* (*Cr.*) *multicontinentalis*, *Cr. ligniota*, *Diatrypella* sp., *Eu. leprosa* and *Eu. australiensis*. Our results also expand host ranges of *Cr. multicontinentalis* to *Salix mucronata* and *Morus* sp., *Eu. australiensis* to *Psidium guajava* and *Dalbergia* sp. and *Eu. microtheca* to *Prunus armeniaca*, *Melia azedarach* and *Morus* sp. Field inoculation on shoots of apricot and plum showed that *E. lata*, *Eu. citricola*, *E. cremea*, *Eu. microtheca* and *C. ampelina* were pathogenic on these hosts and therefore, these fungal species should be considered a threat to stone fruit

production in South Africa. However, the virulence of all these species was less on plum when compared to apricot.

### 3.2 INTRODUCTION

Members of the family Diatrypaceae are widespread globally, occupying a wide range of hosts as saprophytes or plant pathogens infecting natural ecosystems and cultivated crops (Glawe and Rogers, 1984). Examples of Diatrypaceae species causing extensive damage on natural ecosystems include *Eutypella (Eu.) parasitica* R.W. Davidson & R.C. Lorenz and *Cryptosphaeria (Cr.) populina* (Pers.) Sacc. causing cankers on *Acer pseudoplatanus* L. (Jurc *et al.*, 2006) and *Populus tremuloides* Michx. (Hinds, 1981), respectively. *Eutypa (E.) lata* (Pers.) Tul. & C. Tul. is the most well-known pathogen within the family Diatrypaceae, causing cankers and dieback on cultivated crops. *Eutypa lata* was first described as the causal agent of Eutypa dieback on *Prunus (P.) armeniaca* L. (apricots) in Australia (Carter, 1957) and was later associated with Eutypa dieback of *Vitis (V.) vinifera* L. (grapevine) (Moller and Kasimatis, 1978) and other *Prunus* species including *Prunus salicina* Lindl. (Japanese plum) (Carter, 1982), *Prunus avium* L. (sweet cherry) (Munkvold and Marois, 1994), *Prunus domestica* L. (European plum) (Carter, 1995) and *Prunus dulcis* (Mill.) D.A. Webb (almond) (Rumbos, 1997). The host range and distribution of *E. lata* and other Diatrypaceae species is extending as investigations on this fungal family continue worldwide.

Different species of Diatrypaceae have recently been isolated from multiple hosts including grapevines, fruit and ornamental trees as well as natural ecosystems in areas including California (Trouillas and Gubler, 2010a; Trouillas *et al.*, 2010a), Australia (Pitt *et al.*, 2010; Trouillas *et al.*, 2011) and Brazil (Almeida *et al.*, 2016). Vineyards are usually established in close proximity of natural ecosystems as well as fruit orchards in South Africa and this practice poses risks with regards to availability of Diatrypaceae species inoculum. In addition, the possible inoculum from natural ecosystems and fruit orchards poses problems in efforts to control infections caused by these species in vineyards. Trouillas *et al.* (2010a, 2011) found the same Diatrypaceae species occurring on Eutypa dieback-affected grapevines to also occur abundantly on natural ecosystems. Subsequent pathogenicity tests of isolates of species collected from other woody hosts proved these to be pathogenic to grapevine (Trouillas and Gubler, 2010b; Pitt *et al.*, 2013) which proves that inoculum from neighbouring hosts plays a significant role. In South Africa, the newly discovered Diatrypaceae species, on grapevine, have been isolated from persimmon (Moyo *et al.*, 2016) and stone fruit trees (Damm *et al.*, 2009) with dieback symptoms and cankers, but pathogenicity studies were not conducted for these isolates on grapevine.

The isolation of Diatrypaceae species from necrotic symptoms on declining fruit trees in South Africa indicates the potential involvement of these species in the overall decline and possible death of these hosts. Despite Diatrypaceae species being associated with dieback of stone fruit trees and these hosts being one of the major economically important fruit crops in

South Africa (PHI, 2015), no study has attempted to determine the pathogenicity status of Diatrypaceae species on stone fruit trees, except for *E. lata*, which was shown to be pathogenic on field grown apricot trees and on potted plum and peach trees (Mathee *et al.*, 1974). Stone fruits cultivated in South Africa include the Japanese plum, apricot, sweet cherry, almonds as well as peach and nectarine. The Japanese plum dominates the stone fruit industry in terms of production and export in South Africa and apricots are ranked second with regards to production after plums (Potelwa *et al.*, 2014). Moreover, South Africa is the leading producer of apricots in the Southern Hemisphere (Potelwa *et al.*, 2014). It is therefore, important to investigate the role of Diatrypaceae species in the decline and death of stone fruit trees, which may hinder the production of stone fruit in South Africa.

In light of the discovery of Diatrypaceae species within diverse woody hosts occurring in close proximity to vineyards in South Africa and other countries (Damm *et al.*, 2009; Trouillas and Gubler, 2010a; Trouillas *et al.*, 2010a, 2011; Moyo *et al.*, 2016) and the identification of species such as *Eutypa consobrina* (Mont.) Rappaz and *Cryptovalsa* (C.) *rabenhorstii* (Nitschke) Sacc. from grapevine without finding their fruiting structures (Chapter 2 of this dissertation), it was necessary to investigate other woody hosts in the vicinity of vineyards to determine whether these serve as sources of inoculum to grapevines in South Africa. Furthermore, bearing in mind that *Eutypa dieback* was first detected on apricot and later associated with several other stone fruit trees and the fact that the stone fruit industry contributes significantly to the economy of South Africa, it was therefore, of interest to further determine the role of Diatrypaceae species on dieback and canker development on stone fruit trees. The objectives of this study were therefore to: i) identify species of Diatrypaceae associated with dieback and canker symptoms as well as fruiting bodies on woody hosts occurring in close proximity to vineyards and ii) conduct pathogenicity tests of diatrypaceous fungi on plum and apricot, which are the first and second most produced stone fruits in South Africa, respectively.

### **3.3 MATERIALS AND METHODS**

#### **3.3.1 Collection of samples and fungal isolates**

Samples were collected from diseased twigs, branches and trunks of various fruit and ornamental trees occurring near vineyards in different wine grape producing areas of the Western Cape and Limpopo Provinces of South Africa. Samples were collected from orchards of fruit trees including *Malus domestica* Borkh. (apples), *Pyrus communis* L. (pear), *P. armeniaca*, *P. salicina*, *Prunus persica* (L.) Batsch (peach), *Prunus persica* var. *nucipersica* (Suckow) C.K. Schneid. (nectarine), *P. dulcis* (almond), *P. avium* (cherries), *Punica granatum* L. (pomegranate), *Ficus carica* L. (fig tree), *Citrus limon* (L.) Burm.f. (lemon), *Cydonia oblonga*

Mill. (quince), *Eriobotrya japonica* (Thunb.) Lindl. (loquat), *Morus* sp. (mulberry), *Psidium* (*Ps.*) *guajava* L. (guava), *Diospyros* (*D.*) *kaki* Thunb. (persimmon), *Olea* (*O.*) *europaea* L. (olive) as well as ornamental trees such as *Melia* (*M.*) *azedarach* L. (syringa tree), *Quercus* spp. (oak), *Salix* (*S.*) *mucronata* Andersson (Cape silver willow), *Cinnamomum camphora* (L.) J. Presl. (camphor tree), *Populus* spp. (poplar), *Quercus* sp. (oak), *Quercus suber* L. (cork oak), *Olea oleaster* Hoffmanns. & Link (wild olive), *Callistemon* sp. (bottlebrush), *Curtisia dentata* C.A.Sm. (Assegai tree), *Dalbergia* sp. (blackwood), *Searsia lancea* (L.f.) F.A. Barkley (karee), *Rosa* sp. (rose) and *Schinus* (*Sc.*) *molle* L. (pepper tree). Trees with various disease symptoms including gum formation, cankers, dieback and fruiting bodies were targeted. The sampling methods, however, were not structured in such a way that the same number of samples were collected from each host. Furthermore, the same hosts were also not collected from all the areas where sampling was conducted. This was a result of sampling being conducted on commercial farms as well as gardens of farm workers located in the different grapevine producing areas. Isolations of fungal isolates from collected samples were carried out by cutting wood to reveal necrotic areas after which small pieces of wood were taken from the margins of healthy and diseased tissue and plating onto potato dextrose agar (PDA-C, Biolab, Midrand, South Africa) plates containing chloromycetin (250 mg/L) as described in the previous chapter (Chapter 2). Isolations from fruiting bodies were done as described previously in Chapter 2.

### 3.3.2 Identification of isolates

All isolates collected from symptomatic wood and fruiting bodies of different trees were identified by morphological and molecular techniques. Representative isolates are maintained in the culture collection of the Department of Plant Pathology, University of Stellenbosch (STEU), Stellenbosch, South Africa.

#### 3.3.2.1 Morphological characterisation of sexual morphs

This technique was used to characterise isolates of species found to produce fruiting bodies on wood of different fruit and ornamental trees occurring in close proximity to vineyards. Perithecial contents were mounted in 10% lactic acid, examined microscopically and images were captured with a Nikon DS-Ri2 camera on a Nikon Y-TV55 light microscope at 1000x magnification. Images of stromata and measurements of perithecia diameter were captured using the Nikon DS-Ri2 camera on a Nikon SMZ 1500 stereo microscope. Measurements were made for representative samples of each species (either two or three samples of each species), 5% and 95% confidence intervals were calculated from the measurements of 50 ascospores, 20 asci (length=spore bearing part and width=width at the widest point) and 10 perithecia (diameter). Characteristics such as morphology of perithecia, asci and ascospores;

number of ascospores per ascus as well as colour of ascospores were noted for each species as described by Rappaz (1987), Glawe and Rogers (1984) and Trouillas *et al.* (2011).

### 3.3.2.2 *Morphological and cultural characterisation of asexual morphs*

For characterization of the anamorphic states, one or two isolates were characterized per species (depending on sporulation of the isolate). Three replications of each isolate were inoculated on PDA-C and incubated at 24°C. Visual observations of the colony colour were made, every three days, from the day of inoculation until day 30 after inoculation. Measurements of conidia were carried out at 30 days after inoculation and due to inconsistent sporulation of isolates, conidia size for species was obtained from either one replicate and averaging measurements of up to three replicates, depending on sporulation of isolates.

### 3.3.2.3 *Molecular characterisation*

#### 3.3.2.3.1 DNA extraction

Mycelia from actively growing pure cultures resembling Diatrypaceae species were scraped directly from PDA-C and DNA was extracted using CTAB extraction buffer (2% CTAB, 1 M Tris, pH 7.5; 5 M NaCl; 0.5 M EDTA, pH 8.0) according to the following protocol: Glass beads (0.5 g) and 600 µl of CTAB were added to the mycelium in 2 ml tubes which were then shaken for 7 min at 30 1s<sup>-1</sup> frequency using a Mixer Mill type MM 301 (Retsch GmbH & Co. KG, Germany) and incubated at 65°C for 30 min. Thereafter, 400 µl of chloroform: isoamylalcohol (24:1) was added and tubes were centrifuged at 15 800 x g for 10 min. The supernatant was transferred to fresh tubes and 250 µl of 7.5 M cold ammonium acetate solution and 600 µl cold isopropanol were added. The tubes were inverted to mix the solutions and incubated for 15 min at -20°C before centrifugation at 15 800 x g for 15 min. The supernatant was discarded and 1 ml of cold 70% ethanol added to the pellet before centrifuging at 15 800 x g for 5 min. The supernatant was discarded and the pellets were left to dry at room temperature overnight. Pellets were dissolved in 100 µl double distilled water and stored at 4°C.

#### 3.3.2.3.2 PCR amplification and DNA sequencing

All isolates were first subjected to amplification using species-specific primers to detect *Cryptovalsa ampelina* (Nitschke) Fuckel and *E. lata*. The species-specific primer pair, Camp1/Camp2R designed by Luque *et al.* (2006) was used to detect *C. ampelina* while the primer pair EL1/EL4 (Catal *et al.*, 2007) was used for the detection of *E. lata*. A touch down PCR protocol was used for the amplification of *C. ampelina* and was carried out in a 25 µl reaction containing the following end concentrations: 1X PCR buffer, 1 mM MgCl<sub>2</sub>, 0.2 µM each deoxyribonucleotide triphosphates (dNTPs), 1 mM each primer and 0.65 U Taq polymerase. The touch down PCR protocol consisted of two phases: The first phase included an initial denaturing at 94°C for 5 min, followed by 20 cycles of denaturation at 94°C for 30 s,

annealing at 68°C for 30 s, and extension at 72°C for 30 s. Phase 2 consisted of 10 cycles of 94°C for 30 s, 66°C for 30 s, 72°C for 30 s and a final extension step at 72°C for 5 min. The EL1/EL4 PCR reaction was performed in a total volume of 25 µl consisting of 1X PCR buffer, 3.5 mM MgCl<sub>2</sub>, 0.8 mM dNTPs, 0.5 µM of each primer, 0.65 U Taq Polymerase (Bioline, USA) and 1 µl DNA template. PCR amplification was conducted using the following thermal regime: initial denaturing for 2 min at 94°C, 35 cycles of denaturing at 94°C for 1 min, followed by annealing at 62°C for 30 s, elongation at 72°C for 1 min, final extension at 72°C for 5 min.

For isolates in which amplification could not be obtained using species-specific primers, the internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S ribosomal RNA gene were amplified using primers ITS1/ITS4 (White *et al.*, 1990). Twenty isolates of *C. ampelina* and 25 isolates of *E. lata*, randomly chosen, were also included in this PCR to verify specificity of the species-specific primers. Amplification of the ITS region was performed in a 25 µl reaction volume containing 1X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 1 µg/µl bovine serum albumen, 0.2 mM dNTPs, 0.25 µM each primer and 0.65 U of Bioline Taq polymerase. The PCR reaction consisted of denaturation at 94°C for 3 min, followed by 35 cycles of 30 s at 94°C, 30 s at 52°C, and 60 s at 72°C, and a final extension step at 72°C for 7 min.

Identification of the isolates was based on subjecting the ITS sequence data to the megablast function of the National Center for Biotechnology Information's (NCBI) GenBank nucleotide database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). Depending on the number of isolates identified by the BLAST search, up to 24 representative isolates of each species, including *E. lata* and *C. ampelina*, were selected for amplification of the β-tubulin gene. The partial β-tubulin gene was amplified using the primer set, Bt2a/Bt2b (Glass and Donaldson, 1995). The PCR for the β-tubulin gene consisted of 1X PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.5 µM primers, 0.65 units of Taq DNA polymerase and 1 µl of DNA template and made up to 25 µl with sterile water. The following conditions were set for the thermal cycler: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 5 min.

A GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) was used for all PCR reactions and all PCR products were separated by electrophoresis on 1% agarose gels, stained with ethidium bromide and viewed under UV light. Estimates of amplicon sizes were made against a GeneRuler 100 bp molecular marker (Thermo Scientific, South Africa). The MSB Spin PCRapase kit (Invitex, Berlin, Germany) was used to purify PCR products. The amplicons of both ITS and β-tubulin were sequenced in both directions using an ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA) with the primers used in the initial PCR reactions. The products were then analysed on an ABI Prism 3130XL DNA sequencer (Perkin-Elmer, Norwalk, CN).

### 3.3.2.3.3 *Phylogenetic analysis*

The phylogenetic analyses for the combined datasets (ITS and  $\beta$ -tubulin) were performed using Maximum likelihood (ML) and Bayesian analyses. The ITS and  $\beta$ -tubulin sequences of representative isolates obtained in this study were respectively aligned with sequences of Diatrypaceae taxa retrieved from GenBank as well as the outgroup *Hypoxylon fragiforme*. Sequences were aligned with MAFFT v7.017 (Kato *et al.*, 2002) in Geneious v8.1 (<http://www.geneious.com>, Kearse *et al.*, 2012), using the default parameters. The alignments were run through Gblocks v0.91b, using default parameters (Castresana, 2000) to exclude poorly aligned positions from subsequent phylogenetic inferences.

Maximum likelihood analyses were performed using PhyML (Guindon and Gascuel, 2003) in Geneious v8.1 under the general time reversible (GTR) model. Both the proportion of invariable sites and gamma distribution parameter were estimated and bootstrap support values were calculated from 1000 replicates. Clades with bootstrap support  $\geq 70\%$  were considered significant and highly supported (Hillis and Bull, 1993). Bayesian analyses were done with Mr Bayes v3.2.2 (Huelsenbeck and Ronquist, 2001) using the Markov Chain Monte Carlo (MCMC) method. Four MCMC chains were run simultaneously and trees were saved every 100th generation. The first 25% of generated trees were discarded as the burn-in phase of the analyses. Posterior probabilities were assigned after a 50% majority-rule. Clades with Bayesian posterior probability  $\geq 95\%$  were considered significant (Alfaro *et al.*, 2003).

### 3.3.3 Pathogenicity tests on apricot and plum

A total of 15 isolates, representing five different species of Diatrypaceae (Table 1), were plated on PDA-C and incubated at 24°C for 2 weeks for use in the pathogenicity tests. Inoculations were made on shoots, from the previous year growth, of field-grown apricots (cv. 'Belida') and plums (cv. 'Pioneer'). Non-colonised PDA plugs were used to inoculate control shoots. A total of 10 shoots (per host) were used for each isolate and a 4-mm cork-borer was used to wound the shoots before a colonized agar plug, cut from a 2-week-old culture, was placed in each wound which was then covered with Parafilm. Inoculated shoots were removed from the trees and taken to the laboratory for isolations after 5 months. Before isolation, the shoots were split longitudinally through the inoculation point and the internal discolouration (referred here as lesions) measured from both directions of the inoculation point. Isolations were then carried out by cutting 12 small wood pieces (~ 1x1 mm) along the lesions onto PDA-C in Petri dishes. The dishes with wood pieces were incubated at 24°C for  $\geq 4$  weeks at approximately 12 h of daylight and 12 h of darkness, checked every third day and isolates of Diatrypaceae species were transferred onto new PDA-C dishes. Representative isolates of each species were



subjected to DNA extraction and sequencing of the  $\beta$ -tubulin gene, as described above, to confirm that the species re-isolated were the ones used for inoculation.

### 3.3.3.1 Pathogenicity trial layout and statistical analysis

The layout of the pathogenicity trial was a randomized block design. The data on lesion length was subjected to one-way analysis of variance (ANOVA) using General Linear Model in SAS version 9.2 (SAS Institute, Cary, North Carolina USA). Fischer's least significance Difference (LSD) test was then used to compare means of lesion lengths of isolates and F-values with  $P < 0.05$  were considered significant.

## 3.4 RESULTS

### 3.4.1 Collection of samples and fungal isolates

A total of 289 isolates of Diatrypaceae were obtained from diseased wood of plant hosts occurring adjacent to vineyards in the Western Cape and Limpopo Provinces in South Africa, of which 46 isolates were isolated from wood of stone fruit trees. Of the sampled woody hosts ( $n=39$ ), 82% yielded strains of Diatrypaceae. Peach and nectarine were among woody hosts which did not yield any Diatrypaceae isolates.

### 3.4.2 Identification of isolates

The topology of the trees obtained with ML and Bayesian analyses was similar, with regards to the clades/subclades in which isolates obtained in this study resided, for the individual gene areas as well in combined analyses. Based on phylogenetic analysis, 14 Diatrypaceae species were identified in this study namely *Cryptosphaeria ligniota* (Fr.: Fr.) Auersw., *Cryptosphaeria multicontinentalis* Trouillas, F. Peduto, Inderb. and Gubler, *C. ampelina*, *Diatrypella* sp., *E. consobrina*, *E. lata*, *E. cremea*, *Eutypella australiensis* Trouillas, Sosnowski & Gubler, *Eutypella citricola* Speg., *Eutypella leprosa* (Pers. ex Fr.) Berl., *Eutypella microtheca* Trouillas, W.M. Pitt & Gubler and three unidentified *Eutypella* spp. (Fig. 1).

*Eutypa lata* and *Eu. citricola* were the most commonly isolated species representing 34% and 25% of the total isolates, respectively. The newly described *E. cremea* (Chapter 2) was the third most commonly isolated taxon (15% of total isolates), followed by *Eu. microtheca* (10%) and *C. ampelina* (7%) while 9% was made up of the rest of the taxa together. As expected, *E. lata* was isolated from a large diversity of plant hosts (22 host species) compared to the other taxa, followed by *Eu. citricola* and *C. ampelina* with 15 host species each, *E. cremea* (12) and *Eu. microtheca* (11). *Cryptosphaeria multicontinentalis*, *E. consobrina* and *Eu. australiensis* were each isolated from three plant species whereas *Eu. leprosa* was isolated from two plant species. Diatrypaceae species which were isolated from one host

species each include *Diatrypella* sp., *Cr. ligniota* and the three unnamed *Eutypella* species. The diversity of hosts colonised by each Diatrypaceae species obtained in this study is shown in Fig.1.

The most abundant species obtained from isolations from stone fruit trees was *Eu. lata* with 26 isolates collected, followed by *C. ampelina* with seven isolates. *Eutypella citricola* and *E. cremea* had five isolates each whereas three isolates were obtained for *Eu. microtheca*. Although apricot and plum trees were colonised by the same species of Diatrypaceae, the number of isolates collected from these hosts varied, with higher numbers of isolates obtained from apricot (27 isolates). Plum had the second highest number of isolates (10), followed by almond and cherry with five and four isolates, respectively. No perithecia of any of these fungal species were observed on stone fruit trees in this study.

#### 3.4.2.1 Taxonomy

Eight of the 14 Diatrypaceae species identified were found to produce perithecia on different woody hosts. The morphology of one of the eighth species, namely *E. cremea*, was described in the previous chapter. Below are the descriptions of the remaining seven species:

*Cryptovalsa ampelina* (Nitschke) Fuckel, Symbolae Mycologicae, Beiträge Zur Kenntnis Der Rheinischen Pilze: 212, 1870. (Fig. 2).

*Stromata* poorly developed and embedded on bark and/or wood. *Perithecia* globose to ovoid, (439–) 456–862 (–941)  $\mu\text{m}$  diam, necks infrequently observed protruding singly or in groups. *Asci* polysporous, clavate, long stipitate, have apical invagination, (64–) 73–140 (–147)  $\times$  (8–) 9–13 (–14)  $\mu\text{m}$ . *Ascospores* allantoid, pale brown, (7–) 8–12 (–14)  $\times$  2–3  $\mu\text{m}$ .

*Cultural characteristics on PDA-C*: colonies are white and the underside is cream. Conidia were not seen in this study.

*Hosts*: *Cinnamomum camphora*; *Ficus carica*; *Vitis vinifera*.

*Specimen examined*: SOUTH AFRICA: Western Cape Province, Grabouw, Fraaiuitzchit farm, on dead branch of *Cinnamomum camphora*, December 2014, P. Moyo, culture number STEU 8209.

*Additional specimens*: Durbanville, on dead wood of *Vitis vinifera*, August 2014, P. Moyo, culture number STEU 8120.

*Notes*: A higher extreme asci length was observed compared to that reported by Trouillas *et al.* (2011) and Mostert *et al.* (2004) of 135.80  $\mu\text{m}$  and 125  $\mu\text{m}$ , respectively.

*Eutypa lata* (Pers.) Tul. & C. Tul., Selecta Fungorum Carpologia: Xylariei- Valsei- Spaeriei 2: 56, 1863. (Fig. 3).

*Stromata* on bark and wood. *Perithecia* oval, (307–) 334–497 (–553)  $\mu\text{m}$  diam. *Ostioles* not sulcate. *Asci* spindle shaped, 8-spored, long stipitate, truncate at apex, (26–) 32–69 (–83)  $\times$  6–7 (–8)  $\mu\text{m}$ . *Ascospores* allantoid, 7–10 (–13)  $\times$  1–2  $\mu\text{m}$ .

*Cultural characteristics on PDA-C*: mycelium is cottony which later turns grey to black, the underside of colonies are cream. *Conidia* single-celled, filiform, (13–) 27– 43 (–49)  $\times$  1–2  $\mu\text{m}$ .

*Hosts*: *Morus* sp., *Quercus* sp., *Quercus suber*, *Salix mucronata*, *Vitis vinifera*.

*Specimen examined*: SOUTH AFRICA: Western Cape Province, Durbanville, Morgenster farm, on dead wood of *Quercus* sp., April 2014, P. Moyo, culture number STEU 8174.

*Additional specimens*: SOUTH AFRICA: Western Cape Province, Durbanville, Hooggelegen, on dead wood of *Vitis vinifera*, August 2014, P. Moyo, culture number STEU 8218; Durbanville, Morgenster farm, on dead wood of *Quercus* sp., August 2014, P. Moyo, culture number STEU 8176; Constantia, on dead wood of *Quercus suber*, June 2014, P. Moyo, culture number STEU 8219.

*Eutypa consobrina* (Mont.) Rappaz, Mycologia Helvetica 2: 349, 1987. (Fig. 4).

*Stromata* on decorticated wood. *Perithecia* scattered, globose to ovoid, (249–) 262–451 (–468)  $\mu\text{m}$  diam. *Ostioles* sulcate, singly erumpent or in groups of two. *Asci* clavate, with an apical ring, 8-spored, long-stiped, (25–) 27–69 (–82)  $\times$  5–7  $\mu\text{m}$ . *Ascospores* slightly curved to allantoid, pale brown, 6–10 (–11)  $\times$  (1–) 1.5–3  $\mu\text{m}$ .

*Cultural characteristics on PDA-C*: colonies flat, yellowish in colour and with little mycelium. *Conidia* hyaline, (12–) 13–24  $\times$  1.5–2  $\mu\text{m}$ .

*Hosts*: *Quercus* sp., *Dalbergia* sp.

*Specimen examined*: SOUTH AFRICA: Western Cape Province, Constantia, Buitenverwachting, on dead wood of *Dalbergia* sp., June 2014, P. Moyo, culture number STEU 8155.

*Additional specimen*: SOUTH AFRICA: Western Cape Province, Durbanville, Morgenster farm, on dead wood of *Quercus* sp., April 2014, P. Moyo, culture number STEU 8153.

*Eutypella australiensis* Trouillas, Sosnowski & Gubler, Mycosphere 1: 187, 2010. (Fig. 5).

*Stromata* poorly developed, on decorticated wood, raises substrate surface. *Perithecia* embedded on wood, spherical to ovoid, (260–) 284–451 (–468)  $\mu\text{m}$  diam. *Ostioles* sulcate, emerging singly or in groups of two on the surface. *Asci* clavate, long-stiped, octosporous, (37–) 38–56 (–66)  $\times$  (5–) 6–8  $\mu\text{m}$ . *Ascospores* yellowish to almost olive green, allantoid, 7–10  $\times$  (1–) 2–3 (–3.5)  $\mu\text{m}$ .

*Cultural characteristics on PDA-C*: colonies grow rapidly covering a 90 mm diam. Petri dish within 7 days. Mycelium white but turns grey to black starting at the centre of the colony. *Conidia* filiform, (15–) 16–21 (–26)  $\times$  1–1.5  $\mu\text{m}$ .

*Hosts*: *Dalbergia* sp., Unknown host

*Specimen examined:* SOUTH AFRICA: Western Cape Province, Hout Bay, on dead branch of an unknown host, September 2014, P. Moyo, culture number STEU 8203.

*Additional specimen:* SOUTH AFRICA: Western Cape Province, Constantia, Buitenverwachting, on dead branch of *Dalbergia* sp., June 2014, P. Moyo, culture number STEU 8204.

*Eutypella leprosa* (Pers.) Berl.: 74, 1902. (Fig. 6)

*Stromata* on wood and decorticated wood, raising periderm to appear swollen. *Perithecia* circular to ovoid, surrounded by white entostroma, (498–) 529–796 (–820)  $\mu\text{m}$  diam, necks protruding in groups through the cracked periderm. *Ostioles* sulcate. *Asci* clavate, long stipitate, with an apical ring, 8-spored, (40–) 42–75 (–98)  $\times$  (6–) 7–10 (–11)  $\mu\text{m}$ . *Ascospores* slightly curved to allantoid, yellow to olive green, (7–) 8–11 (–12)  $\times$  2–3  $\mu\text{m}$ .

*Hosts:* *Ficus carica*, Unknown host

*Cultural characteristics on PDA-C:* colonies white, covering 90 mm diam. Petri dish within 7 days. *Conidia* filiform, (17–) 18–25 (–26)  $\times$  1–2  $\mu\text{m}$ .

*Specimen examined:* SOUTH AFRICA: Western Cape Province, Hout Bay, on dead wood of *Ficus carica*, September 2014, P. Moyo, culture number STEU 8189.

*Additional specimen:* SOUTH AFRICA: Western Cape Province, Constantia, Groot Constantia, on dead wood of an unknown host, June 2014, P. Moyo, culture number STEU 8192.

*Eutypella citricola* Speg., Anales del Museo Nacional de Buenos Aires 6: 245, 1898. (Fig. 7).

*Stromata* on wood or bark, in pustules unevenly distributed or grouped. *Perithecia* spherical to ovoid, (280–) 306–585 (–603)  $\mu\text{m}$  diam., surrounded by white powdery layer. *Ostioles* apparent on surface, emerging singly or in groups, sulcate. *Asci* octosporous, clavate, long-stiped, (34–) 36–61 (–73)  $\times$  (5–) 5.5–8 (–9)  $\mu\text{m}$ . *Ascospores* pale yellow, allantoid, (7–) 8–10 (–12)  $\times$  2–3  $\mu\text{m}$ .

*Cultural characteristics on PDA-C:* white mycelium but turning black with age starting from the centre of colony, black colour more apparent from beneath the colony. The colony covers 90 mm diameter Petri dish within 7 days at 24°C. *Conidia* filiform, (13–) 14–19 (–20)  $\times$  1–2  $\mu\text{m}$ .

*Hosts:* *Diospyros kaki*, *Ficus carica*, *Melia azedarach*, *Quercus* sp., *Vitis vinifera*, unknown host.

*Specimen examined:* SOUTH AFRICA: Western Cape Province, Stellenbosch, on dead wood of *Quercus* sp., March 2013, P. Moyo, culture number STEU 8183.

*Additional specimens:* SOUTH AFRICA: Western Cape Province, Bonnievale, Merwespont, on wood of *Diospyros kaki*, June 2014, P. Moyo, culture number STEU 8179,

Durbanville, Diemersdal, on dead wood of *Vitis vinifera*, August 2014, P. Moyo, culture number STEU 8220.

*Eutypella microtheca* Trouillas, W. M. Pitt & Gubler, Fungal Diversity 49: 217, 2011. (Fig. 8)

*Stromata* on wood and bark, pustular and cracking the periderm. *Perithecia* circular to ovoid, surrounded by white powdery layer, (273–) 307–655 (–656)  $\mu\text{m}$  diam. *Ostioles* sulcate, emerging through cracked periderm in groups. *Asci* long stipitate, clavate, octosporous, (33–) 35–65 (–94)  $\times$  6–9  $\mu\text{m}$ . *Ascospores* pale yellow, allantoid to sub allantoid, (6–) 7–11 (–12)  $\times$  2–3  $\mu\text{m}$ .

*Cultural characteristics on PDA-C*: colonies start as white turning pink in colour with age. Colonies cover 90 mm diam. PDA-C dish within 7 days. *Conidia* were not observed.

*Hosts*: *Melia azedarach*, *Morus* sp., *Vitis vinifera*.

*Specimen examined*: SOUTH AFRICA: Western Cape Province, Franschoek, Allee Bleue, on dead branch of *Morus* sp., September 2014, P. Moyo, culture number STEU 8195.

*Additional specimens*: SOUTH AFRICA: Western Cape Province, Calitzdorp, on dead wood of *Vitis vinifera*, October 2014, P. Moyo, culture number STEU 8111; Wellington, on dead wood of *Melia azedarach*, September 2014, P. Moyo, culture number STEU 8196.

*Notes*: A higher extreme spore bearing part for asci (94  $\mu\text{m}$ ) and ascospore (12  $\mu\text{m}$ ) length is reported here compared to that reported by Trouillas *et al.* (2011) of 60  $\mu\text{m}$  and 11  $\mu\text{m}$ , respectively.

### 3.4.3 Pathogenicity tests on apricot and plum

Isolates of Diatrypaceae species inoculated on apricot and plum produced lesions within 5 months and mean comparison tests showed that all isolates, except *Eu. microtheca* STEU 6086 on apricot, produced lesions significantly longer than the controls (Table 1). Lesions produced by isolates of different species, on apricot and plum were brown-red in colour (Figs. 9 and 10) which is characteristic of symptoms of *Eutypa dieback* of apricot (Carter and Moller, 1977). Results showed that apricot was more susceptible to the inoculated diatrypaceous species compared to plum (Table 1). Mean lesion lengths ranged from 28.93 mm to 65.95 mm on apricot and 16.34 mm to 35.49 mm on plum. Significant differences in lesion lengths were found among species as well as within species for both apricot ( $F = 5.87$ ,  $P < 0.0001$ ) and plum ( $F = 5.92$ ,  $P < 0.0001$ ). Not all isolates, however, performed similarly on apricots and plum. For example, *E. lata* (STEU 6084) was among the four most virulent isolates on apricot but was the least virulent isolate on plum and *Eu. microtheca* (STEU 6086) was the least virulent on apricot and produced lesions similar to that of the control, but was the second most virulent on plum. All isolates were re-isolated at frequencies of 50-100% but no Diatrypaceae species were isolated from the control shoots (Table 1).

### 3.5 DISCUSSION

This study presents the first comprehensive investigation and characterisation of the Diatrypaceae, using both morphology and molecular data, on non-grapevine woody hosts in South Africa. Consequently, 14 species residing in five genera were found in the woody hosts sampled and were isolated from dieback symptoms directly associated with pruning wounds and/or natural openings. Of these species, the most frequently encountered were *E. lata*, *Eu. citricola*, *E. cremea*, *C. ampelina* and *Eu. microtheca*, which were also the most abundant species isolated from grapevine (Chapter 2). These findings support previous reports on the capability of Diatrypaceae species to infect and complete their life cycle on multiple hosts (Trouillas *et al.*, 2010a, 2011). The discovery of a single species on multiple hosts provides circumstantial evidence that the inoculum travels between different woody hosts and grapevines. However, it is likely that not only does these woody hosts serve as sources of inoculum to grapevines, but several hosts occurring in close proximity can provide inoculum to each other.

Although there was no structured sampling method used, this study revealed that colonisation by fungal species varied among woody hosts. For instance, the highest level of diversity was found on *Sc. molle* and the least diverse assemblage of species of Diatrypaceae was found on hosts including *O. europaea*, *O. oleaster*, *Rosa* spp. and *P. dulcis*. Apart from the uneven sampling strategy used, the differences in the diversity of Diatrypaceae species colonising different hosts could probably be a result of host defense mechanisms, with some hosts possessing biochemical characteristics that limit extensive colonisation by the fungal species and/or probably the absence of inoculum around certain hosts.

The present study revealed a number of new hosts and also extends the geographical distribution for some of the Diatrypaceae species. For example, *Eu. australiensis* was isolated from necrotic areas inside the wood of *Ps. guajava* as well as perithecia on *Dalbergia* sp. and an unknown host in the current study. This species has only been reported on *Acacia longifolia* (Andr.) Wild. in South Australia (Trouillas *et al.*, 2010b). *Eutypella microtheca* represents another example of Diatrypaceae species that was isolated from new hosts which include *P. armeniaca*, *M. azedarach* and *Morus* sp. This fungus was previously reported from *V. vinifera* in Australia and Mexico (Trouillas *et al.*, 2011; Paolinelli-Alfonso *et al.*, 2015; Chapter 2), *D. kaki* in South Africa (Moyo *et al.*, 2016), *Citrus paradisi* Macfad. and *Ulmus procera* Salisb. in Australia (Trouillas *et al.*, 2011) as well as from an unidentified plant in Brazil (Almeida *et al.*, 2016). *Cryptosphaeria multicontinentalis* was reported on *Populus* spp. from California, Australia and Argentina and was considered to be specific to these hosts (Trouillas *et al.*,

2015). In the present study *Cr. multicontinentalis* was found on three host species namely *Populus alba* L., *S. mucronata* and *Morus* sp., although it was most frequently encountered in necrotic areas inside the wood of *S. mucronata*. This is, therefore, the first report of *Cr. multicontinentalis* on *S. mucronata* and *Morus* sp. Such expansion of host range suggests that broad sampling across the globe might be required to fully comprehend the host associations and distribution of the Diatrypaceae before any conclusions concerning these two parameters are drawn.

*Eutypa lata* is a well-known pathogen of apricot and other stone fruit tree species in different countries including South Africa (Carter, 1957; Mathee *et al.*, 1974; Munkvold and Marois, 1994; Rumbos, 1997). Results of this study also showed that *E. lata* was the most dominant species isolated from diseased stone fruit trees, mostly from apricot, and the most pathogenic on apricot. These results indicate that *E. lata* is still an important pathogen of apricot in South Africa. Isolates of *C. ampelina*, *E. cremea*, *Eu. citricola* and *Eu. microtheca* were also pathogenic on apricot in this study, with some isolates of these species producing lesions equivalent to those of *E. lata* isolates. Apart from *C. ampelina* being isolated from apricot in California (Trouillas *et al.*, 2010a) and South Africa (Damm *et al.*, 2009), none of the three remaining species found on stone fruit in this study have been linked to these hosts previously. There were differences in virulence among isolates of *C. ampelina* and *Eu. microtheca* on apricots. Differences in pathogenicity among strains of Diatrypaceae species is, however, not surprising given that such differences have been documented for species including *E. lata* and *C. ampelina* on grapevine (Péros *et al.*, 1997; Trouillas and Gubler, 2010b). Regardless, this is the first demonstration of pathogenicity of *C. ampelina*, *E. cremea*, *Eu. citricola* and *Eu. microtheca* on stone fruit trees.

All isolates used in pathogenicity tests were pathogenic on plum however, their aggressiveness on plum was less compared to apricot. Based on the number of isolates obtained and pathogenicity tests in this study, it seems that apricot is more susceptible to Diatrypaceae infection compared to plum in South Africa. Interestingly, it was also noted that apricot was more sensitive to wounding in comparison to plum. The isolation of Diatrypaceae species from necrotic areas of active cankers of stone fruit trees and results of pathogenicity tests in this study indicate the pathogenic status of these species. Thus, these Diatrypaceae species can be considered to be a threat to stone fruit production in South Africa. It would, however, seem that peach and nectarine are not affected by Diatrypaceae species and this is shown by the failure to detect these species from these hosts plants in this study and the lack of reports of natural infection of these two hosts in literature. *Eutypa lata* isolates have, however, been shown to be pathogenic to peach under artificial inoculation (Mathee *et al.*, 1974; English and Davis, 1978; Munkvold, 2001). The results of this study can be used for

future research aimed at managing infections caused by Diatrypaceae species on stone fruit trees in South Africa.

Diatrypaceae species have been isolated from a number of hosts occurring near vineyards in South Africa and several of these species have been proved to be pathogenic to hosts from which they were isolated. Pathogenicity of *E. lata* has been widely established on several hosts in South Africa including pear (Cloete *et al.*, 2010), persimmon (Moyo *et al.*, 2016) as well as stone fruits (Mathee *et al.*, 1974; this study). Results from a previous study by Moyo *et al.* (2016) and the current study, show that some of the newly discovered Diatrypaceae species are pathogenic to persimmon and *Prunus* spp., respectively, in South Africa. Apart from being inoculum sources to adjacent vineyards, these hosts also serve as additional primary hosts for the survival of the pathogens in the absence of vineyards. Results emerging from previous studies have shown that isolates of Diatrypaceae species, such as *Eu. microtheca* and *C. ampelina*, obtained from other woody hosts are pathogenic and able to produce vascular necrosis similar in length to that of *E. lata* and can colonise grapevine tissue to the same extent as *E. lata* (Trouillas and Gubler, 2010b; Pitt *et al.*, 2013). On the other hand, the status of the newly discovered Diatrypaceae species as grapevine pathogens in South Africa is unclear and their relative importance in South African vineyards is unknown.

The purpose of this study was to determine whether woody hosts occurring close to vineyards are colonised by Diatrypaceae species, usually associated with Eutypa dieback of grapevine as well as determine the role of Diatrypaceae species on stone fruit trees in South Africa. By exploring different hosts occurring in gardens and natural ecosystems, 14 Diatrypaceae species were identified. Given that the five most common species identified in this study were also found to be the most dominant species in the grapevine surveys, it is possible that there are infection routes between grapevine and other woody hosts as well as among the different woody hosts. Results of pathogenicity tests in the present study showed that all Diatrypaceae isolates tested could cause brown-red lesions, typical of Eutypa dieback, on apricot and plum shoots. However, no cankers were observed and this could be explained by the short time of the pathogenicity tests. Further experiments with longer incubation periods are, therefore, required to confirm the potential of the tested species to cause cankers as well as foliar symptoms, such as those from which they were isolated.



### 3.6 REFERENCE LIST

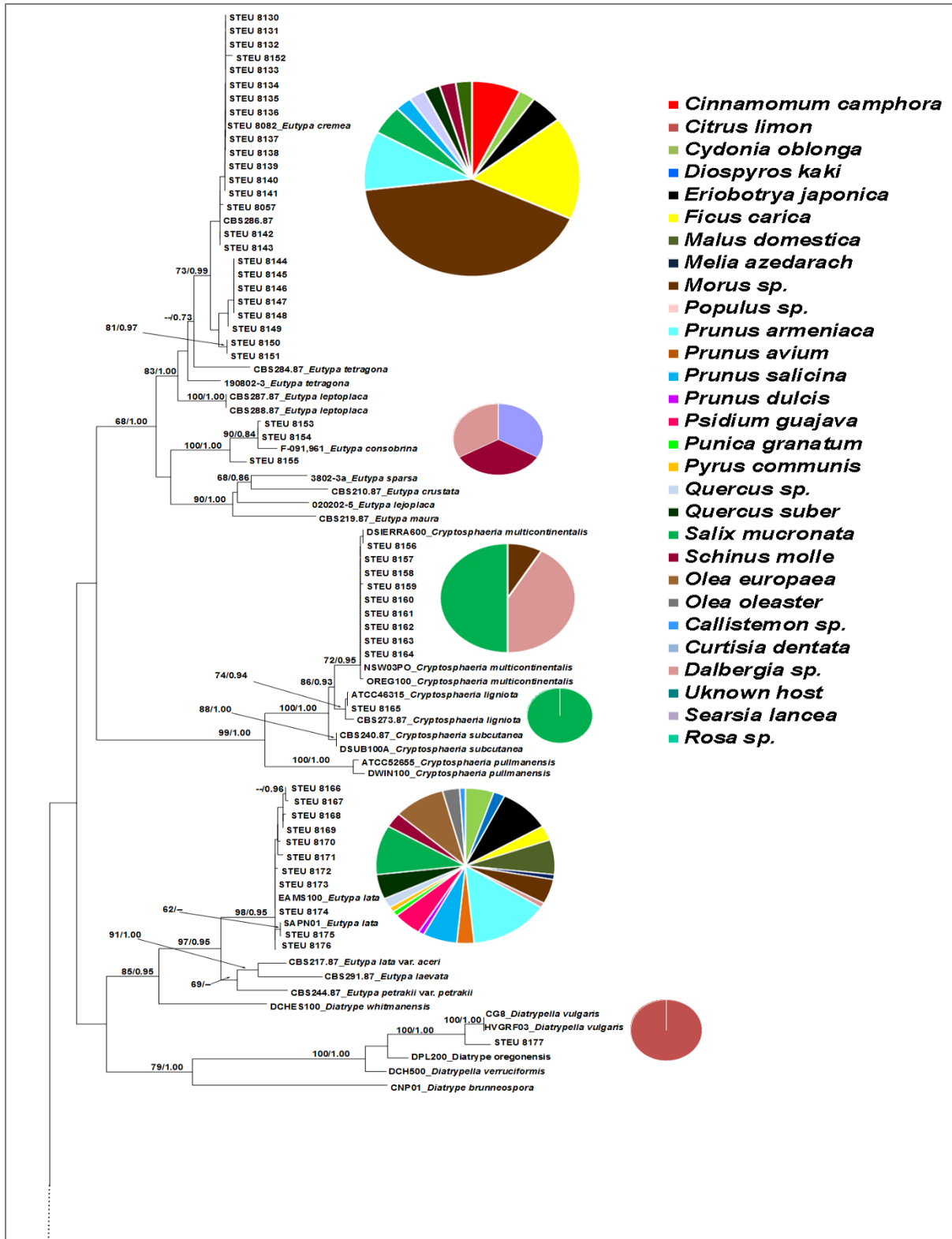
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**Table 1.** Mean lesion lengths on apricot and plum shoots, five months after artificial inoculation of isolates of Diatrypaceae species, and re-isolation percentages of individual isolates from inoculated shoots.

Species	STEU number	Origin of isolate	Host	Apricot		Plum	
				Mean lesion length (mm) <sup>a</sup>	Re-isolation percentage	Mean lesion length (mm) <sup>a</sup>	Re-isolation percentage
<i>Cryptovalsa ampelina</i>	5947	Paarl	Plum	53.37 a-d	100	21.07 c-e	60
<i>Cryptovalsa ampelina</i>	6087	Robertson	Apricot	39.79 ef	90	17.49 de	70
<i>Cryptovalsa ampelina</i>	8056	Bonnievale	Apricot	47.69 b-e	90	24.47 b-d	60
<i>Eutypa lata</i>	6081	Montagu	Apricot	56.52 a-c	80	20.41 c-e	100
<i>Eutypa lata</i>	6082	Robertson	Apricot	65.95 a	90	29.22 ab	90
<i>Eutypa lata</i>	6084	Franschhoek	Plum	58.56 ab	90	16.34 e	100
<i>Eutypa cremea</i>	5832	Stellenbosch	Plum	49.34 b-e	80	20.29 c-e	100
<i>Eutypa cremea</i>	6085	Montagu	Apricot	49.29 b-e	90	20.12 c-e	90
<i>Eutypa cremea</i>	8057	Bonnievale	Apricot	45.44 b-e	100	18.82 de	80
<i>Eutypella citricola</i>	5948	Mookgophong	Apricot	36.95 ef	70	22.30 b-e	80
<i>Eutypella citricola</i>	8054	Bonnievale	Plum	43.33 c-e	70	17.77 de	80
<i>Eutypella citricola</i>	8055	Bonnievale	Apricot	40.49 d-f	50	27.97 a-c	70
<i>Eutypella microtheca</i>	6086	Robertson	Apricot	28.93 fg	70	32.54 a	100
<i>Eutypella microtheca</i>	5949	Mookgophong	Plum	40.56 d-f	100	18.28 de	80
<i>Eutypella microtheca</i>	8058	Calitzdorp	Apricot	63.58 a	100	35.49 a	60
PDA plug	—	—		18.09 g	0	4.82 f	0
LSD ( $P < 0.05$ )				13.21		7.95	

<sup>a</sup>Means followed by the same letter in the same column are not significantly different ( $P < 0.05$ ).



**Figure 1.** Maximum likelihood tree derived from the combined analysis of ITS and  $\beta$ -tubulin sequence data. Maximum likelihood bootstrap support values (calculated from 1000 replicates) and Bayesian posterior probability scores are given at the nodes. *Hypoxylon fragiforme* served as an outgroup. Isolates sequenced in this study are prefixed with STEU. Pie charts indicate the hosts from which species were isolated.

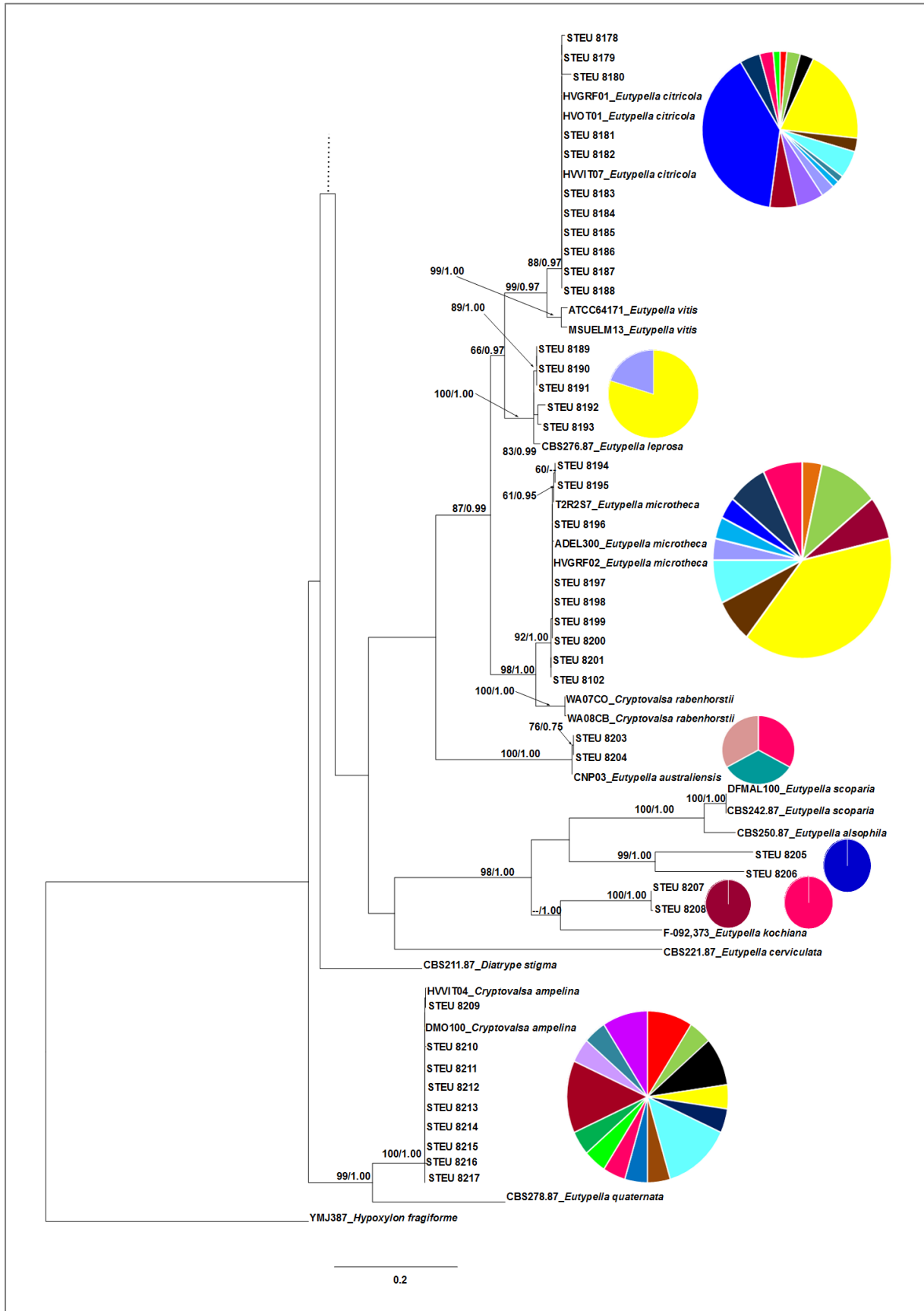
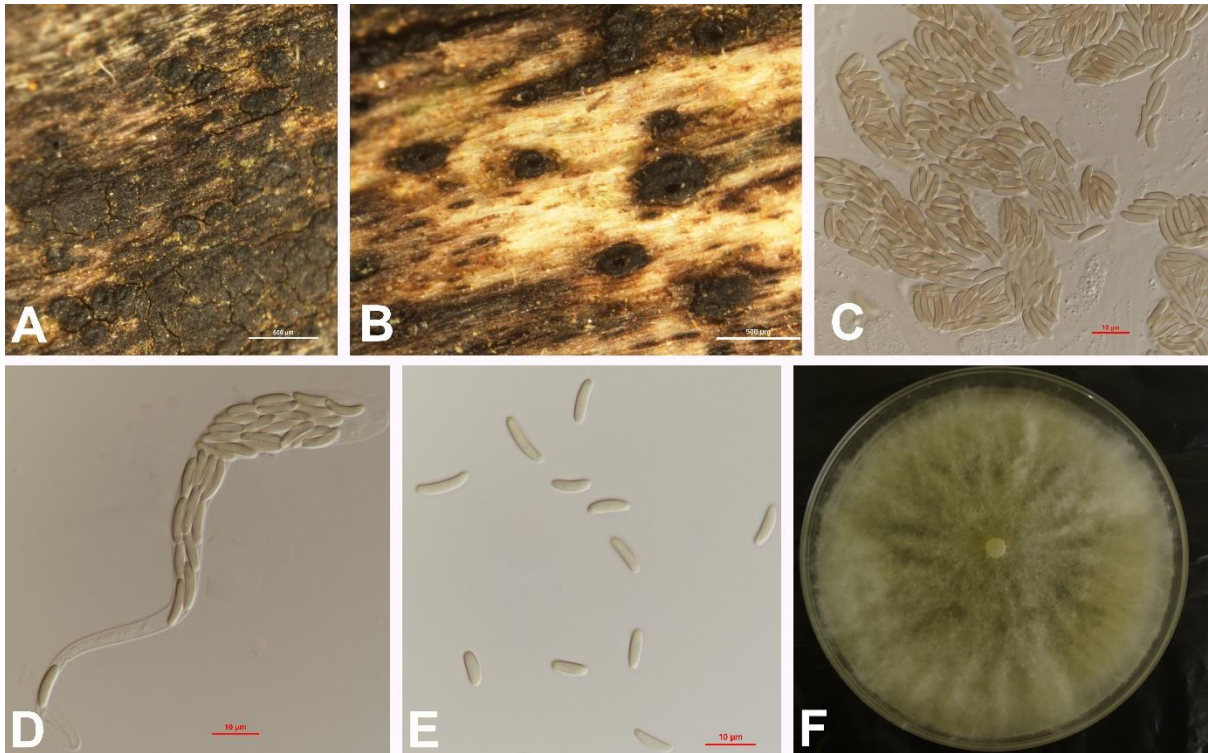
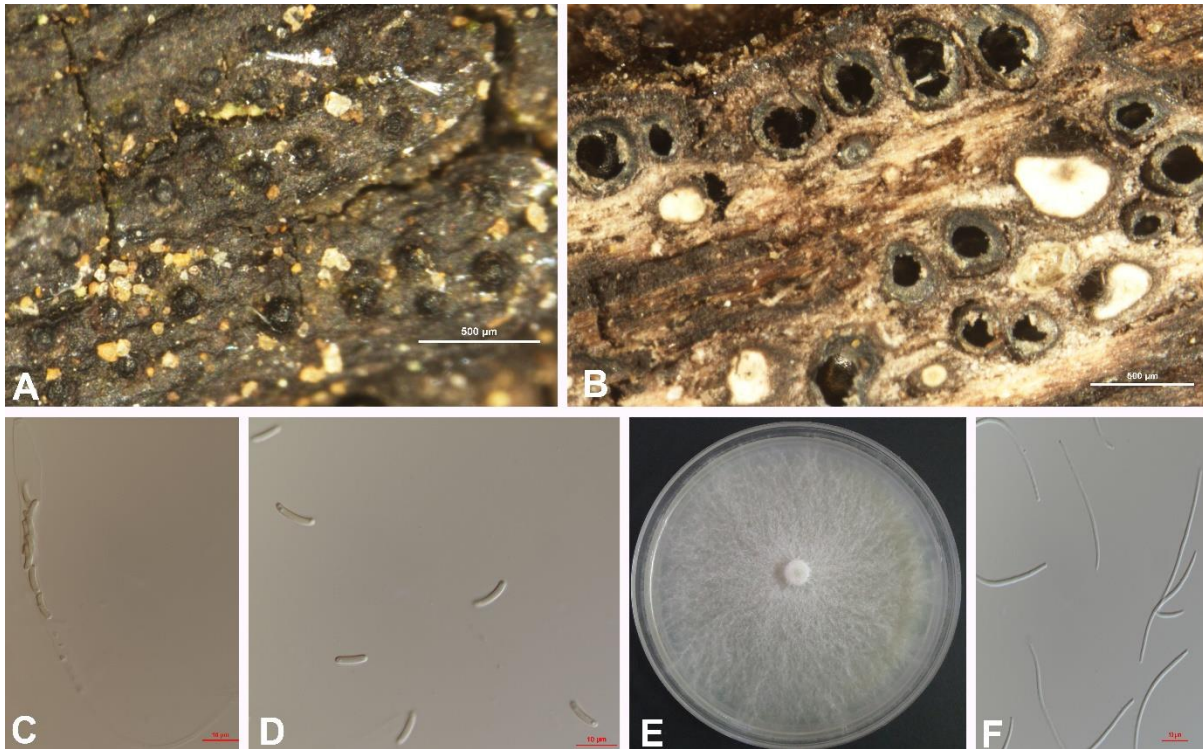


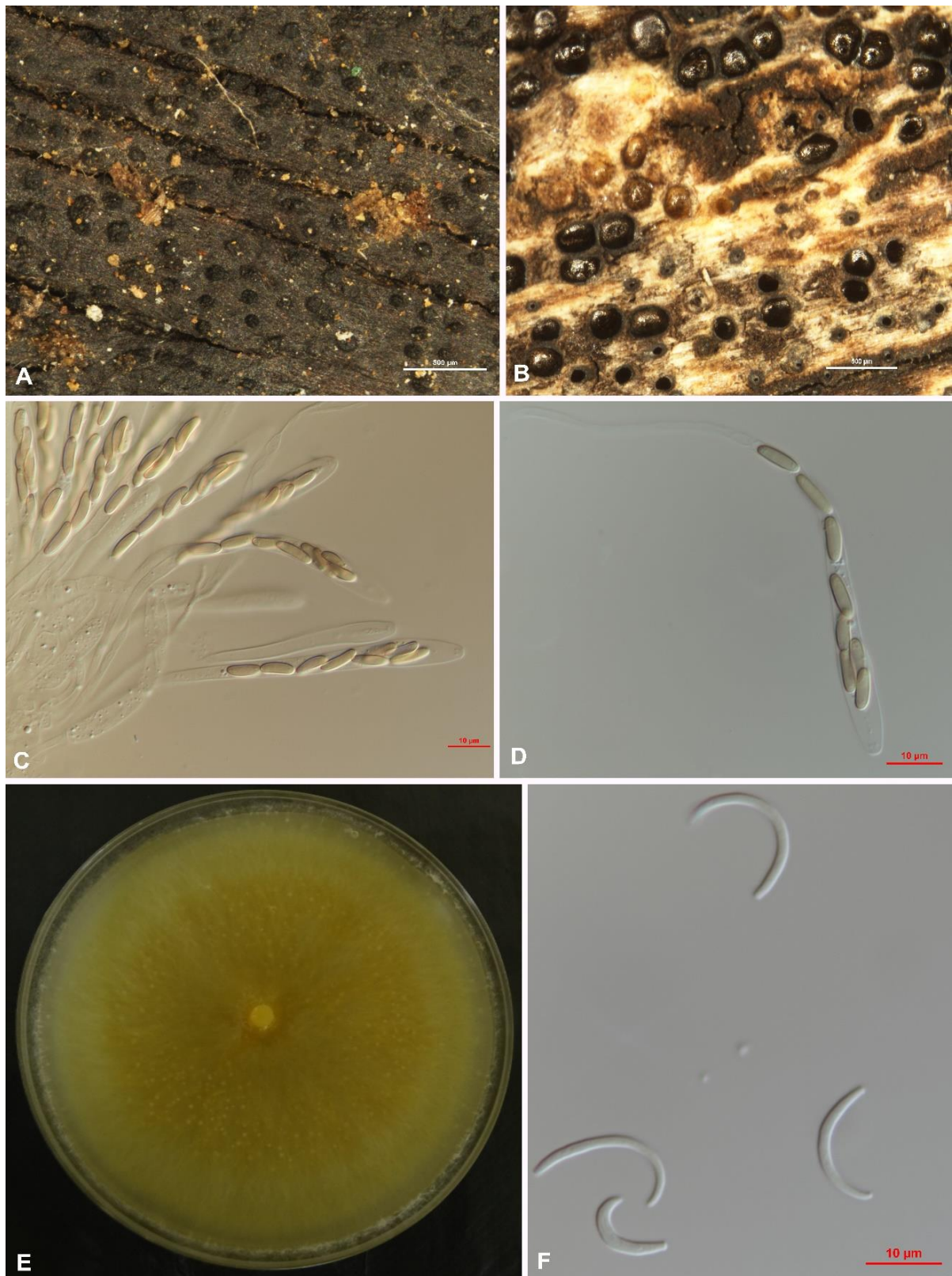
Figure 1. (continued)



**Figure 2.** Morphology of *Cryptovalsa ampelina* on wood of *Cinnamomum camphora*. **A.** Stromata on wood. **B.** Ostioles protruding on wood surface. **C.** Ascospore masses. **D.** Clavate, polysporous ascus. **E.** Allantoid to suballantoid ascospores. **F.** Colony on PDA-C after 15 days growing at 24°C under intermittent light (12 hours).

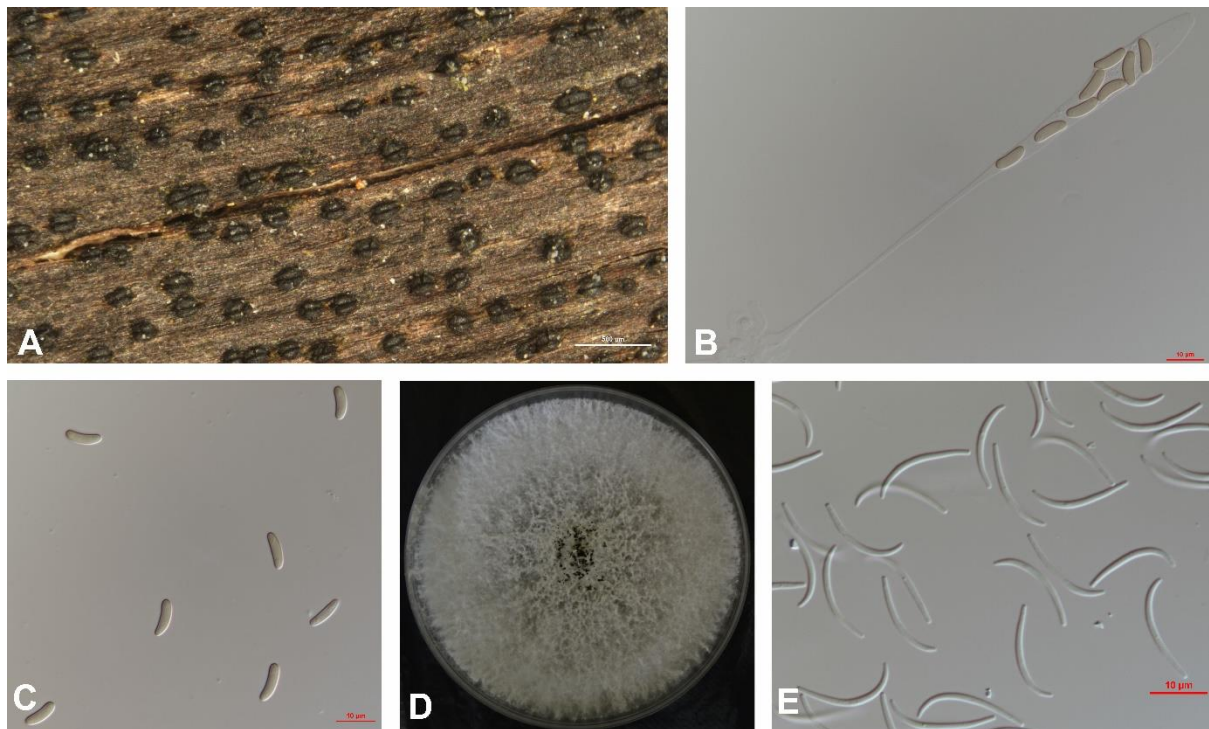


**Figure 3.** Morphology of *Eutypa lata* on dead wood of *Quercus sp.* **A.** Stromata on wood. **B.** Cross-section of stromata. **C.** Octosporous ascus. **D.** Allantoid ascospores. **E.** Colony on PDA-C after 15 days growing at 24°C under intermittent light (12 hours). **F.** Filiform conidia.

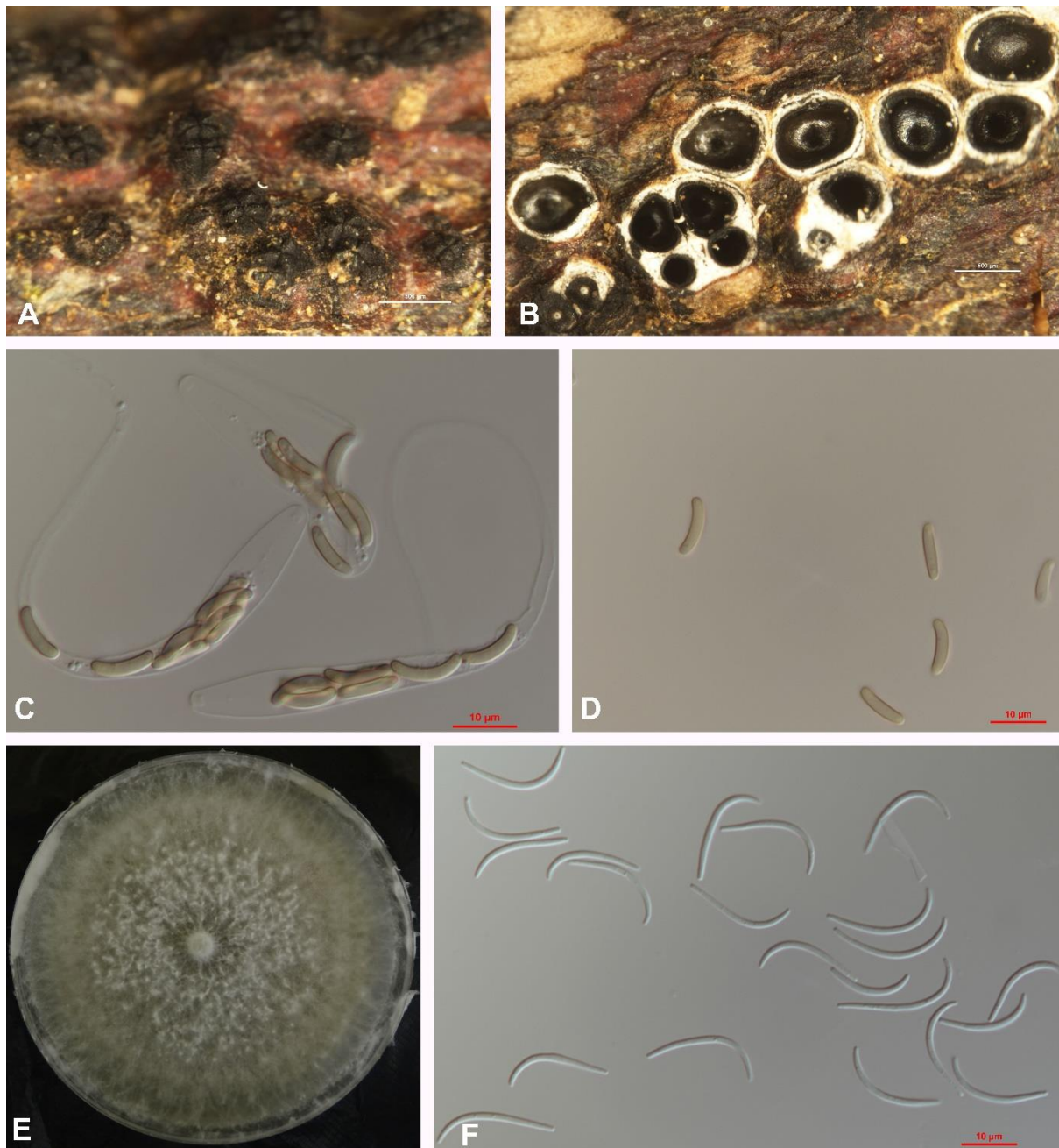


**Figure 4.** Morphology of *Eutypa consobrina* on wood of *Dalbergia* sp. **A.** Stromata with ostioles emerging singly on host tissue. **B.** Perithecial cavities. **C.** Octosporous asci attached to the hymenium. **D.** Long-stipitate, octosporous ascus. **E.** Colony on PDA-C after 15 days growing at 24°C under intermittent light (12 hours). **F.** Curved conidia.

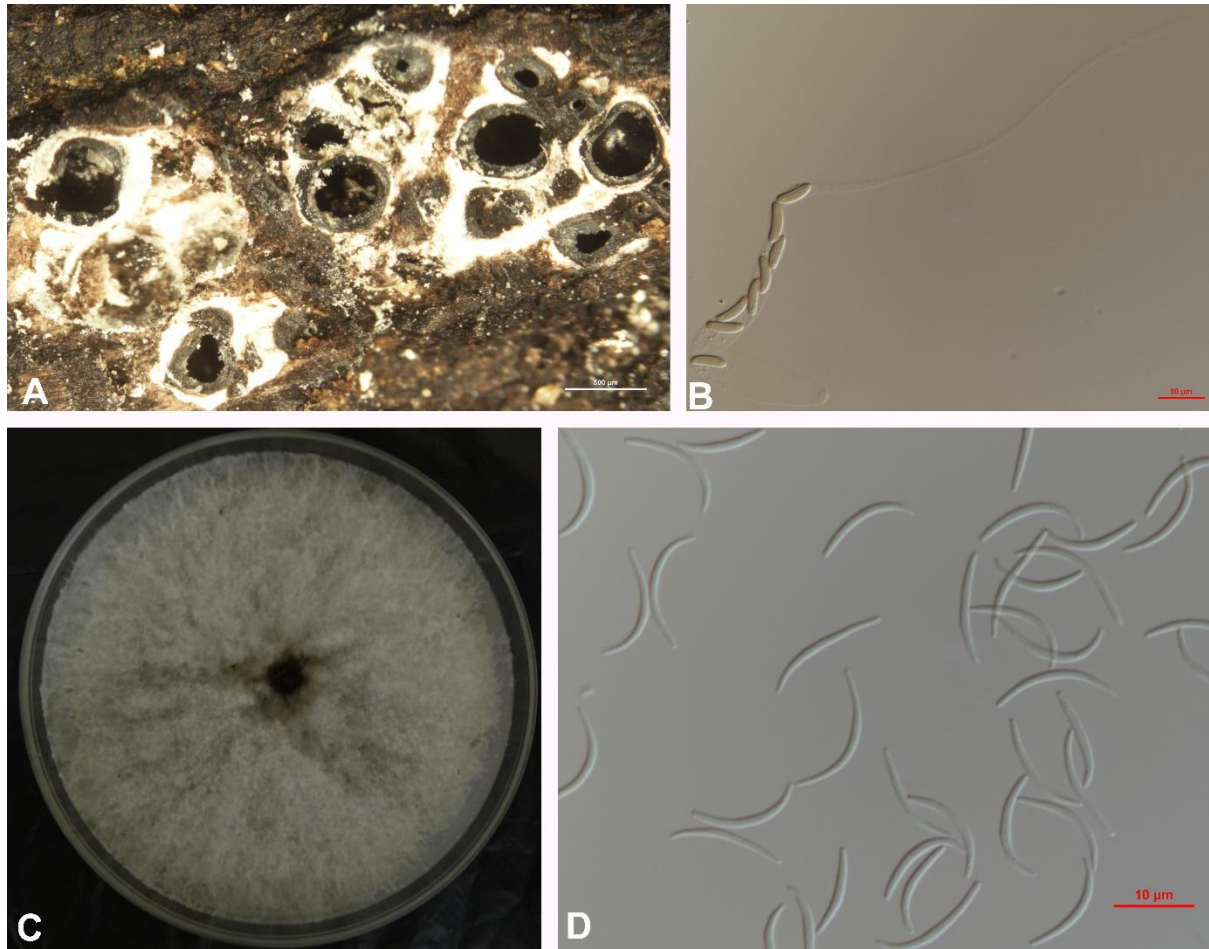




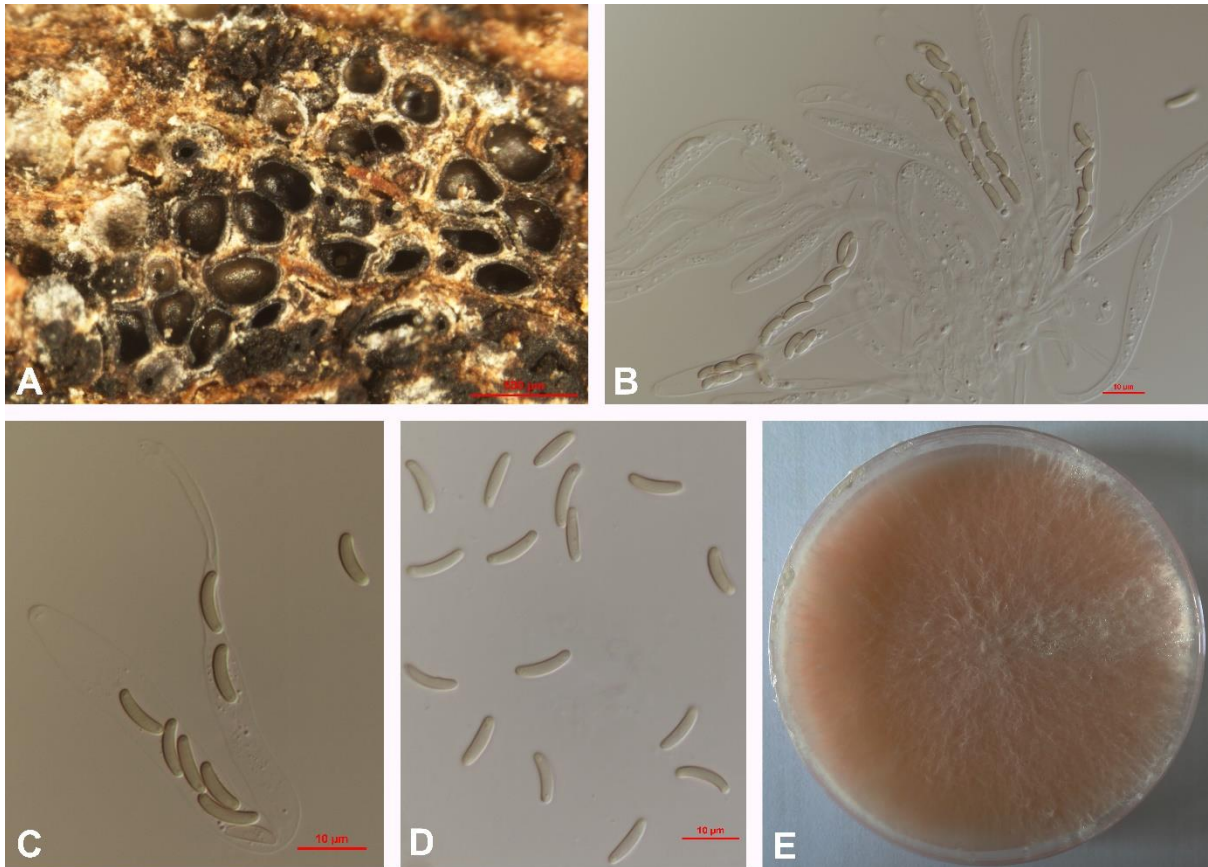
**Figure 5.** Morphology of *Eutypella australiensis* on wood of an unknown host. **A.** Emerging perithecial ostioles. **B.** Long-stipitate, 8-spored ascus. **C.** Allantoid to suballantoid ascospores. **D.** Colony after 15 days on PDA-C dish (90 mm diam.) incubated at 24°C under intermittent light (12 hours). **E.** Filiform conidia.



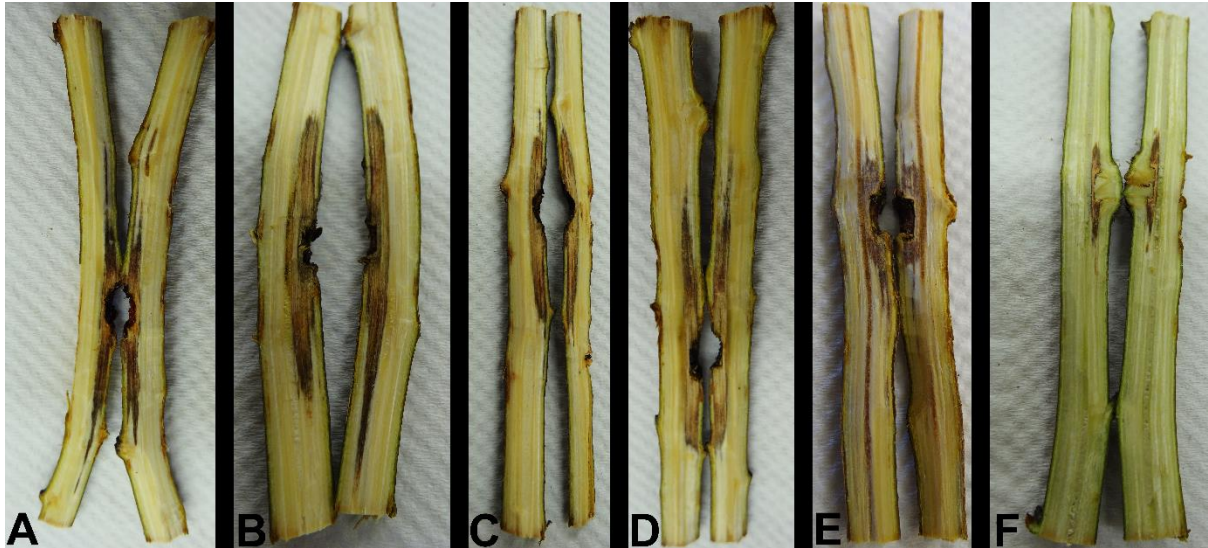
**Figure 6.** Morphology of *Eutypella leprosa* on wood of *Ficus carica*. **A.** Perithecial necks protruding in singly or in groups through the host tissue. **B.** Perithecial cavities surrounded by white entostroma. **C.** Long stalked octosporous asci. **D.** Allantoid ascospores. **E.** Colony on PDA-C 15 days after growing at 24°C under alternating 12 hours of light. **F.** Filiform conidia.



**Figure 7.** Morphology of *Eutypella citricola* on wood of *Quercus* sp. **A.** Perithecial cavities surrounded by white powdery entostroma. **B.** Long-stiped, octosporous ascus. **C.** Colony after 15 days on PDA-C dish (90 mm diam.) incubated at 24°C under intermittent light (12 hours). **D.** Filiform conidia.



**Figure 8.** Morphology of *Eutypella microtheca* on *Morus* sp. **A.** Stromata with a white, powdery layer surrounding the perithecia. **B.** Asci with ascospores. **C.** Octosporous ascus. **D.** Allantoid ascospores. **E.** Colony after 15 days on 90 mm diam. PDA-C dish incubated at 24°C under intermittent light (12 hours).



**Figure 9.** Brown-red lesions caused by Diatrypaceae species on apricot shoots 5 months after inoculation. **A.** *Eutypa cremea*. **B.** *Eutypella citricola*. **C.** *Cryptovalsa ampelina*. **D.** *Eutypa lata*. **E.** *Eutypella microtheca*. **F.** Control.



**Figure 10.** Brown-red lesions caused by Diatrypaceae species on plum shoots 5 months after inoculation. **A.** *Eutypa cremea*. **B.** *Eutypella citricola*. **C.** *Cryptovalsa ampelina*. **D.** *Eutypa lata*. **E.** *Eutypella microtheca*. **F.** Control.

## CHAPTER 4

### Quantitative real-time PCR detection and pathogenicity of Diatrypaceae species on grapevine in South Africa

#### 4.1 ABSTRACT

*Eutypa dieback* causes losses in the grapevine industry worldwide, due to decreases in the quantity and quality of yields as well as costs incurred when vineyards are replanted. Several Diatrypaceae species, including species known to cause decline of different woody hosts, have recently been reported on grapevine and are suspected to be involved in *Eutypa dieback*. In South Africa, 15 Diatrypaceae species were found on grapevine and other woody hosts, but the pathogenic status of the newly discovered species is unknown. In this regard, investigations were conducted to determine if the newly discovered Diatrypaceae species in South Africa are pathogenic to grapevine. Diatrypaceae isolates obtained from grapevine and other woody hosts occurring close to vineyards were used to inoculate fresh pruning wounds, lignified shoots and green shoots of mature grapevines in the field. Since Diatrypaceae species are difficult to distinguish morphologically and the co-occurrence of several species in diseased grapevine wood, could result in misidentification of casual organisms, quantitative real-time polymerase chain reaction (qPCR) assays, targeting the  $\beta$ -tubulin gene, were developed for the detection and quantification of *Cryptovalsa (C.) ampelina* and *Eutypa (E.) lata* in grapevine wood. Pathogenicity trials showed that all isolates of Diatrypaceae species, including those obtained from other woody hosts, were capable of colonising and causing brown discoloration on grapevine wood. The ability of fungal isolates obtained from different woody hosts to cause disease symptoms on grapevine suggests that cross-infections are possible between grapevine and other woody hosts occurring near vineyards. Species including *C. ampelina*, *Eutypella (Eu.) microtheca* and *Eu. citricola* were the most virulent and their virulence was comparable to that of *E. lata*, which has been considered as the sole cause of *Eutypa dieback* for many years. Moderate virulence was observed for species such as *Cryptosphaeria multicontinentalis*. The developed qPCR assays were found to be reliable and specific when validated using grapevine wood samples artificially inoculated with the target species and the detection limit of both assays was 0.01 ng/ $\mu$ l of target DNA in grapevine wood. There are no curative control measures for *Eutypa dieback* and preventative measures are the most reliable methods to control the disease. Thus, the detection of these pathogens in wood samples has great potential for use in early diagnosis of *Eutypa dieback*.

## 4.2 INTRODUCTION

*Eutypa dieback* is responsible for major economic losses to the grapevine industry in South Africa, with losses of approximately R1.7 million estimated for Cabernet Sauvignon vineyards in the Stellenbosch area in South Africa alone, during the 2000/2001 farming season (Van Niekerk *et al.*, 2003). For many years, this disease was believed to be caused by *Eutypa (E.) lata* (Pers.) Tul. & C. Tul., (Ascomycota, Diatrypaceae) alone. Interestingly, recent research conducted in different grape growing areas of the world has shown that several Diatrypaceae species are associated with grapevines exhibiting *Eutypa dieback* symptoms (Trouillas and Gubler, 2004; Jordan and Schilder, 2005; Pitt *et al.*, 2010; Trouillas *et al.*, 2010, 2011; Díaz *et al.*, 2011; Luque *et al.*, 2012; Rolshausen *et al.*, 2014). In South Africa, *Eutypa lata* is still regarded as the sole cause of *Eutypa dieback* of grapevine.

A total of seven Diatrypaceae species have been reported from grapevine exhibiting *Eutypa dieback* symptoms in South Africa (Chapter 2, Mostert *et al.*, 2004; Safodien *et al.*, 2005). These include *Cryptovalsa (C.) ampelina* (Nitschke) Fuckel, *Cryptovalsa rabenhorstii* (Nitschke) Sacc., *Eutypa consobrina* (Mont.) Rappaz, *E. lata*, *Eutypa cremea*, *Eutypella (Eu.) citricola* Speg. and *Eutypella microtheca* Trouillas, W.M. Pitt & Gubler. Furthermore, investigations of non-grapevine woody hosts, occurring in close proximity to vineyards, as potential sources of Diatrypaceae inoculum to adjacent vineyards resulted in 14 Diatrypaceae species being identified, including all species identified from grapevine except *C. rabenhorstii* (Chapter 3). Several of the newly discovered Diatrypaceae species on grapevine, including *C. ampelina*, *C. rabenhorstii*, *Eu. citricola*, *Eu. microtheca*, *Eutypa leptoplaca* (Mont.) Rappaz, *Diatrypella* sp. and *Eutypella leprosa* (Pers. ex Fr.) Berl., have been shown to be pathogenic on grapevines in California, Australia and Chile (Trouillas and Gubler, 2004, 2010; Díaz *et al.*, 2011; Pitt *et al.*, 2013). Nevertheless, despite the existence of several Diatrypaceae species on *Eutypa dieback*-affected grapevine, the biology and pathogenic status of these species on grapevine are currently unknown in South Africa. Furthermore, the potential of isolates of Diatrypaceae species, isolated from other woody hosts in South Africa, to cause disease on grapevine is also unknown. Accordingly, characterisation by pathogenicity testing on grapevine would greatly add to the clarification of the role played by these species on grapevine in South Africa.

The recent discovery of additional Diatrypaceae species on *Eutypa dieback*-affected grapevines has stimulated research into this important disease. An important step going forward, in understanding the aetiology of *Eutypa dieback* would be to improve the detection and identification of the causal organisms. Foliar symptoms of *Eutypa dieback* often appear several years after infection has occurred (Carter, 1988) and thus efficient tools to detect early infection of grapevine in the field are warranted. Early studies that investigated *Eutypa dieback*



on grapevine mainly relied on conventional methods based on isolation of the pathogens from infected plant tissues onto nutrient growth medium and identification of the pathogen microscopically (Lardner *et al.*, 2005). Contemporary approaches to detect and identify causal organisms of Eutypa dieback as well as other plant pathogens have moved from conventional methods to techniques which exploit differences in nucleic acid sequences between pathogenic species. Such techniques include polymerase chain reaction (PCR). Polymerase chain reaction-based methods are advantageous over traditional diagnostic methods because they are quick, able to detect a single target molecule in a complex mixture and they do not require pathogens to be cultured (Schena *et al.*, 2004; Cooke *et al.*, 2007).

The infection of grapevine by a complex of Diatrypaceae species, as seen in Chapter 2 and other studies (Trouillas *et al.*, 2001; Pitt *et al.*, 2010), has provided scope to develop rapid and effective techniques to detect and quantify Diatrypaceae in grapevine wood. A number of studies have reported PCR-based detection and identification of *E. lata*, *C. ampelina* and *Eutypella vitis* (Schwein.: Fr.) Ellis & Everh. (Lecomte *et al.*, 2000; Rolshausen *et al.*, 2004; Lardner *et al.*, 2005; Luque *et al.*, 2006; Catal *et al.*, 2007). Although conventional PCR procedures offer speedy diagnosis in comparison to traditional methods, these procedures have their limitations that include the need for post-PCR sample handling as well as unreliability in quantifying target DNA in samples (Schena *et al.*, 2004; Ward *et al.*, 2004). Quantitative real-time PCR (also known as qPCR) is highly specific, sensitive, accurate and less labour intensive compared to conventional PCR assays. It also offers less risk of cross-contamination and allows detection and quantification of pathogen DNA simultaneously (Gachon *et al.*, 2004; Ward *et al.*, 2004; Cooke *et al.*, 2007). Although qPCR is becoming the choice tool for the identification and quantification of plant pathogens, including Petri disease and esca pathogens (Overton *et al.*, 2004; Aroca *et al.*, 2008; Martín *et al.*, 2012; Pouzoulet *et al.*, 2013), its use on detection and quantification of Eutypa dieback associated pathogens on grapevine has never been reported.

This study therefore, aimed to: i) ascertain the importance of the different Diatrypaceae species on grapevine through pathogenicity trials, ii) develop species-specific qPCR assays for the two most common and important species on grapevine and iii) validate the assays for *in planta* detection and quantification using artificially inoculated grapevine material.

## **4.3 MATERIALS AND METHODS**

### **4.3.1 Pathogenicity trials**

To determine whether Diatrypaceae species found in and around South African vineyards were pathogenic on mature grapevine, three pathogenicity trials were conducted: i) fresh pruning wounds in vineyards inoculated with ascospore suspensions, ii) green shoots in

vineyards inoculated with mycelial plugs, and iii) lignified shoots in vineyards inoculated with mycelial plugs.

#### 4.3.1.1 *Fungal isolates*

Ascospore suspensions of 23 isolates belonging to eight species of Diatrypaceae were used in the first pathogenicity trial. Mycelial plugs of 52 isolates representing 15 species of Diatrypaceae, isolated from grapevine and other woody hosts surrounding vineyards in South Africa were selected for the other two field inoculation trials. The identity of the different species was determined by means of nucleotide sequence analysis of two gene regions as described in Chapter 2 and 3. Isolates for inoculation using fungal mycelium were stored in water as mycelial plugs at 4°C until use.

#### 4.3.1.2 *Preparation of inoculum*

Ascospore suspensions were prepared from perithecia. Stromata were sliced open according to Trouillas *et al.* (2010), under a Nikon SMZ 1500 stereo microscope, with a scalpel to reveal perithecial content. A drop of sterile water was then added on the opened perithecia and perithecial contents were then collected with a needle and placed in sterile water. Ascospore suspensions were adjusted to a final concentration of approximately  $5 \times 10^4$  ascospores/ml using a haemocytometer. Fungal mycelium of each isolate used in mycelial plug inoculation was taken from the margin of colonies growing on potato dextrose agar (PDA-C, Biolab, Midrand, South Africa) plates containing chloromycetin (250 mg/L), incubated at 24°C for 14 days.

#### 4.3.1.3 *Inoculation of pruning wounds with ascospores*

One to four isolates of Diatrypaceae species found to produce perithecia on grapevine (Chapter 2) and other woody hosts surrounding vineyards (Chapter 3) in South Africa were used in this trial. Inoculation was done on pruning wounds of one-year-old lignified canes of a nine-year-old Cabernet Sauvignon vineyard at Nietvoorbij farm in Stellenbosch. The canes were spur-pruned to two buds before inoculation with a 40 µl drop of an ascospore suspension. The ascospore suspensions were applied directly onto the pruning wounds immediately after pruning and each isolate application was replicated 10 times, on shoots of different grapevines. Control canes were inoculated with sterile water. Ten months after inoculation, the inoculated canes were excised and surface sterilised in 70% ethanol for 30 s followed by 1 min in 2.5% sodium hypochlorite and 30 s in 70% ethanol. The canes were then split longitudinally in half through the pruning wounds, under sterile conditions. The length of discolouration (referred to here as lesion length) from the point of infection was measured. To determine the ability of the isolates to grow and colonise pruning wounds, 12 pieces of wood tissue (~ 1×1 mm) were aseptically removed from the entire length of the discolouration

beneath the pruning wound (beneath the border between live and dead tissue), and plating onto PDA-C plates. Four pieces of wood were plated on one plate of PDA-C and all plates incubated at 24°C, exposed to approximately 12 h of daylight and 12 h of darkness, for 4 weeks. The diatrypaceous fungi growing on plates were hyphal-tipped and transferred to new PDA-C plates and isolates were confirmed as the ones used for inoculation by sequencing representative isolates (one or two isolates) of each species.

#### 4.3.1.4 *Inoculation of green shoots with mycelium plugs*

Inoculations were carried out on green shoots of a nine-year-old Cabernet Sauvignon and 14-year-old Sauvignon blanc vineyards at Nietvoorbij farm in Stellenbosch. Green shoots of the new vegetative growth were wounded with a 4mm-cork borer between internodes 3 and 4 and mycelium plugs (4-mm diam.) from 14-day-old cultures were placed on each wound (mycelium side down into the wound) in November of 2015. Control shoots were inoculated with sterile PDA plugs and inoculated wounds were wrapped with Parafilm. Each isolate application was replicated 10 times, on shoots of different grapevines. Five months after inoculation, the inoculated shoots were excised and surface sterilised as described above. After air drying in a lamina flow cabinet, the shoots were cut longitudinally through the inoculation point and the lesion length was measured, both upward and downward directions from point of inoculation, to determine the aggressiveness of each isolate. Isolations were also carried out to determine whether fungal isolates could establish inside the green shoots. The re-isolations were carried out by cutting 12 small wood pieces (~ 1×1 mm) along the entire length of the lesions, from both sides of the inoculation point. Four pieces of wood were plated on one plate of PDA-C and all plates and diatrypaceous fungi were treated as outlined above.

#### 4.3.1.5 *Inoculation of lignified canes with mycelium plugs*

The same inoculation method and fungal isolates used for the inoculation of the green shoots were also used for the lignified cane inoculations. The inoculations were carried out on one-year-old lignified canes of a nine-year old Cabernet Sauvignon vineyard at Nietvoorbij farm in Stellenbosch. Evaluation was carried out after 10 months-incubation period, as described in the green shoot trial.

#### 4.3.1.6 *Trial layout and statistical analysis*

The layout of the trial was a randomized block design. The data for length of discolouration was subjected to one-way analysis of variance (ANOVA) using General Linear Model in SAS version 9.2 (SAS Institute, Cary, North Carolina USA). Fischer's least significance Difference (LSD) test was then used to compare means of lesion lengths of isolates and F-values with  $P < 0.05$  were considered significant.

### 4.3.2 Development and validation of quantitative real-time PCR (qPCR) assays

The qPCR assays were developed using DNA extracted from mycelia of Diatrypaceae isolates collected from perithecia on grapevine. Testing of the assays was performed on DNA extracted from mycelia of isolates obtained from dying spurs, wedge-shaped necrosis and perithecia. Further testing and validation was conducted on grapevine wood inoculated with target species, as described in the pathogenicity trials (inoculation of lignified canes and pruning wounds) as well as on uninoculated grapevine wood.

#### 4.3.2.1 DNA extraction from pure cultures and grapevine wood

Genomic DNA (gDNA) was extracted from mycelia using the CTAB protocol as described in Chapter 2. Subsequently, the gDNA used for standard curves, was sent for purification using the Agencourt AMPure XP beads (Beckman Coulter, Massachusetts) at the Central Analytical Facility (Stellenbosch University). The fungal gDNA was quantified using a Nanodrop ND-1000 Spectrophotometer (Inqaba Biotechnical Industries, South Africa) and stored at -20°C. To extract DNA from grapevine wood, wood chips (~ 10x5 mm) around the inoculation points and along the lesions were excised, placed in 2 ml Eppendorf tubes and stored at -80°C. During the extraction of DNA, the wood chips were frozen in liquid nitrogen and then ground to a fine powder using a mortar and pestle. DNA was extracted from approximately 0.5 g ground wood chips following the protocol described by Retief *et al.* (2005) with one modification: after adding the CTAB extraction buffer, glass beads were added and the tubes were shaken for 7 min using a Mixer Mill Type MM 301 (Retsch GmbH & Co. KG, Germany) before incubation at 65°C for 1 hour. The quantity of DNA was estimated as described above.

#### 4.3.2.2 Development of species-specific primers and probes

To develop potential specific primers and probes for *C. ampelina* and *E. lata*,  $\beta$ -tubulin sequences of these and closely related species within the Diatrypaceae from grapevines and other woody hosts in South Africa (Chapters 2 and 3) were aligned with  $\beta$ -tubulin sequences of Diatrypaceae species from GenBank (National Centre for Biotechnology Information) using the program MAFFT v7.017 (Kato *et al.*, 2002) in Geneious v8.1 (<http://www.geneious.com>, Kearse *et al.*, 2012). Development of candidate species-specific primers and probes was based on regions that were conserved among isolates of each target species, but differed from non-target species. Primers and hydrolysis (Taqman) probes were designed using Primer3 2.3.4 (<http://sourceforge.net/projects/primer3/files/primer3/>, Untergasser *et al.*, 2012) in Geneious v8.1. Primers were selected to be 18-30 bp long, have 40-60% GC content, melting temperature of 60°C ( $\pm 1^\circ\text{C}$ ) and amplify short PCR fragments (<200 bp). Probes were designed to have melting temperatures 10°C higher than the corresponding primer pair and be 18-25 bp in length. Hydrolysis probes for *C. ampelina* and *E. lata* were labelled at the 5'

end with the fluorophores JUN and FAM, respectively. Both probes were labelled at the 3' end with the quencher QSY7. All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, USA), while probes were synthesized by Life Technologies (Carlsbad, USA).

Based on the  $\beta$ -tubulin sequence data, 15 and 5 possible primer/probe combinations were found for *C. ampelina* and *E. lata*, respectively. The candidate primer/probe combinations were screened against sequences of related and unrelated species from GenBank to identify matches that may invalidate the specificity of the primers/probe sets. Only four and two primer/probe combinations showed potential specificity for *C. ampelina* and *E. lata*, respectively. The prospective primers were subjected to preliminary tests using conventional PCR to determine the best primer combinations as well as optimum annealing temperature for each primer set. Each reaction contained 2  $\mu$ l of 10 ng/ $\mu$ l DNA template, 12.5  $\mu$ l of 2 $\times$  KAPA Taq ReadyMix (KAPABiosystems, Massachusetts, United States), 0.4  $\mu$ M of each primer and made up to a final volume of 25  $\mu$ l with sterile water. PCR conditions were: one cycle of initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation (45 s at 94°C), annealing (30 s at 52-64°C) and extension (1 min at 72°C) and a final extension at 72°C for 5 min. Amplification of target DNA varied between different primers sets and annealing temperatures (results not shown), but best amplification was obtained with the primer pairs Camp 363F/Camp 532R and Eut 133F/Eut 273\_22\_R for *C. ampelina* and *E. lata*, respectively. The best amplification was obtained at annealing temperatures of 52°C and 60-64°C, for the Camp 363F/Camp 532R primer set, whereas the Eut 133F/Eut 273\_22\_R set gave good amplification at all the annealing temperatures tested (results not shown). These primer sets were then subjected to preliminary specificity tests using conventional PCR on 52 Diatrypaceae isolates, belonging to 13 species, as well as 15 isolates of 14 other grapevine trunk disease pathogens, obtained from grapevine and other woody hosts in South Africa (results not shown). Finally, these two primer sets were used in the qPCRs in combination with the respective hydrolysis probes. Information regarding the primers and probes used for qPCR for each species are presented in Table 1.

#### 4.3.2.3 qPCR: efficiency, sensitivity and specificity

All qPCR reactions were performed in a Rotor-Gene<sup>TM</sup> 6000 (Corbett Life Science, Whitehead Scientific (Pty) Ltd., South Africa) and had a final volume of 20  $\mu$ l: containing 1 $\times$  KAPA Probefast mix (containing 5 mM MgCl<sub>2</sub>), 300 nM of both forward and reverse primers, 200 nM Taqman probe and 2  $\mu$ l template DNA at 10 ng/ $\mu$ l unless stated otherwise. Cycling conditions were 3 min at 95°C, followed by 40 cycles of 20 s at 95°C and 25 s at 60°C. Individual reactions were performed in triplicate per run. To generate standard curves for the qPCR analyses, isolates STEU 8245 and STEU 8238 were used for *E. lata* and *C. ampelina*, respectively.

To determine the efficiency and limit of detection (LOD) of each qPCR assay, standard curves were generated using 10-fold serial dilutions from 100 ng/μl to 10<sup>-8</sup> ng/μl DNA of fungal gDNA from pure cultures. Furthermore, to evaluate the effect of background grapevine DNA on the detection efficiency of pathogen DNA, DNA samples (10 ng/μl) extracted from uninoculated grapevine wood were spiked with a dilution series of target gDNA (100 ng/μl to 10<sup>-8</sup> ng/μl DNA). The effect of the grapevine DNA was evaluated based on the comparison of standard curves generated with the spiked DNA to those generated from fungal gDNA. All the experiments were repeated once and each dilution run in triplicate.

Samples used to ascertain the specificity of each assay include i) pure culture DNA of eight isolates of each target species collected from vineyards in different areas of the Western Cape of South Africa, ii) pure culture DNA of 13 non-target Diatrypaceae species obtained during surveys of vineyards and non-grapevine woody hosts, iii) pure culture DNA of six fungal taxa often isolated from grapevine wood, iv) uninoculated grapevine DNA, and v) sterile water as a no template control. A total of 20 ng template DNA was included per reaction (2μl of a 10 ng/μl dilution).

#### 4.3.2.4 Detection of *E. lata* and *C. ampelina* in artificially inoculated grapevine wood samples

To evaluate the applicability and reliability of the developed qPCR assays to detect and quantify DNA of the respective target species *in planta*, DNA concentration of all samples were adjusted to 10 ng/μl before qPCR assays were carried out. The qPCR assays were used to detect and quantify target DNA from four (STEU 8240, STEU 8246, STEU 8218 and STEU 8239) and five (STEU 8120, STEU 8232, STEU 8238, STEU 8216 and STEU 8217) DNA extracts obtained from *E. lata* and *C. ampelina* inoculated grapevine wood samples, respectively. Conventional isolation methods of plating wood pieces from observed lesions onto PDA-C, were first carried out on the above grapevine wood samples to confirm colonisation of the wood before DNA extraction and qPCR analyses.

## 4.4 RESULTS

### 4.4.1 Pathogenicity trials

#### 4.4.1.1 Inoculation of pruning wounds with ascospores

Inoculation of grapevine pruning wounds with ascospores of various Diatrypaceae species demonstrated the ability of these species to infect wounds shortly after pruning. All isolates tested caused lesions longer than those of control shoots and lesions were similar in length to those caused by isolates of *E. lata*, except for two isolates of *E. cremea* (STEU 8081 and STEU 8144). The lesions produced were brown-black streaks similar to that observed in the dying spurs examined in Chapter 2. Significant differences ( $P = 0.0011$ ) in mean lesion lengths

were observed among isolates of species including *C. ampelina*, *E. cremea* and *Eu. citricola*. Individual strains belonging to species of *Eutypella australiensis* Trouillas, Sosnowski & Gubler, *Eu. microtheca*, *Eu. leprosa*, *E. lata* and *E. consobrina* behaved similarly (Table 2). Re-isolation percentages of inoculated isolates ranged from 60% (for both *E. consobrina* isolates tested and *Eu. australiensis* isolate STEU 8203) to 100% (for *C. ampelina* isolate STEU 8120, *E. cremea* isolate STEU 8081, *Eu. citricola* isolates STEU 8259 and *Eu. citricola* isolate STEU 8179) and isolations from control shoots did not yield any Diatrypaceae species (Table 2).

#### 4.4.1.2 Inoculation of green shoots with mycelium plugs

Inoculation of green shoots of Cabernet Sauvignon and Sauvignon blanc with isolates of Diatrypaceae species resulted in vascular discoloration on the shoots after five months of incubation in the field. Significant differences in mean lesions lengths within and between species were observed in both Cabernet Sauvignon ( $P < 0.0001$ ) and Sauvignon blanc ( $P = 0.0005$ ) vineyards. Mean lesion lengths differed among isolates of species including *E. lata*, *C. ampelina*, *Eu. microtheca*, *Eu. citricola* and *Cryptosphaeraia (Cr.) multicontinentalis* Trouillas, F. Peduto, Inderb. and Gubler in the Cabernet Sauvignon vineyard. In the Sauvignon blanc vineyard, differences in mean lesion lengths were observed among isolates of *C. ampelina*, *Eu. microtheca*, *Eu. citricola* and *Eu. australiensis*. Overall, isolates produced longer lesions on shoots of the Cabernet Sauvignon vineyard in comparison to Sauvignon blanc vineyard. Mean lesion lengths ranged from 51.63 to 90.62 mm for Cabernet Sauvignon and 45.82 to 80.07 mm for Sauvignon blanc. All isolates produced lesions significantly longer than the control in Cabernet Sauvignon, however, in the Sauvignon blanc vineyard, all but two isolates (*Cr. multicontinentalis* STEU 8164 and *Cr. multicontinentalis* STEU 8158) produced lesions longer than those of the control (Table 3). All isolates inoculated on shoots were re-isolated from the lesions and re-isolations percentages ranged from 40 to 100% and 50 to 100% for Cabernet Sauvignon and Sauvignon blanc, respectively. Isolates of the unnamed *Eutypella* species had the lowest re-isolation percentages from both vineyards. The isolate STEU 8207 had the lowest re-isolation percentage (40%) from shoots of Cabernet Sauvignon while STEU 8208 had the lowest re-isolation percentage (50%) from the Sauvignon blanc vineyard. No Diatrypaceae fungi were isolated from the control shoots.

#### 4.4.1.3 Inoculation of lignified canes with mycelium plugs

All isolates used in this study were pathogenic on lignified shoots of Cabernet Sauvignon and produced brown lesions which extended in both directions from the point of inoculation after an incubation period of 10 months. These lesions were significantly longer than the control. No differences in lesion lengths were observed among isolates of each species, apart from *Eu. citricola* isolate STEU 8186 which produced significantly shorter lesions (46.38 mm)

compared to the *Eu. citricola* isolates STEU 8249 (59.84 mm) and STEU 8182 (58.99 mm). Although *E. lata* isolates produced the longest lesions on lignified canes, these lesions were not significantly different from those produced by isolates of *C. ampelina*, *Eu. leprosa* and *Eu. microtheca* (Table 3). All isolates were re-isolated from lesions but no Diatrypaceae species were isolated from control shoots. Re-isolation percentages of isolates from inoculated shoots ranged from 40 to 100%, with *Eutypella* sp. isolate STEU 8207 having the lowest re-isolation percentage.

#### 4.4.2 Development and validation of qPCR assays

##### 4.4.2.1 qPCR: efficiency, sensitivity and specificity

The sensitivity or limit of detection of the qPCR assays, which was evaluated by use of standard curves generated from gDNA of *C. ampelina* (STEU 8238) and *E. lata* (STEU 8245) isolates, was defined as the minimum concentration of fungal DNA template that could be detected in all three technical replicates, in the different independent repetitions of the qPCR. The limit of detection for both qPCR assays was established at 0.001 ng/μl fungal gDNA and this parameter was defined by *Ct* values of 39.04 and 39.61 for *C. ampelina* and *E. lata*, respectively (Fig. 1). Additional standard curves were constructed using fungal gDNA of these isolates containing 10 ng/μl DNA of uninoculated grapevine wood (spiked DNA). The regression coefficients ( $R^2$ ) of these standard curves were compared to those obtained with fungal gDNA of each species qPCR assay. Results showed that there was no difference between the regression coefficients (all  $R^2 > 0.99$ ) when the different DNA templates were used, which indicates high linear relationships between the *Ct* values and the log of the DNA concentration in each replicate. The detection limit when spiked DNA was used as a template was, however, higher (0.01 ng/μl) for both assays indicating that this parameter was affected by background grapevine DNA (Fig.1). Furthermore, the reaction efficiency when using spiked DNA was also reduced to 84.9% and 76.8% for *C. ampelina* and *E. lata*, respectively.

The specificity of the assays was evaluated against target and non-target samples from pure cultures listed in Table 4. Both qPCR assays were 100% specific, successfully amplifying the target DNA templates of eight isolates of *C. ampelina* and *E. lata*, respectively. All the *C. ampelina* isolates were positively detected within the *Ct* range of 23.23-26.55 while *E. lata* isolates were detected within the *Ct* range of 22.37-27.78 (Table 4). On the other hand, when the assays were applied on the 13 non-target Diatrypaceae species, six other fungal species frequently associated with grapevine wood and uninoculated grapevine DNA, no cross amplification was obtained.



#### 4.4.2.2 Detection of *E. lata* and *C. ampelina* in artificially inoculated grapevine wood samples

The ability of the qPCR assays to detect and quantify the target species in grapevine wood was assessed by performing the assays on DNA of five grapevine wood samples inoculated with different *C. ampelina* isolates as well as on four samples inoculated with different *E. lata* isolates. Both qPCR assays were highly effective in detecting the specific target DNA from the inoculated grapevine wood. No amplification was observed with DNA obtained from the uninoculated wood in both assays (Table 5).

## 4.5 DISCUSSION

Dieback of grapevine has been documented in South Africa and has been associated mainly with *E. lata* (Ferreira *et al.*, 1989; White *et al.*, 2011). This study has, however, shown that all the taxa studied are pathogenic and individually capable of causing brown vascular discolourations when inoculated artificially into grapevine tissues. The pathogenicity trials were not repeated, however, results from the three inoculation trials were comparable. The most virulent species in all three trials include *E. lata*, *C. ampelina*, *Eu. microtheca* and *Eu. citricola* and these results confirm a recent report of pathogenicity of these species in Australia where these were found to be highly virulent on grapevine (Pitt *et al.*, 2013). Although results of this study agree with results of previous studies (Trouillas and Gubler, 2010; Díaz *et al.*, 2011; Pitt *et al.*, 2013) on the high virulence of *C. ampelina*, other studies have reported moderate virulence for this species (Mostert *et al.*, 2004; Luque *et al.*, 2006). Variation in virulence of this species, in the different studies, could be attributed to several factors including variation in isolate virulence, age of host inoculated, variation in cultivar susceptibility and length of incubation periods before evaluation is carried out.

Pathogenicity of *Cryptosphaeria ligniota* (Fr.: Fr.) Auersw., *C. rabenhorstii*, *Diatrypella* sp., *Eu. australiensis*, *Eu. leprosa* and unnamed *Eutypella* spp. on grapevine was also confirmed in the study. This is the first study to demonstrate the pathogenicity of *Eu. australiensis* and *Cr. ligniota* on grapevines worldwide. *Eutypella australiensis* was isolated for the first time on *Acacia longifolia* subsp. *sophorae* (Labill.) Court in South Australia (Trouillas *et al.*, 2010) while *Cr. ligniota* is known to cause *Cryptosphaeria* canker on *Populus tremuloides* Michx. (Hinds, 1981). Pathogenicity of *C. rabenhorstii* has been established on grapevine in Australia (Pitt *et al.*, 2013) while that of *Eu. leprosa* is reported from Chile (Díaz *et al.*, 2011).

Moderate virulence was observed for *Cr. multicontinentalis* which was the only species whose isolates produced lesions not significantly different from the control when inoculated on green shoots of Sauvignon blanc. These results are in line with those of a study in Australia which found *Cryptosphaeria* spp. to produce lesions similar in length to the controls (Pitt *et al.*, 2013). In this regard, this species could be a weak pathogen on grapevine. *Cryptosphaeria*

*multicontinentalis* causes Cryptosphaeria dieback on Fremont cottonwood in California (Trouillas and Gubler, 2016) and was presumed to be specific to species in the Salicaceae (Trouillas *et al.*, 2015) which could also explain the weak pathogenicity on grapevine in this study.

This study has also shown that *E. cremea* and *E. consobrina* are pathogenic on grapevine. These species have been previously reported on *Arundo* species (Rappaz, 1987) and to date, South Africa is the only country from which these two species have been associated with dieback of grapevine. In view of their occurrence and virulence on grapevine observed in this study, the occurrence of these two species on grapevine in other grape growing regions need to be investigated to determine their geographical distribution and confirm their capability to cause dieback and cankers on grapevine.

Results of pathogenicity tests in this study further indicate the existence of intra-specific variation in virulence of species including *C. ampelina*, *Eu. citricola* and *Eu. microtheca*. Variation among isolates of *Cr. multicontinentalis* and *E. lata* were only observed on green shoots of Cabernet Sauvignon whereas variation between isolates of *Eu. australiensis* was observed only on green shoots of Sauvignon blanc. Variability in virulence among isolates of Diatrypaceae has been documented previously. For example, variation in pathogenicity has been reported for isolates of *C. ampelina* (Trouillas and Gubler, 2010) and *E. lata* (Péros *et al.*, 1997). Additionally, in the present study, longer lesions were produced on green shoots of Cabernet Sauvignon in comparison to Sauvignon blanc. These results suggest that Sauvignon blanc is more tolerant to these fungi when compared to Cabernet Sauvignon. This could be true given that Cabernet Sauvignon was shown to have greater susceptibility to *E. lata* compared with Sauvignon blanc (Péros and Berger, 1994).

Although little information is known regarding the epidemiology of the newly discovered Diatrypaceae species infecting grapevines, these species have been viewed to invade plants through wounds. This is because of their ease of isolation from cankers extending from pruning wounds and/or natural openings of grapevine and various hosts (Trouillas *et al.*, 2010, 2011). This opinion is true in South Africa where Diatrypaceae taxa were isolated from dieback and/or canker symptoms associated with pruning wounds and/or natural openings of grapevine and other woody hosts (Chapter 2 and 3). Furthermore, inoculation of fresh pruning wounds of Cabernet Sauvignon with ascospore suspensions of Diatrypaceae species, showed that pruning wounds are subject to attack by these taxa.

While Diatrypaceae species demonstrated the ability to infect grapevine shoots in the inoculation studies, no cankers were observed on the inoculated shoots. It is unclear whether infections by these species would lead to foliar symptoms typical of Eutypa dieback. It usually takes 3-8 years post infection for *E. lata* to cause visible symptoms of Eutypa dieback on grapevine. It is likely that a similar delay in symptom expression on grapevine occurs for the

newly discovered Diatrypaceae species as suggested by Trouillas and Gubler (2004) for *Eutypa leptoplaca* (Mont.) Rappaz. The implications of invasion of green shoots by Diatrypaceae species, as is reported in this study, in the epidemiology of Eutypa dieback are also currently unknown. Additionally, the environmental conditions conducive for spore release and subsequently, periods of low and/or high risk infection during the grape growing season are also currently unknown. Trouillas and Gubler (2010) however, speculated that the epidemiology of *E. leptoplaca* may be similar to that of *E. lata*, after they observed similarities between the stromatic features of the two species.

Species of Diatrypaceae can nonetheless, be considered to be a significant threat to the sustainability of grapevine production. These fungi colonise native and cultivated woody hosts and results of this study show that isolates of Diatrypaceae species collected from non-grapevine hosts are pathogenic to grapevine, reinforcing a previous report that alternative hosts are sources of Diatrypaceae inoculum for grapevines (Trouillas and Gubler, 2010). Management practices for Eutypa dieback should therefore not only focus on vineyards, but also consider non-grapevine hosts occurring in close proximity to vineyards. For instance, in the case of sanitation practices (i.e. removal of dead wood of other hosts of Diatrypaceae species) to reduce inoculum sources. Further strategies to reduce incidences of infection by Diatrypaceae species on grapevine should include protection of pruning wounds with fungicides. A number of fungicides that are effective against *E. lata* were found to be effective against several Diatrypaceae species (Gramaje *et al.*, 2012). The results of this study should be used as a basis for future research aimed at managing Diatrypaceae species causing disease on grapevine in South Africa.

The availability of a molecular tool for detecting organisms associated with Eutypa dieback directly from grapevine wood is important if the epidemiology of these organisms is to be studied comprehensively. Quantitative real-time PCR was favoured as the choice tool because of its greater potential for enhanced through-put, sensitivity, specificity and ability to quantify target DNA. All current PCR primer assays developed for the detection of *E. lata* and *C. ampelina* target the ITS gene region (Lemcote *et al.*, 2000; Lardner *et al.*, 2005; Luque *et al.*, 2006; Catal *et al.*, 2007). Although this region was considered for qPCR assay development in the current investigation, suitable species-specific binding sites for probes could not be identified for either target species (data not shown), consequently the  $\beta$ -tubulin gene was selected as the target region. The  $\beta$ -tubulin gene is present in a single copy in the genome of many fungal species (Ayliffe *et al.*, 2001; Msiska and Morton, 2009) and it has been established that lower detection limits are obtained with the use of multi-copy genes, such as ITS, in comparison to single copy genes in PCR assays (Bilodeau *et al.*, 2007; Tellenbach *et al.*, 2010). Accordingly, the limits of detection (LOD) presented in this study are higher (0.001 ng/ $\mu$ l of pure culture DNA) for both *C. ampelina* and *E. lata* assays in comparison

to those reported for *E. lata* in an ITS-based nested PCR assay described by Catal *et al.* (2007). Amplicons were obtained with <1 fg of pathogen DNA in the nested PCR described by Catal *et al.* (2007). Despite the lower detection limits of the  $\beta$ -tubulin assays, the target species could still be successfully detected and quantified in infected grapevine wood.

Samples obtained from plants are complex and contain substances such as pectin, polyphenols and polysaccharides (Schrader *et al.*, 2012), which can be coextracted with DNA. These substances can inhibit PCR completely or reduce the PCR efficiency (Cankar *et al.*, 2006; Kubista *et al.*, 2006; King *et al.*, 2009; Schrader *et al.*, 2012) which could explain the lower detection limits and reaction efficiencies observed in the presence of grapevine DNA in this study. To reduce effects of these substances on PCR, biological samples may need to be purified and then diluted (Kubista *et al.*, 2006). However, purification of samples can be costly. Nonetheless, the qPCR assays in this study, enabled specific detection and quantification of target DNA in the presence of grapevine DNA, in all samples inoculated with the respective species. The presence of target species in the samples was confirmed by results of conventional isolation methods conducted on the samples before qPCR was carried out. Higher DNA concentrations of *E. lata* were, however, detected from the artificially inoculated samples in comparison to *C. ampelina*. This could mean that *E. lata* colonises the wood faster than *C. ampelina* when inoculated artificially onto grapevine tissues.

Both qPCR assays were able to detect target DNA from all isolates tested for specificity regardless of their geographic origin and symptom type from which they were isolated. This indicates that both assays have great potential for use in early diagnosis of Eutypa dieback infection in vineyards located in different regions. However, wide ranges of *Ct* values were observed among the different DNA samples of the target species, even though all the samples were adjusted to 10 ng/ $\mu$ l. This could have been caused by differing amounts of contaminants (e.g. RNA molecules) in the CTAB extracted DNA since no RNase A treatment was used for RNA removal during extraction and the samples were also not purified as was conducted for isolates used for standard curves.

Although this study was aimed at the detection and quantification of *C. ampelina* and *E. lata* in grapevine tissues, both assays were used successfully to detect target DNA from isolates collected from non-grapevine woody hosts often found adjacent to vineyards (data not shown). Thus, both assays could potentially be used for similar quantifications in other woody hosts. This could be true when bearing in mind that several of the *C. ampelina* and *E. lata* isolates used to develop these assays were obtained from different woody hosts.

The present study provides knowledge on the pathogenicity status of Diatrypaceae species towards grapevine in South Africa. The most common Diatrypaceae species found on grapevine (*E. lata*, *C. ampelina*, *E. cremea*, *Eu. citricola* and *Eu. microtheca*) in South Africa were shown in this study to be the most virulent species. *Cryptovalsa ampelina*, *E. cremea*,

*Eu. citricola* and *Eu. microtheca* were also found to be equally virulent on grapevine as *E. lata*, which indicates their potential involvement in grapevine dieback. The ability of isolates of Diatrypaceae collected from different woody hosts to cause disease symptoms on grapevine, in the present study, further indicates the possibility of cross-infections between grapevine and other woody hosts. This knowledge, together with the developed qPCR assays will be of value in early diagnosis of infection caused by Diatrypaceae species in vineyards. Future studies should determine whether infections caused by the newly discovered Diatrypaceae species on grapevine could lead to cankers and foliar symptoms typical of *Eutypa* dieback.

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**Table 1.** Primers and Taqman probes developed and validated for the detection and quantification of DNA of *Cryptovalsa ampelina* and *Eutypa lata* on grapevine wood.

Target species	Primer/Probe <sup>a</sup>	Sequence (5'-3')	Length of amplicon
<i>Cryptovalsa ampelina</i>	Camp 363F	GCAGCATAGCTACAATGGCAC	170
	Camp 532R	AGGCCTATACGGACAGGAGT	
	Camp 390P	<b>JUN-GCTCCAGCTCGAGCGCATTAAACGTC-QSY7</b>	
<i>Eutypa lata</i>	Eut 133F	TGGGCTCAAATACTACTGGCG	141
	Eut 273_22_R	CCCGCAATGTAATGGTCGATAC	
	Eut 218_P	<b>FAM-AGACCGGCCAATGCGTAAGTGCCT-QSY7</b>	

<sup>a</sup>F = forward primer, R = reverse primer, and P = Taqman probe

**Table 2.** Mean lesion lengths after 10 months of inoculation using ascospores of Diatrypaceae species, on pruning wounds of one-year old canes of nine-year-old Cabernet Sauvignon grapevines.

Species	Strain number	Host	Origin of isolate	Lesion length (mm) <sup>a</sup>	Re-isolation percentage
<i>Cryptovalsa ampelina</i>	STEU 8232	<i>Vitis vinifera</i>	Darling	27.81 b-e	70
<i>Cryptovalsa ampelina</i>	STEU 8120	<i>Vitis vinifera</i>	Durbanville	36.70 a	100
<i>Cryptovalsa ampelina</i>	STEU 8214	<i>Ficus carica</i>	Stellenbosch	25.90 b-e	90
<i>Cryptovalsa ampelina</i>	STEU 8209	<i>Cinnamomum camphora</i>	Grabouw	31.35 a-c	80
<i>Eutypa consobrina</i>	STEU 8153	<i>Quercus</i> sp.	Durbanville	23.89 de	60
<i>Eutypa consobrina</i>	STEU 8155	<i>Dalbergia</i> sp.	Constantia	25.13 b-e	60
<i>Eutypa lata</i>	STEU 8218	<i>Vitis vinifera</i>	Durbanville	31.12 a-d	90
<i>Eutypa lata</i>	STEU 8239	<i>Vitis vinifera</i>	Bonnievale	26.76 b-e	80
<i>Eutypa cremea</i>	STEU 8084	<i>Vitis vinifera</i>	Darling	31.36 a-c	80
<i>Eutypa cremea</i>	STEU 8081	<i>Vitis vinifera</i>	Bonnievale	22.05 ef	100
<i>Eutypa cremea</i>	STEU 8130	<i>Morus</i> sp.	Franschoek	27.92 b-e	90
<i>Eutypa cremea</i>	STEU 8144	<i>Quercus</i> sp.	Durbanville	21.30 ef	80
<i>Eutypella australiensis</i>	STEU 8203	Unknown	Hout Bay	30.21 a-d	60
<i>Eutypella australiensis</i>	STEU 8204	<i>Dalbergia</i> sp.	Constantia	27.48 b-e	70
<i>Eutypella citricola</i>	STEU 8220	<i>Vitis vinifera</i>	Durbanville	32.46 ab	80
<i>Eutypella citricola</i>	STEU 8259	<i>Vitis vinifera</i>	Durbanville	30.87 a-d	100
<i>Eutypella citricola</i>	STEU 8179	<i>Diospyros kaki</i>	Bonnievale	24.28 c-e	100
<i>Eutypella citricola</i>	STEU 8260	<i>Ficus carica</i>	Bonnievale	26.59 b-e	70
<i>Eutypella leprosa</i>	STEU 8189	<i>Ficus carica</i>	Hout Bay	29.64 a-d	70
<i>Eutypella leprosa</i>	STEU 8192	<i>Quercus</i> sp.	Constantia	30.17 a-d	80
<i>Eutypella microtheca</i>	STEU 8111	<i>Vitis vinifera</i>	Calitzdorp	24.96 c-e	70
<i>Eutypella microtheca</i>	STEU 8195	<i>Morus</i> sp.	Franschoek	30.67 a-d	90
<i>Eutypella microtheca</i>	STEU 8196	<i>Melia azedarach</i>	Wellington	25.10 b-e	70
PDA plug				14.74 f	—
LSD ( $P < 0.05$ )				7.41	

<sup>a</sup>Means followed by the same letter are not significantly different ( $P < 0.05$ ).

**Table 3.** Mean lesion lengths caused by Diatrypaceae species from South Africa on green shoots of both Cabernet Sauvignon (CS) and Sauvignon blanc (SB) after 5 months as well as on lignified canes of CS after 10 months.

Species	Isolate	Host origin	Origin of isolate	Lesion lengths (mm) <sup>a</sup>			
				Lignified canes		Green shoots	
				CS		CS	SB
<i>Cryptovalsa ampelina</i>	STEU 8238	<i>Vitis vinifera</i>	Rawsonville	64.05 a-e		74.93 b-l	70.99 a-h
<i>Cryptovalsa ampelina</i>	STEU 8216	<i>Prunus armeniaca</i>	Bonnievale	66.94 ab		87.19 a-c	80.07 a
<i>Cryptovalsa ampelina</i>	STEU 8217	<i>Psidium guajava</i>	Klawer	66.48 a-c		79.61 a-j	68.81 a-j
<i>Cryptovalsa ampelina</i>	STEU 8213	<i>Punica granatum</i>	Bonnievale	63.45 a-e		82.77 a-h	61.96 b-n
<i>Cryptovalsa ampelina</i>	STEU 8261	<i>Schinus molle</i>	Durbanville	61.48 a-h		88.15 ab	76.70 ab
<i>Cryptovalsa ampelina</i>	STEU 8212	<i>Eriobotrya japonica</i>	Darling	59.13 a-k		83.87 a-f	65.03 a-m
<i>Cryptovalsa ampelina</i>	STEU 8211	<i>Rosa</i> sp.	Vredendal	61.98 a-g		71.57 e-l	68.33 a-k
<i>Cryptovalsa rabenhorstii</i>	STEU 8112	<i>Vitis vinifera</i>	Stellenbosch	60.31 a-i		69.15 g-m	65.91 a-m
<i>Cryptosphaeria ligniota</i>	STEU 8165	<i>Salix mucronata</i>	Stellenbosch	50 g-n		68.42 i-m	65.32 a-m
<i>Cryptosphaeria multicontinentalis</i>	STEU 8262	<i>Salix mucronata</i>	Constantia	48.88 g-n		68.80 h-m	56.29 h-o
<i>Cryptosphaeria multicontinentalis</i>	STEU 8158	<i>Salix mucronata</i>	Stellenbosch	53.91 d-n		76.62 a-l	48.63 n-p
<i>Cryptosphaeria multicontinentalis</i>	STEU 8164	<i>Salix mucronata</i>	Stellenbosch	46.79 k-n		69.88 f-m	45.82 op
<i>Cryptosphaeria multicontinentalis</i>	STEU 8163	<i>Salix mucronata</i>	Stellenbosch	57.23 a-n		65.63 j-n	53.71 k-o
<i>Cryptosphaeria multicontinentalis</i>	STEU 8162	<i>Morus</i> sp.	Calitzdorp	45.71 n		51.63 n	51.85 l-o
<i>Diatrypella</i> sp.	STEU 8177	<i>Citrus limon</i>	Stellenbosch	53.79 d-n		80.74 a-i	58.94 e-o
<i>Eutypa consobrina</i>	STEU 8092	<i>Vitis vinifera</i>	Grabouw	56.66 a-n		74.98 b-l	57.96 f-o
<i>Eutypa consobrina</i>	STEU 8154	<i>Schinus molle</i>	Durbanville	46.61 l-n		75.29 b-l	61.02 c-n
<i>Eutypa lata</i>	STEU 3055	<i>Vitis vinifera</i>	Hermanus	67.94 a		68.79 h-m	68.34 a-k
<i>Eutypa lata</i>	STEU 8246	<i>Psidium guajava</i>	Constantia	68.40 a		90.62 a	75.41 a-c

**Table 3.** (continued)

Species	Isolate	Host origin	Origin of isolate	Lesion lengths (mm) <sup>a</sup>		
				Lignified canes		Green shoots
				CS	CS	SB
<i>Eutypa cremea</i>	STEU 8081	<i>Vitis vinifera</i>	Bonnievale	57.06 a-n	81.87 a-i	55.02 j-o
<i>Eutypa cremea</i>	STEU 8139	<i>Prunus armeniaca</i>	Bonnievale	58.27 a-m	77.71 a-k	67.21 a-k
<i>Eutypa cremea</i>	STEU 8135	<i>Ficus carica</i>	Grabouw	53.31 d-n	81.20 a-i	67.49 a-k
<i>Eutypa cremea</i>	STEU 8149	<i>Malus domestica</i>	Bonnievale	53.89 d-n	80.39 a-i	59.63 d-o
<i>Eutypa cremea</i>	STEU 8150	<i>Salix mucronata</i>	Constantia	57.85 a-n	77.03 a-l	60.81 c-o
<i>Eutypa cremea</i>	STEU 8138	<i>Schinus molle</i>	Wellington	47.59 j-k	75.62 b-l	73.38 a-e
<i>Eutypa cremea</i>	STEU 8132	<i>Eriobotrya japonica</i>	Reebok	53.98 d-n	76.55 a-l	69.01 a-j
<i>Eutypella australiensis</i>	STEU 8248	<i>Psidium guajava</i>	Stellenbosch	49.33 h-n	82.78 a-h	57.46 g-o
<i>Eutypella australiensis</i>	STEU 8204	<i>Dalbergia</i> sp.	Constantia	52.66 e-n	83.68 a-f	56.73 g-o
<i>Eutypella australiensis</i>	STEU 8203	Unknown	Hout Bay	47.35 k-n	88.30 ab	72.57 a-f
<i>Eutypella citricola</i>	STEU 8263	<i>Vitis vinifera</i>	Hermanus	55.37 b-n	75.04 b-l	54.41 j-o
<i>Eutypella citricola</i>	STEU 8247	<i>Prunus armeniaca</i>	Bonnievale	48.58 i-n	81.13 a-i	71.51 a-g
<i>Eutypella citricola</i>	STEU 8249	<i>Prunus salicina</i>	Bonnievale	59.84 a-i	86.32 a-d	56.84 g-o
<i>Eutypella citricola</i>	STEU 8250	<i>Psidium guajava</i>	Paarl	52.77 e-n	77.61 a-k	70.41 a-i
<i>Eutypella citricola</i>	STEU 8181	<i>Eriobotrya japonica</i>	Grabouw	51.91 e-n	80.34 a-i	68.60 a-k
<i>Eutypella citricola</i>	STEU 8186	<i>Melia azedarach</i>	Hout Bay	46.38 nm	71.05 e-l	65.27 a-m
<i>Eutypella citricola</i>	STEU 8182	<i>Punica granatum</i>	Bonnievale	58.99 a-l	64.92 k-n	67.56 a-k
<i>Eutypella citricola</i>	STEU 8251	<i>Schinus molle</i>	Durbanville	50.95 f-n	74.75 b-l	64.57 b-m
<i>Eutypella leprosa</i>	STEU 8193	<i>Ficus carica</i>	Hout Bay	56.19 a-n	88.60 ab	66.81 a-l

**Table 3.** (continued)

Species	Isolate	Host origin	Origin of isolate	Lesion lengths (mm) <sup>a</sup>		
				Lignified canes	Green shoots	
				CS	CS	SB
<i>Eutypella leprosa</i>	STEU 8191	<i>Ficus carica</i>	Hout Bay	62.65 a-f	77.07 a-l	71.50 a-g
<i>Eutypella leprosa</i>	STEU 8190	<i>Ficus carica</i>	Hout Bay	63.35 a-f	87.65 ab	62.62 b-n
<i>Eutypella microtheca</i>	STEU 8108	<i>Vitis vinifera</i>	Robertson	58.49 a-m	79.11 a-k	64.70 b-m
<i>Eutypella microtheca</i>	STEU 8201	<i>Prunus armeniaca</i>	Calitzdorp	62.22 a-g	76.66 a-l	66.29 a-l
<i>Eutypella microtheca</i>	STEU 8252	<i>Ficus carica</i>	Lutzville	57.57 a-n	80.90 a-i	50.95 m-o
<i>Eutypella microtheca</i>	STEU 8253	<i>Morus</i> sp.	Franschoek	66.75 a-c	83.22 a-g	58.23 f-o
<i>Eutypella microtheca</i>	STEU 8197	<i>Cydonia oblonga</i>	Bonnievale	65.71 a-d	80.19 a-i	58.75 e-o
<i>Eutypella microtheca</i>	STEU 8199	<i>Pyrus communis</i>	Calitzdorp	62.33 a-g	84.38 a-e	74.26 a-d
<i>Eutypella microtheca</i>	STEU 8202	<i>Schinus molle</i>	Calitzdorp	58.14 a-n	86.58 a-d	58.17 f-o
<i>Eutypella microtheca</i>	STEU 8194	<i>Diospyros kaki</i>	Stellenbosch	61.64 a-h	62.88 k-n	59.42 d-o
<i>Eutypella</i> sp. 1	STEU 8206	<i>Psidium guajava</i>	Calitzdorp	63.91 a-e	72.90 c-l	55.84 i-o
<i>Eutypella</i> sp. 2	STEU 8205	<i>Diospyros kaki</i>	Bonnievale	48.34 i-n	56.38 nm	57.71 f-o
<i>Eutypella</i> sp. 3	STEU 8208	<i>Schinus molle</i>	Oudtshoorn	54.40 c-n	80.50 a-i	66.44 a-l
<i>Eutypella</i> sp. 3	STEU 8207	<i>Schinus molle</i>	Oudtshoorn	54.63 b-n	72.62 d-l	65.02 a-m
PDA plug				20.88 o	23.78 o	34.45 p
LSD (P < 0.05)				12.49	14.30	15.09

<sup>a</sup>Means followed by the same letter in the same column are not significantly different ( $P < 0.05$ ).

**Table 4.** Specificity validation of qPCR methods to detect the target species (*Cryptovalsa ampelina* and *Eutypa lata*) and non-target fungal pathogens usually found colonising grapevine tissues, from isolates collected from different regions of the Western Cape Province.

Sample number	Isolate identity	Isolate number	Origin of isolate	Host	Symptom	<i>Cryptovalsa ampelina</i>	<i>Eutypa lata</i>
						qPCR assay (Ct values) <sup>a</sup>	qPCR assay (Ct values) <sup>a</sup>
1	<i>Cryptovalsa ampelina</i>	STEU 8232	Darling	<i>Vitis vinifera</i>	Perithecia	+(23.23±0.01)	ND
2	<i>Cryptovalsa ampelina</i>	STEU 8120	Durbanville	<i>Vitis vinifera</i>	Perithecia	+(24.82±0.01)	ND
3	<i>Cryptovalsa ampelina</i>	STEU 8233	Barrydale	<i>Vitis vinifera</i>	Wedge-shaped necrosis	+(25.98±0.03)	ND
4	<i>Cryptovalsa ampelina</i>	STEU 8234	Robertson	<i>Vitis vinifera</i>	Dying spur	+(24.08±0.06)	ND
5	<i>Cryptovalsa ampelina</i>	STEU 8235	Constantia	<i>Vitis vinifera</i>	Dying spur	+(24.72±0.03)	ND
6	<i>Cryptovalsa ampelina</i>	STEU 8236	Grabouw	<i>Vitis vinifera</i>	Dying spur	+(24.77±0.05)	ND
7	<i>Cryptovalsa ampelina</i>	STEU 8237	Riebeeck-Wes	<i>Vitis vinifera</i>	Wedge-shaped necrosis	+(26.55±0.07)	ND
8	<i>Cryptovalsa ampelina</i>	STEU 8238	Rawsonville	<i>Vitis vinifera</i>	Perithecia	+(25.65±0.04)	—
11	<i>Eutypa lata</i>	STEU 8239	Bonnievale	<i>Vitis vinifera</i>	Perithecia	ND	+(24.41±0.05)
12	<i>Eutypa lata</i>	STEU 8218	Durbanville	<i>Vitis vinifera</i>	Perithecia	—	+(24.79±0.03)
13	<i>Eutypa lata</i>	STEU 8240	Hermanus	<i>Vitis vinifera</i>	Wedge-shaped necrosis	ND	+(24.06±0.08)
14	<i>Eutypa lata</i>	STEU 8241	Slanghoek	<i>Vitis vinifera</i>	Perithecia	ND	+(24.43±0.19)
15	<i>Eutypa lata</i>	STEU 8242	Constantia	<i>Vitis vinifera</i>	Dying spur	ND	+(22.37±0.04)
16	<i>Eutypa lata</i>	STEU 8243	Sommerset West	<i>Vitis vinifera</i>	Wedge-shaped necrosis	ND	+(22.90±0.11)
17	<i>Eutypa lata</i>	STEU 8244	Barrydale	<i>Vitis vinifera</i>	Wedge-shaped necrosis	ND	+(27.78±0.04)
18	<i>Eutypa lata</i>	STEU 8245	Rawsonville	<i>Vitis vinifera</i>	Perithecia	ND	+(25.86±0.09)
19	<i>Cryptosphaeria multicontinentalis</i>	STEU 8164	Stellenbosch	<i>Salix mucronata</i>	Orange discolouration	—	—
20	<i>Cryptosphaeria ligniota</i>	STEU 8165	Stellenbosch	<i>Salix mucronata</i>	Orange discolouration	—	—

**Table 4.** (continued)

Sample number	Isolate ID	Strain number	Origin of isolate	Host	Symptom	<i>Cryptovalsa ampelina</i>	<i>Eutypa lata</i>
						qPCR assay (Ct values) <sup>a</sup>	qPCR assay (Ct values) <sup>a</sup>
21	<i>Cryptovalsa rabenhorstii</i>	STEU 8112	Stellenbosch	<i>Vitis vinifera</i>	Dying spur	—	—
22	<i>Diatrypella</i> sp.	STEU 8177	Stellenbosch	<i>Citrus limon</i>	Brown discolouration	—	—
23	<i>Eutypa consobrina</i>	STEU 8092	Grabouw	<i>Vitis vinifera</i>	Dying spur	—	—
24	<i>Eutypa cremea</i>	STEU 8083	Constantia	<i>Vitis vinifera</i>	Dying spur	—	—
25	<i>Eutypella australiensis</i>	STEU 8203	Hout Bay	Unknown host	Black spot	—	—
26	<i>Eutypella citricola</i>	STEU 8254	Constantia	<i>Vitis vinifera</i>	Dying spur	—	—
27	<i>Eutypella leprosa</i>	STEU 8190	Hout Bay	<i>Ficus carica</i>	Brown discolouration	—	—
28	<i>Eutypella microtheca</i>	STEU 8108	Robertson	<i>Vitis vinifera</i>	Dying spur	—	—
29	<i>Eutypella</i> sp.	STEU 8207	Oudtshoorn	<i>Schinus molle</i>	Brown-orange discolouration	—	—
30	<i>Eutypella</i> sp.	STEU 8206	Calitzdorp	<i>Psidium guajava</i>	Brown/black discolouration	—	—
31	<i>Eutypella</i> sp.	STEU 8205	Bonnievale	<i>Diospyros kaki</i>	Black spot	—	—
32	<i>Diaporthe ambigua</i>	STEU 8255	Stellenbosch	<i>Vitis vinifera</i>	Dying spur	—	—
33	<i>Fomitiporia capensis</i>	STEU 7922	Bonnievale	<i>Diospyros kaki</i>	Brown rot	—	—
34	<i>Ilyonectria liriodendri</i>	STEU 8256	Wellington	<i>Vitis vinifera</i>	Brown discolouration	—	—
35	<i>Neofusicoccum australe</i>	STEU 8257	Grabouw	<i>Vitis vinifera</i>	Dying spur	—	—
36	<i>Phaeoacremonium minimum</i>	STEU 8258	Grabouw	<i>Vitis vinifera</i>	Dying spur	—	—
37	<i>Phaeomoniella chlamydospora</i>	STEU 7538	Stellenbosch	<i>Vitis vinifera</i>	Dying spur	—	—

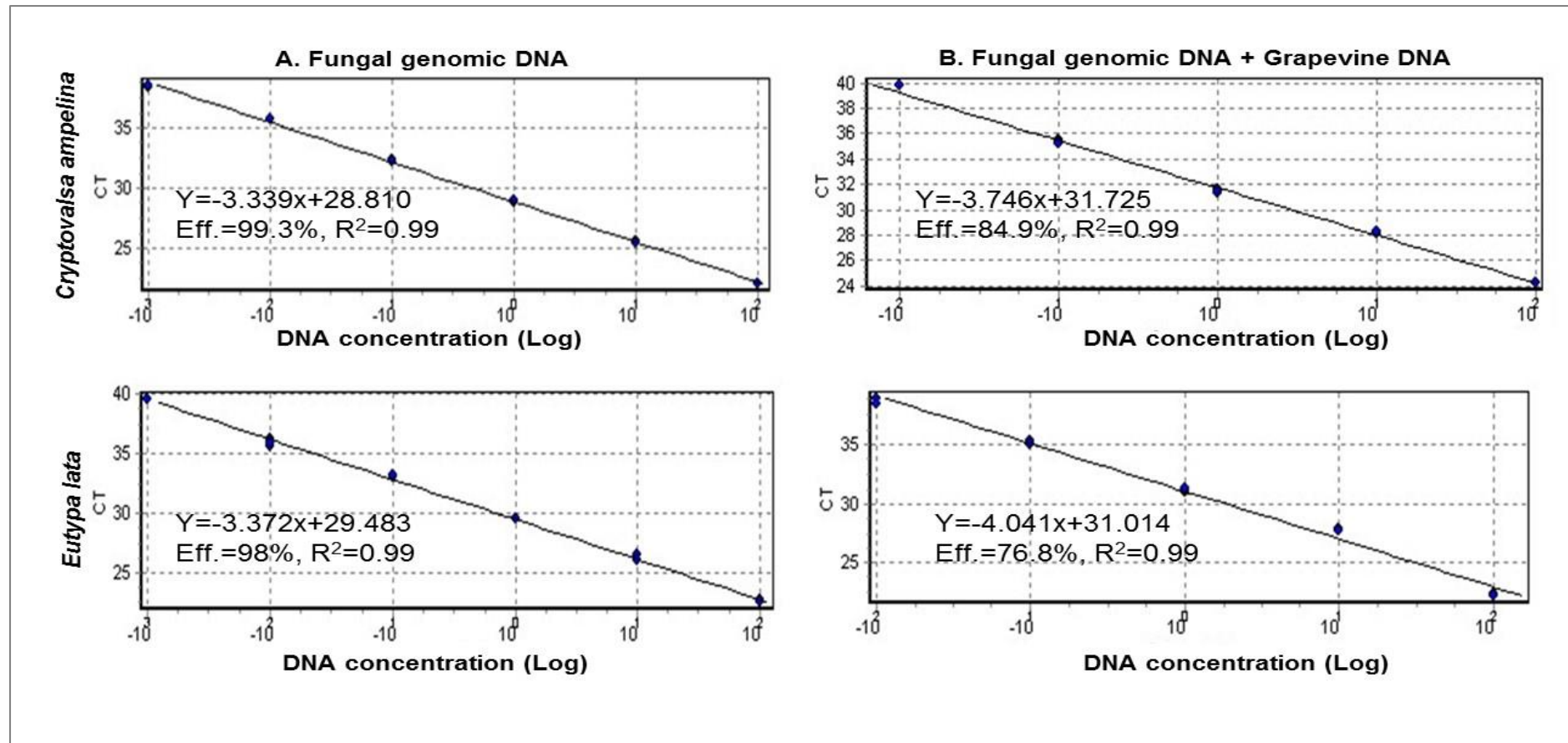
<sup>a</sup> + = positive detection; — = negative detection; the mean Ct of three replicates ± standard deviation are given in brackets; ND=Not determined



**Table 5.** Detection of *Cryptovalsa ampelina* and *Eutypa lata* from grapevine samples, artificially inoculated with target species, using qPCR assays developed in this study.

Isolate identity	Isolate number	<i>Cryptovalsa ampelina</i> qPCR assay		<i>Eutypa lata</i> qPCR assay	
		(Ct values) <sup>a</sup>	Target DNA detected (ng/μl)	(Ct values) <sup>a</sup>	Target DNA detected (ng/μl)
<i>Cryptovalsa ampelina</i>	STEU 8232	+(33.18±0.09)	0.067	ND	ND
<i>Cryptovalsa ampelina</i>	STEU 8120	+(37.10±0.26)	0.0045	ND	ND
<i>Cryptovalsa ampelina</i>	STEU 8238	+(35.24±0.23)	0.016	ND	ND
<i>Cryptovalsa ampelina</i>	STEU 8217	+(35.88±0.27)	0.010	ND	ND
<i>Cryptovalsa ampelina</i>	STEU 8216	+(36.49±0.13)	0.0069	ND	ND
<i>Eutypa lata</i>	STEU 8239	ND	ND	+(29.94±0.26)	2.587
<i>Eutypa lata</i>	STEU 8218	ND	ND	+(33.23±0.09)	0.274
<i>Eutypa lata</i>	STEU 8240	ND	ND	+(34.76±0.48)	0.096
<i>Eutypa lata</i>	STEU 8246	ND	ND	+(25.64±0.08)	4.841
Uninoculated wood		–	0	–	0

<sup>a</sup> + = positive detection; – = negative detection, the mean Ct of three replicates ± standard deviation are given in brackets; ND=Not determined



**Figure 1.** Standard curves from qPCR reactions of 10-fold dilution series of DNA of *Cryptovalsa ampelina* (isolate STEU 8238) and *Eutypa lata* (isolate STEU 8245), starting with 100 ng/ $\mu$ l of: **A.** fungal genomic DNA and **B.** fungal genomic DNA containing 10 ng/ $\mu$ l of DNA of un-inoculated grapevine wood. Standard curves are as produced by the Rotor-Gene Software 2.0.2.4. Regression coefficients (R<sup>2</sup>) and the slope of each regression curve are shown. The results presented are based on the means of three replicates. CT means cycle threshold and Eff. refers to PCR reaction efficiency.

## CHAPTER 5

### General discussion

Eutypa dieback is an important disease of grapevine, worldwide. For many decades, this disease has been attributed to the Diatrypaceae fungus, *Eutypa (E.) lata* and hence this fungus was the focus of many studies, which led to much work being published on its identification, epidemiology and management. The recent evaluation of grapevines exhibiting Eutypa dieback symptoms, in different grape growing areas including South Africa, Australia, Spain and the United States of America, has revealed that infected grapevines are colonised by several species belonging to the family Diatrypaceae. The possible involvement of several species of Diatrypaceae in Eutypa dieback has stimulated more research into the disease, in different grape growing areas around the world. The research has mainly focused on the taxonomy, inoculum sources and the capability of the newly discovered Diatrypaceae species to have a severe impact on the sustainability of the grapevine industry.

Several aspects of the Diatrypaceae and Eutypa dieback are discussed in Chapter 1 of this dissertation. Prior to the present investigation, no extensive work was done on the taxonomy and the role of the Diatrypaceae in grapevine decline in South Africa. Molecular detection tools have been limited to conventional PCR. It is necessary that several aspects regarding the Diatrypaceae including the diversity, host range, ecology, detection and pathogenicity are investigated in order to define and address any threat that these species may pose to the grapevine industry in South Africa and to contribute to management strategies to combat this disease.

#### 5.1. Diversity of Diatrypaceae species associated with declining grapevines

Isolations were carried out from dying spurs, wood samples showing wedge-shaped necrosis in cross section and single ascospore isolates were also obtained from perithecia on dead grapevine wood, in order to identify Diatrypaceae species associated with declining grapevines. Seven Diatrypaceae species were identified in this study and these findings were consistent with other studies which demonstrated that several Diatrypaceae species are associated with declining grapevines (Trouillas and Gubler, 2004; Pitt *et al.*, 2010; Trouillas *et al.*, 2010, 2011; Díaz *et al.*, 2011; Luque *et al.*, 2012). The frequency of isolation of the Diatrypaceae species between the different symptom types examined differed, which suggests that different symptoms can be attributed to different fungal species. For example, *Cryptovalsa (C.) ampelina* was the most predominant species isolated from dying spurs whereas *E. lata* was the most common pathogen isolated from wedge-shaped necrosis. However, in some instances, more than one fungal species was isolated from a single

symptom which suggests that interactions may also be occurring leading to the observed symptom.

Multiple infections may affect virulence and have the potential to influence epidemiology of pathogens (Bose *et al.*, 2016). Understanding the interactions between Diatrypaceae species (and/or between Diatrypaceae species and other grapevine trunk pathogens) should thus, be addressed if the grapevine lifespan is to be prolonged. There is, however, also a possibility that pathogens may not act differently in multiple compared to single infection of grapevine tissues, but the grapevine may represent a limited resource and therefore, competition between the cohabiting Diatrypaceae species within the grapevine is most likely. Competition could result in increased enzyme production within each species to facilitate the exploitation of host resources (Brown *et al.*, 2002). Bose *et al.* (2016) postulates that if, for instance, a host is co-infected by two species with different virulence strategies, the more virulent species may grow faster than its competitor and in doing so, it kills the host even quicker.

Despite evidence showing the involvement of several Diatrypaceae species occurring in Eutypa dieback-affected grapevines, the contribution of each species in the disease expression or mortality of the host is unclear. Studies have only focused on single inoculation of Diatrypaceae species on grapevine to evaluate their pathogenicity behaviour and therefore, the extent of combined inocula of pathogens associated with Eutypa dieback in the development of the disease is unknown. To fully understand the role of each Diatrypaceae species in Eutypa dieback development, it is important to assess not only the ability of the pathogen to infect and cause a symptom, but also its effect when in association with other pathogens. The order in which pathogens infect the host has also been found to influence the interaction of fungal species in a complex disease (Lamichhane and Venturi, 2015). For instance, when *Mycosphaerella pinodes* and *Phoma medicaginis* var. *pinodella*, which are associated with Ascochyta blight disease complex, were simultaneously inoculated, disease development was limited. However, when the pathogens were inoculated one after the other, a marked increase in severity of the disease was observed (Le May *et al.*, 2009). Furthermore, because both hosts and pathogens each have their own responses to the environment, as does their interaction (Barret *et al.*, 2009), studies therefore, need to further address the interactions between the different fungal species and grapevine, together with environmental conditions that may alter the nature of their interactions. Such studies are obviously complex, but these interactions need to be taken into consideration in the development of more effective control measures against the disease.

The Diatrypaceae has been plagued by taxonomic complexities, but the recent advances in molecular tools have been crucial in the correct identification of species in this group. The Diatrypaceae was first subjected to molecular phylogenetic analysis, based on the internal

transcribed spacer regions (ITS1 and ITS2) and the 5.8S rRNA gene, by Acero *et al.* (2004). Results of this study could not find any correlation between the clades and the traditional classification scheme of the Diatrypaceae, which is based on morphological characteristics and evolutionary relationships among the different taxa could, therefore, not be clarified. Trouillas *et al.* (2010, 2011) and Almeida *et al.* (2016) conducted phylogenetic studies on this fungal group, based on ITS and the partial  $\beta$ -tubulin gene. New species were described in these studies. Both studies suggested wider sampling of taxa and using multiple genes to help elucidate relationships among genera of this fungal group. Trouillas *et al.* (2015) investigated the taxonomy of *Cryptosphaeria* (*Cr.*) species using multilocus phylogenetic analyses based on ITS,  $\beta$ -tubulin gene, DNA-dependent RNA polymerase II subunit 2 (*RPB2*) and partial 28S coding region (28S). They showed that *Cr. multicontinentalis* and *Cr. lignyota* are sister species which are closely related to *Cr. subcutanea* although more distantly related to *Cr. pullmanensis*. The focus of the present study was not to comprehensively evaluate the biological and taxonomic aspects of the Diatrypaceae, however, the application of phylogenetic analysis necessitated the assignment of a new name to isolates previously identified as *Eutypa leptoplaca*. Furthermore, Acero *et al.* (2004) found that isolates designated as *Diatrype flavovirens* were distinct from other *Diatrype* species when phylogenetically analysed. The authors suggested that these isolates require to be assigned to a new genus, but several genes would need to be analysed. Isotypes need to be recollected for herbarium specimens for which there is no living cultures or sequence data available. This will aid in obtaining a more complete phylogeny of the Diatrypaceae.

## 5.2. Diatrypaceae species associated with woody hosts occurring adjacent to vineyards

The first phase of the work (Chapter 2) revealed the presence of *Cryptovalsa rabenhorstii* and *Eutypa consobrina*, on declining grapevines, but no perithecia of these species were found. Speculations were that the presence of these species was influenced by the occurrence of other woody hosts in the vicinity of vineyards. In that regard, the first objective of Chapter 3 was to investigate non-grapevine woody hosts occurring in close proximity to vineyards, as potential sources of inoculum of Diatrypaceae species to adjacent vineyards. Fourteen Diatrypaceae species were subsequently, identified from the different woody hosts examined and the most frequently encountered species were *E. lata*, *C. ampelina*, *Eutypella* (*Eu.*) *citricola*, *Eutypella microtheca* and *Eutypa cremea*, which were also the most abundant species isolated from declining grapevines in Chapter 2. The presence of the same Diatrypaceae species on grapevine and other woody hosts reinforce results from previous studies (Trouillas *et al.*, 2010, 2011) and highlights the importance of non-grapevine woody hosts surrounding vineyards as inoculum sources to nearby vineyards in South Africa.

Although studies have shown that Diatrypaceae isolates collected from different woody hosts have the potential to infect grapevine (Trouillas and Gubler, 2010a, b; Travadon and Baumgartner, 2015), the importance of woody hosts in the spread of species associated with Eutypa dieback to healthy vineyards is unclear. Studying the genetic relatedness of populations of the newly discovered Diatrypaceae species that occur in vineyards and compare with populations on other woody hosts would determine whether there is gene flow between these hosts and grapevine or not. Genetic diversity is an important feature for fungal populations with respect to dispersal and certain traits such as virulence and resistance to control strategies (Cortesi and Milgroom, 2001). Therefore, an in-depth understanding of population dynamics of the Diatrypaceae pathogens, which have been shown to infect multiple woody hosts, is necessary especially with the growing concerns about the newly discovered species infecting grapevines. Travadon and Baumgartner (2015) found no substantial genetic differentiation of *E. lata* populations between grapevine, apricot and willow in California proving that high gene flow occurred between these hosts. The possibility of gene flow among different woody hosts and grapevine needs to be ascertained and plant hosts contributing more to disease need to be identified for the purpose of developing effective disease management strategies (Gandon, 2004).

Perithecia of Diatrypaceae species found on grapevine were also found on several other woody hosts which indicates that these species can survive in the absence of grapevines. The availability of Diatrypaceae inoculum from a broad range of hosts around vineyards presents complications in the efforts to control any infections that maybe caused by these species in the vineyards. This therefore, implies that management practices for Eutypa dieback will not only have to focus on vineyards alone, but also consider non-grapevine hosts occurring around vineyards. A number of strategies including sanitation, protection of host plants, development of resistance in the host plants and treatment of infected plants are used for disease management (Ploetz, 2007). Sanitation, (the removal of infected debris and host material) is a common practice in the grapevine industry. This practice could also be used in cases where inoculum reservoirs are significant. Thus, the removal and destruction of other woody hosts for Diatrypaceae species would reduce the inoculum pressure on vineyards. However, this strategy is most feasible for pathogens with limited host ranges rather than wide host ranges, as discovered in this study and its effectiveness also depends on the size of the woody hosts (Ploetz, 2007).

Since a wide range of reservoirs were found for the Diatrypaceae, certainly it is going to be difficult to eradicate all infected hosts around vineyards and therefore, other methods of disease management such as the protection of grapevine pruning wounds might be of significant value. A number of chemical, physical and biological pruning wound protectants have been studied in the efforts to manage Eutypa dieback. Except for a few fungicides tested

by Gramaje *et al.* (2012), none of these protectants have been evaluated for the potential to control or prevent any infection that could be caused by the newly discovered Diatrypaceae species on grapevine. Because of the unlimited Diatrypaceae inoculum around vineyards in South Africa, future studies should therefore, focus on testing different pruning wound protectants against the Diatrypaceae fungi.

Since stone fruits are one of the most economically important fruit crops in South Africa (PHI, 2015) and were found to be colonised by the Diatrypaceae, the next objective of this study was to determine the potential role of Diatrypaceae species in the decline of stone fruit orchards by means of pathogenicity tests. Pathogenicity tests performed on apricot and plum demonstrated that the newly discovered Diatrypaceae species on stone fruit trees can cause vascular discolouration characteristic of *Eutypa* dieback of apricot and they produced lesions equivalent in length to those produced by *E. lata*. Given the results of this study and the ease of isolation of Diatrypaceae species from dieback symptoms and cankers on stone fruit trees, further research is needed to determine the distribution and epidemiology of these species on stone fruits. This is necessary in efforts to find effective management strategies against these fungi in stone fruit orchards.

### **5.3 qPCR detection and pathogenicity of Diatrypaceae species on grapevine**

Results obtained in Chapters 2 and 3 provided confirmation that declining grapevines are colonised by several Diatrypaceae species and non-grapevine woody hosts are important sources of inoculum to adjacent vineyards in South Africa. However, the status of the newly discovered Diatrypaceae species as grapevine pathogens or their relative importance in South African vineyards was unknown before this study. The first objective of Chapter 4 was therefore, to characterise Diatrypaceae species isolated from grapevine by means of pathogenicity testing on mature grapevines. The capacity of the Diatrypaceae isolates obtained from other hosts to infect grapevine was also tested. Pathogenicity tests performed in this study confirmed previous reports about the ability of newly discovered Diatrypaceae species to colonize grapevine wood and cause lesions (Trouillas and Gubler, 2010a, b; Pitt *et al.*, 2013). Results presented in this study also demonstrated that several species including *C. ampelina*, *Eu. citricola* and *Eu. microtheca* caused vascular discoloration similar in length to that caused by *E. lata*. Results further showed that isolates of Diatrypaceae species collected from non-grapevine hosts are pathogenic to grapevine, reinforcing the importance of additional hosts as importance sources of Diatrypaceae inoculum for grapevines.

Cell wall degrading enzymes and phytotoxins are involved in the breakdown of plant tissues and the induction of cell death during pathogenicity (Saldanha *et al.*, 2007). *Eutypa lata* is known to slowly kill its host tissues by producing cell wall degrading enzymes and

phytotoxins (English and Davis, 1978; Tey-Rulh *et al.*, 1991). Pildain *et al.* (2005) showed that species in the Diatrypaceae were capable of producing ligninolytic and cellulolytic enzymes and postulated that species in the Diatrypaceae might therefore, be capable of causing wood decay. However, the roles of extra cellular compounds and toxicity of the potential metabolites produced by the newly discovered Diatrypaceae species in the expression of Eutypa dieback symptoms has not been studied and may need to be investigated.

Recently, DNA based techniques have been used increasingly to identify Diatrypaceae from grapevine wood. The infection of grapevine by a complex of Diatrypaceae species, as seen in Chapter 2 and other studies (Trouillas *et al.*, 2001; Pitt *et al.*, 2010), has provided scope to develop rapid and effective techniques to detect and quantify Diatrypaceae in grapevine wood. The second objective of Chapter 4 was to develop species-specific primers in a real-time quantitative polymerase chain reaction (qPCR) approach to detect and quantify DNA of *E. lata* and *C. ampelina* on grapevine wood. Both methods developed had a detection limit of 0.001 ng/ $\mu$ l fungal genomic DNA and 0.01 ng/ $\mu$ l DNA extracted from grapevine wood. Both procedures were specific since they did not yield any detection signal when applied to non-target fungal species often found colonising grapevine wood. Their reliability was confirmed by the positive detection of several isolates of each target species, collected from different grape growing areas in the Western Cape Province, as well as grapevine wood samples artificially inoculated with isolates of each species, respectively. Identification of Eutypa dieback involves the recognition of symptoms on grapevines. However, Eutypa dieback symptoms are unreliable because they only appear a long time after infection has occurred. The qPCR assays will therefore, be of significant value in the early detection and accurate identification of these pathogens in symptomless grapevines.

#### **5.4 Conclusion and future prospects**

The primary step in managing plant diseases is to identify the causal agent before any control strategies are implemented. To that end, this study succeeded in identifying Diatrypaceae species associated with declining grapevines and identified other woody hosts as potential sources of inoculum for these species, to vineyards in South Africa. The pathogenicity of several species on grapevine and stone fruit trees was also confirmed in this study. Similar to the present study, many studies around the world have largely focused on host range as well as species diversity and pathogenicity of Diatrypaceae on grapevine (Pitt *et al.*, 2010, 2013; Trouillas and Gubler, 2010a, b; Trouillas *et al.*, 2010; Chapter 2 and 3). Thus, much is still unknown with regards to the biology and epidemiology of the Diatrypaceae on grapevine except for *E. lata*.



The grapevine pathosystem is complex with a diversity of trunk pathogens and environmental conditions involved in disease development and expression. Sosnowski *et al.* (2007) suggested that the variation in severity of Eutypa dieback from one year to another could be attributed to environmental factors. Future studies should therefore, focus on unravelling the potential effects of environmental conditions on the pathogenicity and disease expression caused by the Diatrypaceae. The interactions between the Diatrypaceae and other fungal groups within the grapevine wood will also make an interesting topic for future research. The fact that Diatrypaceae species cohabit grapevine tissues with other trunk disease pathogens (Úrbez-Torres *et al.*, 2009; Pitt *et al.*, 2010), could have effects on the virulence of the colonising species. Interactions among different grapevine trunk disease pathogens should be assessed.

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