Universidade de Aveiro Departamento de Biologia
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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Artur Jorge da Costa Peixoto Alves, Investigador Principal do Departamento de Biologia da Universidade de Aveiro, do Professor Doutor António Carlos Matias Correia, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e do Doutor Alan John Lander Phillips, Investigador Principal da Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa

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o júri

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palavras-chave

Botryosphaeriaceae, eucaliptos, alterações climáticas, stress hídrico, patogenicidade, stress combinado, espetrospopia de infravermelho, fisiologia vegetal.

resumo

O eucalipto é das espécies florestais mais plantadas devido à sua importância económica. Em Portugal, (principalmente *E. globulus*) representa atualmente 26% da área total, sendo a espécie florestal mais abundante no país. Membros da família Botryosphaeriaceae podem ocorrer como endofíticos ou patogénios latentes numa variada gama de hospedeiros lenhosos. Várias espécies têm sido associadas a eucaliptos em todo o mundo. Apesar da sua importância económica, não existem estudos relacionados com a ocorrência de espécies de Botryosphaeriaceae associadas a eucaliptos em Portugal, nem sobre o impacto que as alterações climáticas possam ter no desenvolvimento de doenças.

Foi estudada a comunidade de espécies de Botryosphaeriaceae que ocorre tanto em plantações saudáveis como doentes de *E. globulus* por todo o país. Nove espécies pertencentes a três géneros (*Botryosphaeria*, *Diplodia* e *Neofusicoccum*) foram identificadas, sendo o género *Neofusicoccum* claramente dominante tanto em plantas doentes como saudáveis. *Neofusicoccum algeriense*, *D. corticola* e *D. seriata* foram descritos pela primeira vez em *E. globulus*, enquanto *N. algeriense*, *N. kwambonambiense* e *N. eucalyptorum* correspondem aos primeiros registos em Portugal.

É fundamental detetar precocemente estes fungos de modo a evitar surtos de doenças que possam resultar em elevadas perdas económicas. A sua identificação, de modo geral, baseia-se em técnicas moleculares que embora muito poderosas na discriminação de espécies podem ser demoradas e dispendiosas. A técnica de espectroscopia de infravermelho médio (MIR) permitiu a discriminação de espécies de Botryosphaeriaceae com base no seu perfil de “fingerprint” de infravermelho. Esta técnica revelou potencial para ser uma alternativa eficaz, rápida e económica aos métodos de identificação convencionais.

Em ensaios de inoculação artificial foram encontradas diferenças claras na agressividade destes fungos. *Neofusicoccum kwambonambiense* e *D. corticola* foram as espécies mais virulentas, em contraste com *B. dothidea* e *D. seriata*. Apesar de algumas diferenças nos parâmetros morfo-fisiológicos não foi encontrada qualquer relação direta entre o tamanho da lesão (agressividade) e as respostas fisiológicas da planta. Considerando o perfil fisiológico global e as dimensões das lesões notou-se uma clara variação na suscetibilidade entre os diferentes genótipos de plantas testadas.

As alterações climáticas influenciam a ocorrência e gravidade das doenças nas plantas. Os nossos resultados indicam que as plantas em stress hídrico são mais suscetíveis a espécies de Botryosphaeriaceae. Esta resposta foi particularmente relevante quando os fungos foram inoculados em plantas que já se encontravam em privação de água. Além disso, o pré-condicionamento das plantas a condições de seca levou a um ligeiro aumento da resistência à infeção fúngica.

Os nossos resultados realçam o facto de as estratégias de gestão para as plantações não poderem ignorar o impacto que as doenças associadas a Botryosphaeriaceae podem ter num cenário de alterações climáticas.

keywords

Botryosphaeriaceae, eucalypts, climate changes, drought stress, pathogenicity, stress interaction, infrared spectroscopy, plant physiology.

abstract

Eucalypts are one of the most widely planted forest trees due to their economic importance. In Portugal, they (mostly *E. globulus*) represent currently 26% of the total forest area, being the most abundant forest tree in the country. Botryosphaeriaceae species occur as endophytes or latent pathogens on a diverse range of woody hosts, including eucalypts. Despite the economic importance of these plants, there are no studies related to the occurrence of Botryosphaeriaceae species associated with them in Portugal or the impact of climate changes in the triggering of diseases.

The community of Botryosphaeriaceae species occurring on diseased and healthy *E. globulus* trees was studied on several plantations throughout the country. Nine species from three different genera (*Botryosphaeria*, *Diplodia* and *Neofusicoccum*) were identified being the genus *Neofusicoccum* clearly dominant on both diseased and healthy trees. *Neofusicoccum algeriense*, *D. corticola* and *D. seriata* were reported for the first time on *E. globulus*, while *N. algeriense*, *N. eucalyptorum* and *N. kwambonambiense* correspond to the first reports in Portugal.

The early detection of Botryosphaeriaceae species could allow preventing disease outbreaks that may result in significant economic losses. The identification of these fungal species is, in general, based on molecular techniques, that although being very powerful in discriminating species, can be time consuming and still quite expensive. Mid-infrared spectroscopy (MIR) technique allowed the discrimination of species of Botryosphaeriaceae based on their infrared fingerprint profile, being a powerful, cost-effective and faster alternative method to conventional identification techniques.

In artificial inoculation trials, marked differences in aggressiveness between these fungi were reported. *Neofusicoccum kwambonambiense* and *D. corticola* were the most virulent species while *B. dothidea* and *D. seriata* were the less ones. Despite some differences in morpho-physiological parameters no direct relation was found between lesion sizes (aggressiveness) and plant morpho-physiological responses. Considering the global physiological profile and lesion sizes, a clearly variation in susceptibility between different genotypes of eucalypts in study was shown.

It is known that climate changes influence the occurrence and severity of plant diseases. Our results indicate that water stressed plants are more susceptible to Botryosphaeriaceae diseases. This response was particularly relevant when the plant was inoculated while water deprivation was already occurring. Moreover, drought primed plants presented a slightly increased resistance to fungal infection.

Our results reinforce the fact that management strategies for plantations should not overlook the impact that Botryosphaeriaceae diseases can have in a climate change scenario.

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Thesis outline

The present thesis documents the research work carried out in the scope of evaluating the potential impact that climate changes, especially drought, would have on the development of Botryosphaeriaceae diseases on *Eucalyptus*.

In Portugal, the forestry sector has a great economic relevance and *Eucalyptus* species are one of the most widely planted and commercially exploited forest species in this country. Members of the family Botryosphaeriaceae are known to cause diseases in a wide range of plants including eucalypts and mostly when the host is exposed to physiological stress. Thus, it is expected that these pathogens will be favored by climatic changes, especially drought. However, virtually nothing is known regarding the occurrence and severity of Botryosphaeriaceae species associated with eucalypts in Portugal, as well as the effect of climate changes on diseases of eucalypts.

This document is organised in six chapters:

The first chapter comprises the general review of literature to provide background for the work carried out, and to state the objectives of the research.

In chapter 2, the diversity and potential impact of Botryosphaeriaceae species associated with *Eucalyptus globulus* plantations in Portugal are presented. In this chapter the fungal species associated with these plants as well as their aggressiveness to them were identified. The fungal species obtained in this chapter were used in the subsequent work.

In chapter 3, an alternative method for fungal identification is presented based on infrared spectroscopy (MIR) coupled with multivariate analysis. Molecular techniques, although very powerful in discriminating species, still have some limitations and MIR have proved to be a good alternative.

In chapter 4, the pathogenicity of the Botryosphaeriaceae species identified in chapter 2 was evaluated towards three *Eucalyptus* species.

In chapter 5, the concepts of multiple stress (fungal disease and drought), priming and predisposition were tested. This information allows a better understanding of fungi-drought interaction to improve the establishment and productivity of *Eucalyptus* species in Portugal.

Finally, Chapter 6 presents the main conclusions of this work, as well as some ideas for future work.

CHAPTER 1

Introduction

The host: *Eucalyptus* species

Characteristics and importance

Eucalyptus sensu lato (Myrtaceae) is a species-rich group that includes the genera *Eucalyptus*, *Angophora*, and *Corymbia* (Paine et al. 2011) and according to The Plant List (2013) more than 800 species are accepted. These plants are mostly Australian natives (Du et al. 2015; Wingfield et al. 2015) but they can also be found as native trees in Indonesia, Philippines, and New Guinea (Paine et al. 2011).

In spite of their origin, eucalypts are the most widely planted hardwood trees and the total area planted is estimated at 20 million ha (Du et al. 2015; Wingfield et al. 2015). For instance, Brazil (21%), India (19%), and China (13%) are the three non-native countries with the largest planted area (Paine et al. 2011; Wingfield et al. 2015).

Eucalypts are an important economic resource for the forest-related industries (Paine et al. 2011; Slippers et al. 2004b) since they exhibit fast-growth rates, excellent pulp properties, short rotation, easy vegetative propagation and wide adaptability to soils and climates (Brondani et al. 2012; Old et al. 2003). Furthermore, eucalypts are also planted as ornamental trees namely in North America (Paine et al. 2011).

Currently, more than ten species and their hybrids are well established in commercial plantations around the world (Wingfield et al. 2015). *Eucalyptus grandis* W. Hill is more commonly exploited in tropical/subtropical areas while *Eucalyptus globulus* Labill. is widely planted in temperate zones (Carocha et al. 2015), including Mediterranean climates. For instance, in the Southern Hemisphere tropics and subtropical regions, eucalypts plantations are an important source of fibre (Slippers et al. 2004b). In China, eucalypts plantations have been increasing, and currently there are about 2-6 million ha of established plantations that have an important role in paper and structural timber industries (Chen et al. 2011). In Colombia, *Eucalyptus* species are used by private companies and in government projects to produce timber and pulp, and protect soils from erosion (Rodas et al. 2009). In Ethiopia, eucalypt plantations represent around 100 000 ha and supply wood for fuel, construction and production of poles and posts (Gezahgne et al. 2004). Several species and hybrids of *Eucalyptus* have been extensively planted in Mexico where they cover around 25 000 ha of commercial plantations (de la Mora-Castañeda et al. 2014). *Eucalyptus globulus* is well adapted to the Mediterranean climate where it is exploited mainly for the production of pulp and currently occupies approximately 812 000 ha in Portugal (ICNF 2013) and 760 000 ha in Spain (Gominho et al. 2014).

Eucalyptus globulus plantations in Portugal

In Portugal, the forest sector is one of the pillars of the economy since this activity generates numerous jobs (3 % of the total employment) and contributes widely to the national exports (10 % of the Portuguese exports values) (Louro et al. 2014).

Eucalyptus species were introduced in Portugal in the middle of the 19th century (Águas et al. 2014; Bragança et al. 2015) and, nowadays, are the most abundant forest trees in the country (Figure 1.1), representing approximately 26% of the total forest area (ICNF 2013).

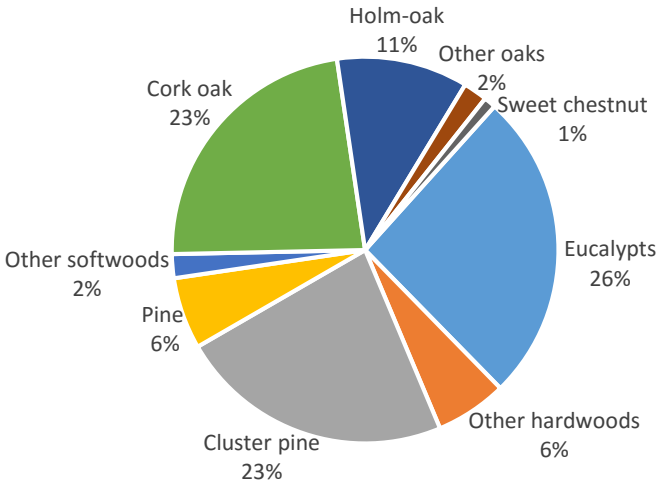

Figure 1.1: Distribution of the species plants for total area in Portugal (adapted from ICNF (2013)).

Eucalyptus globulus or blue gum, which is well adapted to Mediterranean climate (Granda et al. 2014), is the dominant species in Portugal, both in commercial and non-commercial plantations. Since these plants have high productivity of about 16 m^3 ha^{-1} $year^{-1}$ (reaching 30 m^3 ha^{-1} $year^{-1}$), short rotation (12 years), and excellent pulp properties, they are widely exploited by pulp industries (Águas et al. 2014; Pita et al. 2011). In spite of the dominance of *E. globulus*, other *Eucalyptus* species have been introduced in Portugal also. For instance, *E. camaldulensis* is more common in urban parks or growing as arboreta and roadside trees (Pessoa et al. 2014). According to Decreto-Lei nº565/99 there are more 21 species and hybrids in Portugal whose occurrence is infrequent or even rare.

Pests and diseases of *Eucalyptus* plants

Eucalypts are distributed worldwide and their total plantation area has increased in the last centuries (Paine et al. 2011; Roux et al. 2005). Despite their high adaptability to different environments, the incidence of pests and pathogens associated with these hosts is increasing (Naidoo et al. 2014; Paine et al. 2011; Roux et al. 2005).

The distribution of *Eucalyptus* pests currently is not restricted to Australia and it is possible to find Australian insect herbivores colonizing new environments around the world, although the patterns of colonization are not yet clear (Paine et al. 2011; Phillips 2008; Zhou and Wingfield 2011).

Among pathogens, fungal species are the most frequently found associated with *Eucalyptus* trees, although some bacteria (Coutinho et al. 2002) and oomycetes (Wingfield and Knox-Davies 1980) have also been reported. Some fungal species are endophytes,

living inside the plants without causing any symptoms, whereas others are important pathogens that cause diseases on these plants (Old et al. 2003; Slippers and Wingfield 2007; Slippers et al. 2004c). Diseases can appear in different stages of the plant growth, from seedlings in nurseries to trees after out-planting (Old et al. 2003). Moreover, some fungi spend part of their life-cycles as endophytes within healthy plant tissue making the detection very difficult and increase the possibility of their transmission to different areas through introduction of germplasm (Old et al. 2003; Slippers et al. 2004c, 2009).

Pests and pathogens found in eucalypt plantations may come from their native environment (through seeds and plants) or can be introduced as result of host shifts (Naidoo et al. 2014; Paine et al. 2011). For instance, host jumps of fungal species between native Myrtaceae and *Eucalyptus* have previously been reported (Pérez et al. 2010; Slippers et al. 2005) as well as host shifts of native pests (Paine et al. 2011; Wingfield et al. 2008). In their native environment, the variability of host phenotype provides significant protection against pests and pathogens, but in commercial plantations the genetic uniformity of the plants increases the risk of diseases leading to high economic losses (Chen et al. 2011; Naidoo et al. 2014; Old et al. 2003; Slippers et al. 2009).

Several authors have focused on the most frequent and severe pests and pathogens that globally affect eucalypts plants (Armengol et al. 2008; Crous et al. 1989; de la Mora-Castañeda et al. 2014; Roux et al. 2005; Silva et al. 2014; Zhou and Wingfield 2011). Thus, bacterial wilt *Ralstonia solanacearum* (Smith) Yabuuchi et al., bacterial dieback *Xanthomonas eucalypti* (Coutinho et al. 2002), root rot *Phytophthora cinnamomi* Rands (Oomycete) (Naidoo et al. 2014; Wingfield and Knox-Davies 1980), myrtle rust *Puccinia psidii* G. Winter (Coutinho et al. 1998; Naidoo et al. 2014), and stem canker *Chrysoporthe austroafricana* Gryzenhout & M.J. Wingf. (Naidoo et al. 2014; Wingfield et al. 1989) and Botryosphaeriaceae (Rodas et al. 2009; Smith et al. 1994), are some examples of the most frequently reported pathogens. Concerning insect pests, blue gum psyllid *Ctenarytaina eucalypti* Maskell, psyllid *Glycaspis brimblecombei* Moore, snout beetle *Gonipterus scutellatus* Gyllenhal, galling wasp *Leptocybe invasa* Fisher & LaSalle, longhorned borer *Phoracantha semipunctata* Fab. (Naidoo et al. 2014) are also recognized as a threat to eucalypts.

Pests and diseases of *Eucalyptus* plants in Portugal

When eucalypts were introduced in Portugal, as it was observed in other countries where these plants are exotic species, they have been almost free from pests and diseases (Bragança et al. 2015; Wingfield et al. 2008). However, in the last decades, it has been verified an increasing tendency in damage caused by pests and pathogens in Portuguese eucalypt forests (Bragança et al. 2015; Reis et al. 2012; Silva et al. 2012) and this is correlated with the marked increase in occupied area by *Eucalyptus* species (Branco et al. 2014).

Concerning insect pests, most of them are invasive species which are native to Australia (Branco et al. 2014). This is the case of *Gonipterus plantensis* Marelli, the major problem in Portuguese eucalypt plantations and the species responsible for tree growth losses in the Centre and North of Portugal (Reis et al. 2012). *Ctenarytaina eucalipti* Maskell, which feeds on the buds of the juvenile leaves, constitutes a problem in nurseries, however, in the field they have low impact because of the presence of many native predators (Azevedo and Figo 1979). *Phoracantha semipunctata* Fab. mainly attacks weakened trees under water stress, leading to plant death (Caldeira et al. 2002), and *P. recurva* Newman, which causes less severe injuries, is more restricted to the southern areas (Valente and Ruiz 2002).

There are some other Australia natives pest species, but their entrance in Iberian Peninsula was not through a direct pathway, for example, *Ctenraytaina spatulata* Taylor, *Glycaspis brimblecombei* Moore, *Blastopsylla occidentalis* Taylor (Pérez-Otero et al. 2011; Valente et al. 2004), *Rhombacus eucalypti* Ghosh & Chakrabarti (Ferreira et al. 2006), *Leptocybe invasa* Fisher & La Salle, *Ophelimus maskelli* Ashmead (Branco et al. 2006), and *Thaumastocoris peregrinus* Carpintero & Dellapé (Garcia et al. 2013).

Although, e*ucalypts* plantations were almost free from fungal diseases for a long period after their introduction in Portugal, *Botryosphaeriaceae* and *Mycosphaerella* spp. were considered the most important agents in earlier studies. In recent decades, other pathogens were also associated with dieback, canker and mortality observed on eucalypt stands, namely *Phomopsis* spp., *Teratosphaeria* spp., *Cytospora* spp., *Pestalotiopsis* spp., *Phytophthora* spp., *Sporothrix* spp., *Phoma* sp., *Harknessia* sp., *Cylindrocarpon* sp., *Biscogniauxia mediterranea* (De Not.) Kuntze, *Teratosphaeria gauchensis* (M.N. Cortinas, Crous & M.J. Wingf.) M.J Wingf. & Crous, and *Quambalaria eucalypti* (M.J. Wingf., Crous & W.J. Swart) J.A. Simpson (Bragança et al. 2015; Branco et al. 2014; Silva et al. 2014). However, studies related with the importance of these species to *Eucalyptus* diseases as well as their distribution remain scarce in Portugal (Branco et al. 2014).

Considering the objective of this thesis, the following sections will focus on the Botryosphaeriaceae.

The pathogen: Botryosphaeriaceae species

Characteristics and importance

Members of the family Botryosphaeriaceae have a cosmopolitan distribution, apparently with the exception of the polar regions, and are reported as occurring on monocotyledonous, dicotyledonous and gymnosperm hosts (Barr 1987; Crous et al. 2006; Phillips et al. 2013). This family is a comprehensive group that includes pathogens, endophytes and saprophytic species that globally infect a wide range of woody plants

(Phillips et al. 2013; Slippers et al. 2007; Smith et al. 1996; von Arx 1987). Opportunistic human infections have also been reported for some species (Saha et al. 2012).

Botryosphaeriaceae species are often referred to as weak or opportunistic pathogens because they cause diseases mainly on stressed or wounded plants after drought, hail, wind, frost or insect damage (Chen et al. 2011; Mohali et al. 2007; Slippers and Wingfield 2007). Nevertheless, they can penetrate healthy plants through lenticels, open stomata, wounds, or other openings on twigs, stems, roots and leaves, and can remain in a latent stage (Old et al. 1990; Rodas et al. 2009). Consequently, they can live as endophytes for long periods without apparent damage or disease symptoms (Burgess et al. 2005; Chen et al. 2011; Smith et al. 1996) until some stress triggers the infection, so they become active and cause serious diseases (Old et al. 1990; Rodas et al. 2009). Several species can be found as saprophytes on dead wood or other material (Fisher et al. 1993; Old et al. 1990; Rodas et al. 2009).

Despite the efforts of several authors (Gezahgne et al. 2004; Lynch et al. 2013; Mohali et al. 2009; Mullerin 2013; Pérez et al. 2010; Rodas et al. 2009; Slippers et al. 2004c; van Niekerk et al. 2004), the interactions between Botryosphaeriaceae species and their hosts are not yet completely clear. In other words, some Botryosphaeriaceae species can be isolated both from healthy and diseased tissues, even for the same host (Pavlic et al. 2007; Piškur et al. 2011) and it is known that these fungi are able to perform a switching of their lifestyle, from endophytic to parasitic and *vice versa*, in response to hosts or environmental factors (Rai and Agarkar 2014). *Diplodia sapinea*, contrary to what happens for almost of these species, its ecological role to *Pinus* trees is well studied (Bihon et al. 2011; Swart and Wingfield 1991) and understood (Slippers et al. 2013). Considering the current knowledge about the contribution of these species on plant diseases (Dakin et al. 2010; Piškur et al. 2011) and predicting an increase in their importance as result of climate change (Desprez-Loustau et al. 2006), the need to better understand the diversity, distribution and pathogenicity of the species should be reinforced.

Diversity of genera and species

The family Botryosphaeriaceae was firstly introduced as a sub-family in the Pseudosphaeriaceae by Theissen and Sydow (1918). After almost a century of controversy of revisions it was finally established by Schoch et al. (2006) as the unique family within the order Botryosphaeriales (Dothideomycetes, Ascomycota). However, based on recent extensive phylogenetic studies, several species were excluded or allocated to other families within the same order (Liu et al. 2012; Phillips et al. 2013; Slippers et al. 2013). Even so, this family is rich in genera/species and is the largest family of the order Botryosphaeriales (Trakunyingcharoen et al. 2015).

When Phillips et al. (2013) reviewed the taxonomy of this family, at least 78 genera were legitimate according to MycoBank (http://www.mycobank.org). In this study, in the

light of the abolished dual nomenclature, many genera were reduced to synonyms, some new genera were introduced and some old genera were resurrected. Currently, according to more recent studies (Crous et al. 2013; Jami et al. 2014; Liu et al. 2012; Phillips et al. 2013) at least 19 genera (*Alanphillipsia*, *Aplosporella*, *Barriopsis*, *Botryosphaeria*, *Botryobambusa*, *Cophinforma*, *Diplodia*, *Dothiorella*, *Endomelanconiopsis*, *Lasiodiplodia*, *Macrophomina*, *Neodeightonia*, *Neofusicoccum*, *Neoscytalidium*, *Phaeobotryon*, *Pseudofusicoccum*, *Spencermartinsia*, *Sphaeropsis*, *Tiarosporella*) are accepted in this family that includes more than 100 species.

How to identify species?

The identification of Botryosphaeriaceae species was, for many years, based on the morphological features of the spores (size, shape, septation, wall thickness and texture of the conidia as well as details of conidiogenesis) (Denman et al. 2000; Jacobs and Rehner 1998; Slippers et al. 2009). In certain species, culture morphology, coloration, and growth rates at different temperatures can also be helpful to species identification (Denman et al. 2000; Pennycook and Samuels 1985).

Despite microscopy upgrade in the last century (Crous et al. 2015), the identification of closely related or cryptic species remained complicated based only in the morphological characters, because species exhibit extensive morphological plasticity and fungal morphology can be influenced by the substrate on which it is grown (Alves et al. 2007; Denman et al. 2000; Pennycook and Samuels 1985). The use of host association is also not a valid taxonomic character while a single species can be found associated to a wide range of hosts and the same host can be colonized by various fungal species (Alves et al. 2007; Crous et al. 2015).

Since the emergence of DNA based techniques several methodologies have been successfully applied to fungi identifications (Alves et al. 2007; Crous et al. 2015). Nowadays, molecular tools are used, in combination with morphological characters, to distinguish members of Botryosphaeriaceae (Crous et al. 2006; Phillips et al. 2013; Slippers et al. 2009). Sequences of ITS region (internal transcribed spacer region) of ribosomal DNA are successfully used to discriminate fungi at different taxonomic level (Crous et al. 2015; White et al. 1990) and was proposed by Schoch et al. (2012) as the official DNA barcode for fungi. However, for some cryptic species ITS sequence data seems not to be enough. In this cases, different protein coding genes (translation elongation factor 1-alpha (tef1), beta-tubulin (tub2) gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin (act) and histone H3 (his3) and more conserved gene regions (large subunit (LSU), small subunit (SSU), and RNA polymerase II (RPB2) gene) might help the identification (Crous et al. 2015; Phillips et al. 2013).

In spite of DNA sequencing being the broadly accepted methodology for fungal identification, other techniques as micro-satellite loci (de Wet et al. 2003), PCR-RFLPs

(Dreaden et al. 2014; Slippers et al. 2004c), species-specific primers (Luchi et al. 2005), ISSRs (Zhou et al. 2001), RAPDs (Smith and Stanocz 1995), SSRs (Burgess et al. 2004) and ARDRA (Alves et al. 2005) have been successfully applied for characterization and identification of Botryosphaeriaceae species. In addition, fingerprinting techniques can be powerful tools to rapidly and reliably screen a large number of isolates when species have been identified from a particular host or area (Alves et al. 2007).

Molecular approaches, for instance, allowed to separate and characterize important closely related or cryptic species such as *Diplodia sapinea* and *D. scrobiculata* (de Wet et al. 2003) *Neofusicoccum eucalyptorum* and *N. eucalypticola* (Slippers et al. 2004c), *N. parvum* and *N. ribis* (Slippers et al. 2004a), *N. luteum* and *N. australe* (Slippers et al. 2004b), and resolve species complexes in *Diplodia* (Alves et al. 2014; Alves et al. 2004; Phillips et al. 2012). Taking this into account, molecular tools have greatly contributed to species separation and geographical distribution, which are very important to understand the evolution and ecology of Botryosphaeriaceae species (Slippers et al. 2009).

DNA-based techniques, although very powerful in discriminating species, still have some limitations: they can be sensitive to mutations or do not reflect the phenotypic diversification, the choices of specific primers can be a hard task, protocols are complex and time consuming and reagents are expensive (Mancini et al. 2013; Santos et al. 2010). In the recent years some alternative techniques have increased their importance in order to overcome these limitations.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is presently the most promising method for routine identification, differentiation and classification of microorganisms (Chalupová et al. 2014; Mancini et al. 2013), including filamentous fungi (Chalupová et al. 2014; Lecellier et al. 2015) even though these are one of the most challenging ones (Mancini et al. 2013). This is a reliable, fast, easy, low labor and consumable cost methodology and allows a direct identification of phytopathogenic fungi without need to isolate them from their hosts (Chalupová et al. 2014; Lecellier et al. 2015). The technique relies on the principle that metabolites composition is characteristic for each species, since the samples were maintained under the same experimental conditions (Chalupová et al. 2014). For instance, Mancini et al. (2013) found that spectra are clearly dependent on mycelial age when used MALDI-TOF to distinguish three monophyletic species of *Diplodia* (*D. sapinea*, *D. seriata* and *D. scrobiculata*).

Mid-infrared spectroscopy (MIR) is a powerful technique that is fast, effective, needs only small sample quantities, is reagent-free, and normally do not require sample pre-treatment (Schmitt and Flemming 1998). Moreover, the recent infrared spectrometers are quite inexpensive, unlike MALDI-TOF equipments. It is an analytical technique based on the vibrations between atoms in a molecule (Stuart 2004) and such vibrations are likely to conform to a 'fingerprint' of the molecule as a whole, rather than a specific group within the molecule (Smith 2011). In terms of the electromagnetic spectrum, MIR comprises the

infrared radiation between 4000-200 cm^{-1} (Santos et al. 2010), which gives information about some important cell macromolecules like as proteins, lipids, nucleic acids and carbohydrates (Lecellier et al. 2015). Thus, MIR is a perfect tool to assess the overall molecular composition of the microbial cells in a fast and non-destructive manner. Hence, it is one of the most promising techniques in microbiology since it allows the detection, identification, characterization and authentication of several microorganisms including filamentous fungi (Erukhimovitch et al. 2005; Fischer et al. 2006; Lecellier et al. 2015; Naumann et al. 2005; Santos et al. 2010). However, these works are focused manly in fungi with clinical, food and industry importance and no studies were addressed to Botryosphaeriaceae species.

MALDI-TOF MS and MIR, in order to allow species identification as molecular methodologies, depend on a good and complete database. Although, spectral libraries for filamentous fungi are not yet completely build (Lecellier et al. 2014, 2015).

Pathogenicity and hosts range

Botryosphaeriaceae species are frequently associated with disease symptoms observed in plants with high agricultural, forestry, ecological and economic value (Barr, 1972, 1987; Alves et al. 2005). Diseases caused by these species include fruit rots, leaf spots, seedling damping-off and collar rot, cankers, blight of shoots and seedlings, gummosis, blue-stain of the sapwood, dieback, and tree death (Rodas et al. 2009; Slippers et al. 2007). Further, their pathogenicity is typically associated with biotic and abiotic stresses, in which drought stress is the most frequently reported (Chen et al. 2011; Mohali et al. 2007; Old et al. 1990; Phillips et al. 2013).

Members of Botryosphaeriaceae can be found associated with a wide range of woody hosts, namely eucalypts (Chen et al. 2011; Mohali et al. 2009; Pérez et al. 2010; Rodas et al. 2009; Slippers et al. 2004c), pines (Alves et al. 2013; Mohali et al. 2007), grapevines (Urbez-Torres et al. 2010; van Niekerk et al. 2004), oaks (Alves et al. 2004; Linaldeddu et al. 2014), mango (Marques et al. 2013), and Rosaceae (Phillips et al. 2012) Even though, some species seem to be specific to the host (*Neofusicoccum eucalyptorum* appears to be almost exclusive to *Eucalyptus* spp. among other Myrtaceae plants (Pillay et al. 2013) and *Diplodia corticola* is specialized on cork oak and other oak species (Alves et al. 2004; Linaldeddu et al. 2014; Lynch et al. 2013)), a consistent pattern of pathogen-host association has not been verified for the majority of the species (Pillay et al. 2013).

Recent studies demonstrate that the distribution of Botryosphaeriaceae species has been highly influenced by anthropogenic activity. Owing to their endophytic nature, these species were introduced trough the germplasm into new environments (Slippers et al. 2004c). For instance, species that were common on eucalypts in their native environment, as *N. parvum* and *N. eucalyptorum*, were also found on Myrtaceae plants growing nearby

eucalyptus plantations. Host jumps between native Myrtaceae and non-native Eucalyptus plantations has also been reported (Pérez et al. 2010).

Host-pathogen interactions

Plants response to biotic and abiotic stresses

Plant hosts, owing to their sessile lifestyle, are continually exposed to a broad range of environmental stresses, namely abiotic (drought, salinity, heat, cold, chilling, freezing, nutrient, high light intensity, ozone (O_3) and anaerobic stresses) and biotic (bacteria, oomycetes, fungi, viruses, nematodes, and attack by herbivore pests) stresses (Atkinson and Urwin 2012; Naidoo et al. 2014; Suzuki et al. 2014). So, they have developed strategies that allow them to recognize stress source minimizing the possible damage which compromise growth and reproduction. Thus, plants respond through activation of a complex biochemical and molecular response systems (Atkinson and Urwin 2012; Ramegowda and Senthil-Kumar 2015) and adjust their physiological mechanisms to preserve homeostasis during the stress occurrence (Bostock et al. 2014).

In the field, plants are exposed not only to an isolated stress but to a set of them. Therefore, the plant response to multiple and simultaneous stresses is unique and cannot be directly extrapolated from the response to each individual stress alone (Atkinson and Urwin 2012; Suzuki et al. 2014). Moreover, combined stresses can have either a negative (i.e., susceptibility) or positive (i.e., tolerance) effect depending on various factors as the abiotic stress type, the pathogen in cause, the severity of stresses, and which one starts first (Jactel et al. 2012; Ramegowda and Senthil-Kumar 2015). In this respect, it is difficult to anticipate which would be the plants response considering only the pathogen species, while pathogen-host interaction depends on various factors. Even so, some studies were addressed in order to understand which modifications occur in plants physiology as well as in biochemical and molecular response system in case of fungal infection (Alves et al. 2011; Bettucci and Alonso 1997; Desprez-Loustau et al. 2006; Luque et al. 1999; Mayek-Pérez et al. 2002; Pinkard and Mohammed 2006). Further, it is recognized that water stress can influence the appearance and development of fungal diseases by disturbing the physiological status of the host plants and, consequently, their capacity to resist fungal infection, being this the case of Botryosphaeriaceae diseases (Desprez-Loustau et al. 2006).

Fungal effect on plant response

Plants have to deal permanently with environmental challenges and are not able to escape physically. Even so, during fungal attack, plants activate several defense strategies with the objective to limit or prevent the pathogen entry and spread (Atkinson and Urwin 2012; Naidoo et al. 2014; Torres 2010). These strategies are divided into constitutive and inducible defences (Freeman and Beattie 2008). Constitutive defences are the first line of defense against fungal invasions and include many preformed barriers such as outer bark,

leaf cuticle, plant cell walls composition (pectin and lignin) (Freeman and Beattie 2008; Kovalchuk et al. 2013; Naidoo et al. 2014), and secretory cells, glands and ducts (where defensive substances are produce, transport and storage) (Naidoo et al. 2014). While constitutive defences are continuous, preformed and non-specific barriers, inducible defences are activated during pathogens invasion and consist in the production of chemical compounds, as antimicrobial and toxic secondary metabolites (Freeman and Beattie 2008; Kovalchuk et al. 2013; Naidoo et al. 2014), antioxidant, low-molecular- weight (LMW) compounds (such as phenolic compounds, terpenoids, and alkaloids) (Kovalchuk et al. 2013), pathogen-degrading enzymes, and apoptosis induction (Freeman and Beattie 2008; Z. Li et al. 2014).

Considering eucalypts, beyond the mechanisms described for plants in a global way, these plants present secretory cavities, mainly located on leaves, which produce and store essential oils and resinous substance with unknown function (Naidoo et al. 2014). Further, the formation of barrier zones or reaction zones to prevent fungal spread is also described for some species of eucalypts (Naidoo et al. 2014; Tippett and Shigo 1981).

Inducible defences normally are only activated when constitutive mechanism fail because chemicals production and maintenance have high energy costs (Freeman and Beattie 2008; Naidoo et al. 2014). The success of these responses are related with plants ability to differentiate self from non-self. Hormone signalling pathways, phenolic compounds production, pathogen associated molecular patterns (PAMPs) and reactive oxygen species (ROS) play a very important role in plant responses at this level (Atkinson and Urwin 2012; Kovalchuk et al. 2013; Naidoo et al. 2014; Torres 2010).

Plant physiological parameters are also frequently affected by fungal activity. For instance, some endophytic fungi are able to promote the plants growth by production of enzymes that degrade substances which have negative impacts on the growth of the plants (e.g. phenolic acid allelochemicals), secretion of plant growth promoters (e. g. gibberellins (GAs) and indoleacetic acid) or contribution to nitrogen uptake (Zhou et al. 2014). On the other hand, several pathogenic fungi, including some Botryosphaeriaceae species, produce phytotoxins and secondary metabolites which are spread in the whole plant and have a negative effect on photosynthesis and transpiration rate (Berger et al. 2007; Linaldeddu et al. 2009; Luque et al. 1999). The decreased photosynthesis rate leads to a decline in enzymes and proteins production that play an important role in the plant defense against the pathogen (van Niekerk et al. 2011). Conversely, some fungi do not produce these compounds, being restricted to the infection zone and, consequently, do not affect the leaf gas exchange parameters (Linaldeddu et al. 2009). Li et al. (2014) observed a decrease of total chlorophyll amount in plants infected with fungi as a result of down-regulation of the gene expression for the chlorophyll a,b binding proteins. Modifications in the accumulation of soluble sugars depend on the fungal species, while in some cases occur an increment in the accumulation of sugars, in other situations sugar content presents no alterations or even decreases (Berger et al. 2007). During fungal infection, the amount of ROS, which play

a crucial role in signalling, is higher (Berger et al. 2007). Some fungal species are able to successfully colonize xylem leading to vessel obstruction and consequently plant wilt (Polle and Luo 2014). Unfortunately, neither the same plant responds in a similar way facing different fungi nor the same fungi induces the same plant response. Thus, the lack of patterns in plants responses difficults our understanding about plant-fungi interaction. Recently, the application of omics technologies have been improved our knowledge about this subject (Kovalchuk et al. 2013).

Climate changes

Climate has a great influence not only on plant and animal distribution but also on their parasites and pathogens. Thus, the distribution patterns and the severity of pathogens can be directly or indirectly affected by climate, since this factor also affects host physiology and parasite-associated organisms (Desprez-Loustau et al. 2007).

According to the report released by IPCC (2013), it is expected an increase in global temperature and frequency of extreme events (higher or lower precipitation events in different areas and extended dry periods) in the 21st century. Thus, the occurrence of abiotic stresses will be intensified by climate changes which will also lead to shifts in the distribution of species, expand the host range of pathogens and increase in pathogens aggressiveness (Desprez-Loustau et al. 2007; Ramegowda and Senthil-Kumar 2015). Consequently, the frequency and severity of plant diseases should be favoured in a scenario of climate changes (Lindner et al. 2010; Ramegowda and Senthil-Kumar 2015; Sturrocka et al. 2011). Among abiotic factors, drought stress is the one which is most frequently associated with emergence of fungal diseases, namely Botryosphaeriaceae diseases (Pérez et al. 2010; Slippers and Wingfield 2007; Smith et al. 1994).

However, as mentioned above, it should not be overlooked that the response of plants to multiple simultaneous stresses is complex and depends on the source of stress and the pathogen involved (Atkinson and Urwin 2012; Jactel et al. 2012; Ramegowda and Senthil-Kumar 2015; Suzuki et al. 2014). Moreover, the reason why some host-pathogen interactions resulted in tolerance while others lead to susceptibility is not already clear and no general trend can be assumed (Desprez-Loustau et al. 2007; Jactel et al. 2012; Ramegowda and Senthil-Kumar 2015).

Botryosphaeriaceae diseases in *Eucalyptus* sp.

Eucalypt plantations have increased in extent and economic relevance, so the study of their pathogens is crucial.

In early studies, Botryosphaeriaceae species have been associated to eucalypts in their native environment (Burgess et al. 2006b; Old et al. 1990; Slippers et al. 2004c). Nowadays, these fungi are considered to be a threat to the sustainability and production of eucalypts both in native and non-native plantations (Chen et al. 2011; de la Mora-

Castañeda et al. 2014; Mohali et al. 2009; Rodas et al. 2009). The genetic uniformity of the commercial plantations increases the risk of diseases (Chen et al. 2011; Old et al. 2003; Slippers et al. 2009) and the presence of these plants close to native plants may have a negative effect, considering that host jumps are common in the Botryosphaeriaceae species (Pavlic et al. 2007; Pérez et al. 2009, 2010).

Some members of Botryosphaeriaceae family can occur as endophytes in healthy eucalypts for a long period without producing any symptoms (Pavlic et al. 2009; Smith et al. 1996). On the other hand, they may also occur as pathogenic and cause different disease symptoms, such as dieback of shoots and branches, cankers on the stems followed by kino exudation, coppice failure and even host death (Chen et al. 2011; Gezahgne et al. 2004; Slippers et al. 2009). Further, Rodas et al. (2009) verified that young eucalypts are more susceptible than older ones. When these authors observed plants aged between 6 to 36 months they found that the prevalence of the symptoms were most common on plants aged between 18 to 26 months.

In early studies, investigators believed that *Botryosphaeria dothidea* and *Neofusicoccum ribis* were the most abundant species in eucalypts growing in temperate areas, causing stem cankers and dieback (Chen et al. 2011; Pérez et al. 2010; Slippers et al. 2009). However, in the light of the most recent studies, it is known that these species are not common on *Eucalyptus* plants. Nowadays, at least 29 Botryosphaeriaceae species have been confirmed as occurring in eucalypts (Phillips et al. 2013; Pillay et al. 2013; Barradas et. al. 2016) as shown on table 1.1.

Although efforts have been made to comprehensively understand which species are associated with eucalypts, little is known about their pathogenicity. Thus, pathogenicity tests conducted both in greenhouses and field trial allowed to identify some of the causal agents of diseases. For instance, pathogenicity tests confirmed the ability of *N. parvum* to cause stem canker on *Eucalyptus* in Ethiopia (Gezahgne et al. 2004) and *N. eucalyptorum* on *E. grandis* in Uruguay (Pérez et al. 2009). Pavlic et al. (2007) inoculated *Eucalyptus grandis* × *camaldulensis* clones and found that *N. ribis*, *N. parvum* and *L. theobromae*, were the most aggressive while *B. dothidea* was less pathogenic to the plants. Pathogenicity tests performed in *E. grandis* also found *N. ribis* as the most aggressive and *B. dothidea* as the less one (Rodas et al. 2009). The species *N. parvum* and *N. ribis* were the most aggressive species in a study conducted in Venezuela, in which differences in clones tolerance were also verified (Mohali et al. 2009). Pérez et al. (2010) inoculated *E. grandis* plants with species isolated both from introduced eucalypts and native Myrtaceae trees and found that *L. pseudotheobromae*, *N. eucalyptorum* and the *N. parvum*-*N. ribis* complex are the most aggressive to the plants. In artificial inoculation trials conducted in Portugal it was found that *D. corticola* and *N. kwambonambiense* were the most aggressive fungi, while *B. dothidea* and *D. seriata* were the least aggressive ones. Further, differences in plant tolerance were also observed among the *Eucalyptus* species (Mohali et al. 2009; Chapter 4).

Table 1.1: Botryosphaeriaceae species confirmed to be associated to eucalypts plants, distribution and associated symptoms. Ex-type strains in bold face.

Genus	Specie	*Eucalyptus* sp.	Distribution	Pathogenic	Symptoms	References
Botryosphaeria	***B. dothidea***	*Eucalyptus* spp.	Cosmopolitan	Weak	Unknown	(Farr and Rossman 2015; Phillips et al. 2013)
	B. fabicerciana	*Eucalyptus* sp.	China	Weak	Unknown	(Chen et al. 2011; Farr and Rossman 2015; Phillips et al. 2013)
	B. ramosa	*E. camaldulensis*	Western Australia	No	Unknown	(Farr and Rossman 2015; Pavlic et al. 2008; Phillips et al. 2013)
Cophinforma	***C. atrovirens***	*Eucalyptus* sp.	Thailand	Yes	Dead branch	(Liu et al. 2012; Phillips et al. 2013; Farr and Rossman 2015)
		E. urophylla	Venezuela	Unknown	Unknown	(Farr and Rossman 2015; Xu et al. 2014)
Diplodia	*D. corticola*	*E. globulus*	Portugal	Yes	Canker and dieback	This study
	D. seriata	*E. globulus*	Portugal	Latent	Unknown	This study
Lasiodiplodia	*L. crassispora*	*E. urophylla*	Uruguay	Unknown	Unknown	(Farr and Rossman 2015; Pérez et al. 2010; Phillips et al. 2013)
	L. iraniensis	*Eucalyptus* sp.	Iran	Yes	Canker and dieback	(Abdollahzadeh et al. 2010; Li et al. 2015; Phillips et al. 2013)
	L. rubropurpurea	*E. grandis*	Australia	Yes	Canker	(Burgess et al. 2006a; Farr and Rossman 2015; Phillips et al. 2013)
	L. theobromae	*E. urophylla* × *grandis*	China	Yes	Dieback	(Li et al. 2015)
Macrophomina	*M. phaseolina*	*Eucalyptus* spp.	Cosmopolitan	Yes	Seedling blight	(Farr and Rossman 2015; Kaur et al. 2012; Phillips et al. 2013)
Neofusicoccum	*N. andinum*	*Eucalyptus* sp.	Venezuela	No	Unknown	(Burgess et al. 2006a; Farr and Rossman 2015; Mohali et al. 2009; Phillips et al. 2013)

Genus	Specie	*Eucalyptus* sp.	Distribution	Pathogenic	Symptoms	References
Neofusicoccum	*N. australe*	*Eucalyptus* spp.	Australia	Weak	Unknown	(Farr and Rossman 2015; Phillips et al. 2013; Taylor et al. 2009)
		E. globulus	Spain	Yes	Canker and dieback	(Armengol et al. 2008; Farr and Rossman 2015)
		E. globulus	Portugal	Yes	Canker and dieback	This study
	N. algeriense	*E. globulus*	Portugal	Yes	Canker and dieback	This study
	N. eucalypticola	*Eucalyptus* spp.	Eastern Australia	Unknown	Unknown	(Farr and Rossman 2015; Phillips et al. 2013; Slippers et al. 2004c)
	N. eucalyptorum	*Eucalyptus* spp.	Australia	Yes	Canker and dieback	(Phillips et al. 2013; Slippers et al. 2004c)
		Eucalyptus spp.	South Africa	Yes	Canker and dieback	(Farr and Rossman 2015; Phillips et al. 2013; Smith et al. 2001)
		Eucalyptus spp.	Uruguay	Yes	Bark lesions	(Pérez et al. 2010; Phillips et al. 2013)
		E. grandis	Zimbabwe	Yes	Stem canker	(Jimu et al. 2015)
		E. globulus	Portugal	Latent	Unknown	This study
	N. kwambonambiense	*Eucalyptus* spp.	Australia	Yes	Unknown	(Pillay et al. 2013; Sakalidis et al. 2013)
		Eucalyptus spp.	Uganda	Yes	Canker	(Sakalidis et al. 2013)
		E. globulus	Portugal	Yes	Canker	This study
	N. luteum	*Eucalyptus* spp.	Eastern Australia	Yes	Stem canker	(Denman et al. 2003; Phillips et al. 2013)

Genus	Specie	*Eucalyptus* sp.	Distribution	Pathogenic	Symptoms	References
Neofusicoccum	*N. macroclavatum*	*Eucalyptus* spp.	Western Australia	Yes	yes	(Burgess et al. 2005; Phillips et al. 2013)
	N. mediterraneum	*Eucalyptus* sp.	(California) USA	Unknown	Unknown	(Farr and Rossman 2015; Inderbitzin et al. 2010; Phillips et al. 2013)
		Eucalyptus sp.	Greece	Unknown	Unknown	(Farr and Rossman 2015; Inderbitzin et al. 2010; Phillips et al. 2013)
	N. occulatum	*Eucalyptus* spp.	Australia	yes	Unknown	(Farr and Rossman 2015; Phillips et al. 2013; Sakalidis et al. 2011a)
		Eucalyptus sp.	(Hawaii) USA	Unknown	Unknown	(Sakalidis et al. 2013)
		Eucalyptus sp.	Uganda	Unknown	Unknown	(Sakalidis et al. 2013)
		E. grandis	Uruguay	Unknown	Unknown	(Sakalidis et al. 2013)
	N. parvum	*Eucalyptus* spp.	Australia	Unknown	Unknown	(Barber et al. 2005; Phillips et al. 2013; Sakalidis et al. 2013)
		Eucalyptus spp.	China	Unknown	Unknown	(Sakalidis et al. 2013)
		Eucalyptus sp.	Colombia	Unknown	Unknown	(Sakalidis et al. 2013)
		Eucalyptus spp.	Ethiopia	Yes	Dieback and death	(Gezahgne et al. 2004; Phillips et al. 2013; Sakalidis et al. 2013)
		Eucalyptus sp.	(Hawaii) USA	Unknown	Unknown	(Sakalidis et al. 2013)
		Eucalyptus spp.	Indonesia	Unknown	Unknown	(Sakalidis et al. 2013)
		E. camaldulensis	Kenya	Unknown	Unknown	(Sakalidis et al. 2013)

Genus	Specie	*Eucalyptus* sp.	Distribution	Pathogenic	Symptoms	References
Neofusicoccum	***N. parvum***	*E. globulus*	Portugal	Yes	Canker and dieback	This study
		Eucalyptus spp.	Spain	Yes	Canker and dieback	(Farr and Rossman 2015; Iturritxa et al. 2011; Sakalidis et al. 2013)
		E. smiithi	South Africa	Yes	Unknown	(Farr and Rossman 2015; Sakalidis et al. 2013; Xu et al. 2015)
		E. grandis	Swaziland	Unknown	Unknown	(Sakalidis et al. 2013)
		E. obliqua	Thailand	Unknown	Unknown	(Farr and Rossman 2015; Sakalidis et al. 2013; Trakunyingcharoen et al. 2015)
		E. grandis	Uganda	Unknown	Unknown	(Sakalidis et al. 2013)
		E. urophylla	Venezuela	Unknown	Unknown	(Farr and Rossman 2015; Mohali et al. 2007; Phillips et al. 2013)
		E. grandis	Zambia	Unknown	Unknown	(Sakalidis et al. 2013)
		E. grandis	Zimbabwe	Yes	Stem canker	(Jimu et al. 2015)
	N. ribis	*E. camaldulensis*	Australia	Unknown	Unknown	(Farr and Rossman 2015; Sakalidis et al. 2011b)
		E. grandis	Australia	Unknown	Unknown	(Farr and Rossman 2015; Urbez-Torres et al. 2012)
		E. camaldulensis x E. grandis	Australia	Unknown	Unknown	(Farr and Rossman 2015; Inderbitzin et al. 2010)
		E. pellita	Australia	Unknown	Unknown	(Farr and Rossman 2015; Xu et al. 2015)
		E. urophylla	Venezuela	Unknown	Unknown	(Farr and Rossman 2015; Mohali et al. 2007)

Genus	Specie	*Eucalyptus* sp.	Distribution	Pathogenic	Symptoms	References
Neofusicoccum	*N. vitifusiforme*	*Eucalyptus* spp.	Australia	Yes	Leaf lesions	(Phillips et al. 2013; Taylor et al. 2009)
Neoscytalidium	***Ne. hyalinum***	*Eucalyptus* spp.	worldwide	Yes	gummosis, dieback, wilt and cankers	(Phillips et al. 2013)
Pseudofusicoccum	*P. adansoniae*	*Eucalyptus sp.*	Australia	Unknown	Unknown	(Pavlic et al. 2007; Phillips et al. 2013; Sharma et al. 2013)
	P. ardesiacum	*Eucalyptus sp.*	Western Australia	Unknown	Unknown	(Pavlic et al. 2008; Phillips et al. 2013; Sharma et al. 2013)
	P. kimberleyense	*Eucalyptus sp.*	Western Australia	Unknown	Unknown	(Pavlic et al. 2008; Phillips et al. 2013; Sharma et al. 2013)
	P. stromaticum	*Eucalyptus spp.*	Venezuela	Unknown	Unknown	(Mohali et al. 2006; Phillips et al. 2013; Sharma et al. 2013)
Sphaeropsis	*S. eucalypticola*	*Eucalyptus sp.*	Thailand	Unknown	Collected from a dead twig	(Liu et al. 2012; Phillips et al. 2013)

Aims of the work

Climate change is a major challenge for forest health and productivity since it is expected to impose a drastic modification of growth conditions, and probably an increase in the occurrence of diseases. In fact, environmental conditions are recognized to strongly influence plant diseases, affecting the host, the pathogen, and the interactions between them, however these subjects are not completely understood. Hence, researches carried out in order to identify and clarify the interaction between the different factors of decline (biotic/abiotic) should be privileged.

In Portugal, forest activity is an important economic sector, generating many jobs. Indeed, Portugal extracts more benefits from one hectare of forest than any other country in the Mediterranean area. To maintain these economic values and ensure competitiveness, forest research in Portugal must focus on the most representative (both in area and economy) forest species, namely *Eucalyptus globulus*, *Pinus pinaster* and *Quercus suber*. *Eucalyptus globulus* is at present the most abundant tree species in Portuguese forests. It is known that water availability limits the growth and survival of these plants and consequently, Botryosphaeriaceae diseases can be favoured. Taking this into account, the main goal of this thesis was to evaluate the potential impact of climate change on Botryosphaeriaceae-related diseases of eucalypts in Portugal.

For that, this work aimed to answer the following questions:

1. Which species of Botryosphaeriaceae occur in association with *Eucalyptus* species in Portugal?
2. Which species are actually pathogenic to *Eucalyptus* species?
3. Is there variation in susceptibility to these pathogens among *Eucalyptus* species and clones?
4. How does water stress affect Botryosphaeriaceae-related diseases of eucalypts?

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CHAPTER 2

Diversity and potential impact of Botryosphaeriaceae species associated with *Eucalyptus globulus* plantations in Portugal

Diversity and potential impact of Botryosphaeriaceae species associated with *Eucalyptus globulus* plantations in Portugal

Abstract

Eucalyptus globulus, a non-native species, is currently the most abundant forest species in Portugal. This economically important forest tree is exploited mainly for the production of pulp for the paper industry. The community of Botryosphaeriaceae species occurring on diseased and healthy *E. globulus* trees was studied on plantations throughout the country. Nine species from three different genera were identified, namely *Botryosphaeria* (*B. dothidea*), *Diplodia* (*D. corticola* and *D. seriata*) and *Neofusicoccum* (*N. australe*, *N. algeriense*, *N. eucalyptorum*, *N. kwambonambiense*, *N. parvum* and *Neofusicoccum* sp.). Of these, *N. algeriense*, *D. corticola* and *D. seriata* are reported for the first time on *E. globulus*, while *N. algeriense*, *N. eucalyptorum* and *N. kwambonambiense* correspond to first reports in Portugal. The genus *Neofusicoccum* was clearly dominant with *N. australe* and *N. eucalyptorum* being the most abundant species on both diseased and healthy trees. In artificial inoculation trials representative isolates from all nine species were shown to be pathogenic to *E. globulus* but there were marked differences in aggressiveness between them. Thus, *D. corticola* and *N. kwambonambiense* were the most aggressive while *B. dothidea* and *D. seriata* were the least aggressive of the species studied.

Keywords: *Botryosphaeria*, *Diplodia*, *Neofusicoccum*, canker, dieback, endophytic.

Introduction

Eucalyptus species are mostly native to Australia but due to their economic importance they have been planted extensively outside their native range. Eucalypts were introduced in Portugal more than 100 years ago and are nowadays widespread throughout the country. Eucalypt plantations are a major component of the forestry industry. According to the most recent National Forest Inventory released in 2013 (ICNF 2013), eucalypts occupy 812,000 ha corresponding to 26% of the total forest area. Commercial and non-commercial plantations are almost exclusively composed of *Eucalyptus globulus* Labill. (Blue gum), which is native to Tasmania and Southeastern Australia. This species is well adapted to the Portuguese climate with Mediterranean influence and is exploited mainly for the production of pulp for the paper mill industry.

Despite the economic importance of *E. globulus* plantations in Portugal very few studies have addressed the fungal diseases affecting them. For example, MLD (*Mycosphaerella* leaf disease) caused by several *Teratosphaeria* and related species has

been reported on young *E. globulus* trees (Silva et al. 2009). In the case of canker and dieback diseases very little is known about the pathogens causing them.

The fungal family Botryosphaeriaceae (Botryosphaeriales, Ascomycetes) includes a large number of species with a worldwide distribution in mostly woody hosts. These fungi are usually considered as opportunistic pathogens that elicit disease symptoms on plants exposed to stress conditions such as drought, frost, hail and damage caused by other pathogens and pests (Phillips et al. 2013; Slippers and Wingfield 2007). In addition the Botryosphaeriaceae are known to occur in asymptomatic plant tissues as endophytes and latent pathogens on a variety of tree species including eucalypts (Pérez et al. 2010; Phillips et al. 2013; Slippers and Wingfield 2007; Smith et al. 1996).

Eucalypts are hosts to a wide range of Botryosphaeriaceae species both in native and non-native environments. Until now more than 25 species in several genera of Botryosphaeriaceae have been reported from *Eucalyptus* spp. in their native range and in plantations worldwide (Chen et al. 2011; Phillips et al. 2013; Slippers et al. 2009). Botryosphaeriaceae species cause various disease symptoms in eucalypts but are most frequently associated with dieback of shoots and branches, cankers on the stems followed by kino exudation, coppice failure and in more severe cases host death (Chen et al. 2011; Slippers et al. 2009). Although not as aggressive as other primary pathogens, under certain conditions diseases caused by these fungi represent a significant threat to the productivity and sustainability of *Eucalyptus* spp. plantations, especially in non-native environments (Chen et al. 2011; Slippers et al. 2009).

Although canker and dieback symptoms typical of Botryosphaeriaceae have been observed in eucalypt plantations in Portugal, nothing is known about which species are associated with these symptoms, if they occur also as endophytes and what is their pathogenic potential. Therefore, the aims of this study were to identify the Botryosphaeriaceae species associated with diseased and asymptomatic *E. globulus* plants in Portugal and determine their pathogenicity to this host.

Materials and Methods

Fungal isolation and morphological characterization

Plant material was collected between 2012 and 2013 from *E. globulus* plantations from several regions in Portugal. Samples (n = 82) were taken from stems and trunks of trees showing disease symptoms such as canker and dieback. From asymptomatic trees 39 samples were collected from stems (n = 21), leaves (n = 14) and roots (n = 4). The samples from symptomatic plants were initially screened for the presence of fruiting bodies (ascomata and conidiomata) and when present single spore isolations were made as described previously (Phillips et al. 2013). In the absence of fruiting bodies, and for asymptomatic material, isolations were made by plating pieces of plant tissues following surface sterilization as described by Alves et al. (2013).

Isolates were induced to sporulate by plating them on 2% water agar or ¼ strength PDA (Merck, Germany) containing sterilised pine needles and incubating at room temperature (about 20–25ºC) under diffused daylight. Pycnidia were mounted in 100% lactic acid and morphological characters of the conidia (shape, colour, septation) and mode of conidiogenesis were observed with a Nikon 80i microscope.

Molecular characterization

Genomic DNA was extracted from mycelium by the procedures described by Alves et al. (2004). PCR reactions were prepared with NZYTaq 2× Green Master Mix (2.5 mM MgCl2; 200 µM dNTPs; 0.2 U/µL DNA polymerase) (NZYtech, Lisbon, Portugal).

BOX-PCR fingerprinting was done as described previously (Alves et al. 2007) using primer BOXA1R. Primers ITS1 and ITS4 (White et al. 1990) were used for amplification and sequencing of the ITS region of the ribosomal RNA gene as described by Alves et al. (2004). Part of the translation elongation factor 1-alpha (EF1- α) gene was amplified and sequenced using primers EF1-728F and EF1-986R (Carbone and Kohn, 1999) as described by Phillips et al. (2005). PCR amplicons were purified with the DNA Clean & Concentrator™-5 kit (Zymo Research, CA, USA). Both strands of the PCR products were sequenced at GATC Biotech (Cologne, Germany). The nucleotide sequences were read and edited with FinchTV 1.4.0 (Geospiza Inc. http://www.geospiza.com/finchtv). All sequences were checked manually and nucleotide arrangements at ambiguous positions were clarified using both primer direction sequences.

Sequences were aligned with ClustalX v 1.83 (Thompson et al. 1997), using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 25%). Alignments were visually inspected and adjustments were made where necessary with BioEdit v 7.2.5.

Phylogenetic analyses of sequence data were done with MEGA6 (Tamura et al. 2013). Trees were visualised with TreeView (Page 1996). Maximum likelihood (ML) analyses were performed on a neighbor-joining (NJ) starting tree automatically generated by the software. The DNA model of evolution used for each dataset was determined with MEGA6. All gaps were included in the analyses. Nearest-Neighbour-Interchange (NNI) was used as the heuristic method for tree inference and 1000 bootstrap replicates were performed.

Pathogenicity trials

Pathogenicity of selected isolates representative of each species was tested on 6 months old potted plants of a commercial *E. globulus* clone AL-18. Six plants were inoculated with each isolate. Isolates were grown on PDA at 25°C for 7 days prior to inoculation. For inoculation a cut was made at the base of the stem and a colonized agar disc (5 mm diam.) was placed on top of the damaged area, which was then covered with

wet sterilized cotton wool and sealed with Parafilm. Controls were inoculated with pieces of non-colonized PDA. The plants were maintained in a greenhouse at room temperature for 2 months after which the aggressiveness of the isolates was determined by evaluating the size of the necrotic lesions.

Data from pathogenicity tests was subjected to one-way ANOVA (analysis of variance). Prior to analysis data were checked for normality and heteroscedasticity. Significance of differences between means was determined by Least Significant Difference (LSD). Statistical analyses were performed on the software Minitab® and a significance level of 0.05 was used.

Results

Fungal isolation and morphological characterization

A total of 121 samples of *E. globulus* plants (82 symptomatic and 39 asymptomatic) were collected during this study. From these samples 215 isolates (Table 2.1) were obtained of which 173 (80.5%) were from symptomatic and 42 (19.5%) from asymptomatic plant material (Table 2.1). On the basis of cultural and micromorphological characters (conidial size, shape, colour, septation) these isolates where assigned to three genera within the family Botryosphaeriaceae namely *Botryosphaeria*, *Diplodia* and *Neofusicoccum*.

Table 2.1: Relative abundance of Botryosphaeriaceae species obtained from *E. globulus* plantations. SB – stem or branch, L – leaf, R – root.

	Occurrence per sample						**Nº of isolates**					
Origin	**Symptomatic**			**Asymptomatic**			**Symptomatic**			**Asymptomatic**		
Species	**SB**	**L**	**R**	**SB**	**L**	**R**	**SB**	**L**	**R**	**SB**	**L**	**R**
B. dothidea	1/82			2/21			1			2		
D. corticola	2/82						3					
D. seriata	2/82						4					
N. australe	26/82			13/21	8/14		83			14	10	
N. eucalyptorum	26/82			6/21	2/14		76			6	8	
N. kwambonambiense	1/82						1					
N. parvum	1/82					2/4	2					2
N. algeriense	2/82						2					
***Neofusicoccum* sp.**	1/82						1					

Molecular characterization

Cluster analysis of the BOX-PCR fingerprints divided the 215 isolates into 9 clusters that are deemed to represent distinct species. Isolates representative of the overall diversity of each cluster were selected for further characterization. Thus, the ITS region and part of the EF1-α gene of 62 isolates were sequenced and new sequences deposited in GenBank (Table 2.2). BLASTn searches confirmed the previous morphology-

based assignment of the isolates to the genera *Botryosphaeria*, *Diplodia* and *Neofusicoccum*. To accomplish a reliable species-level identification the sequences of the isolates obtained in this study were aligned with sequences from all known species of *Botryosphaeria*, *Diplodia* and *Neofusicoccum* available in GenBank. For each genus a combined ITS and EF1-α phylogenetic analysis was performed and the resulting ML trees are shown in figures 2.1, 2.2 and 2.3.

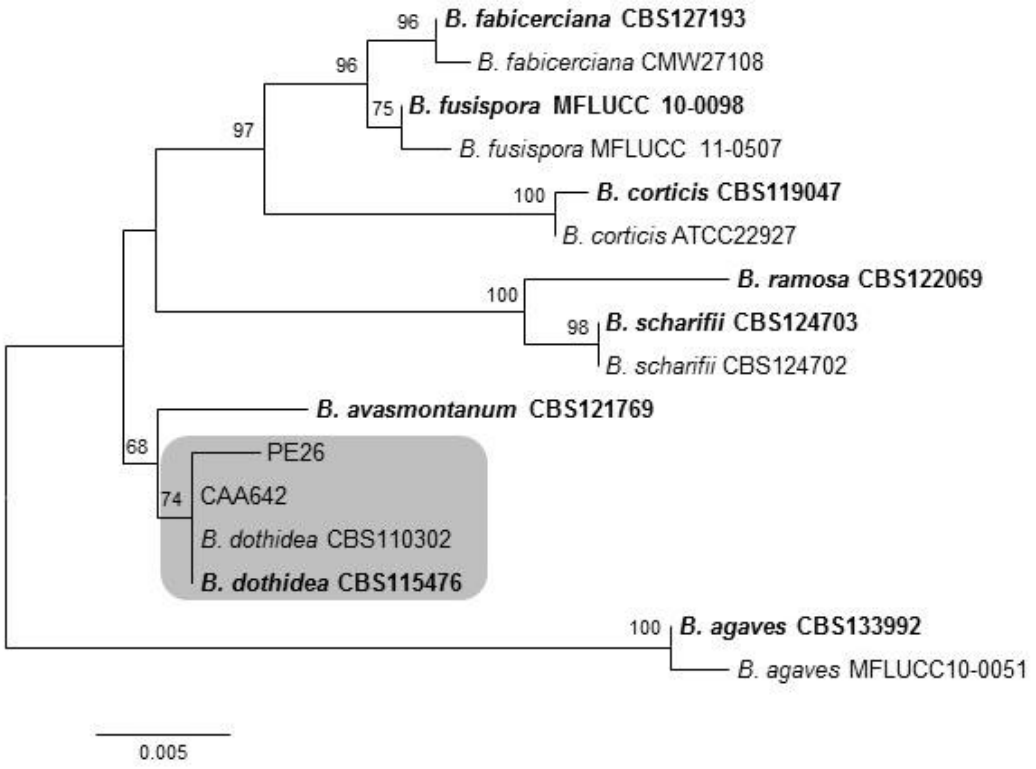
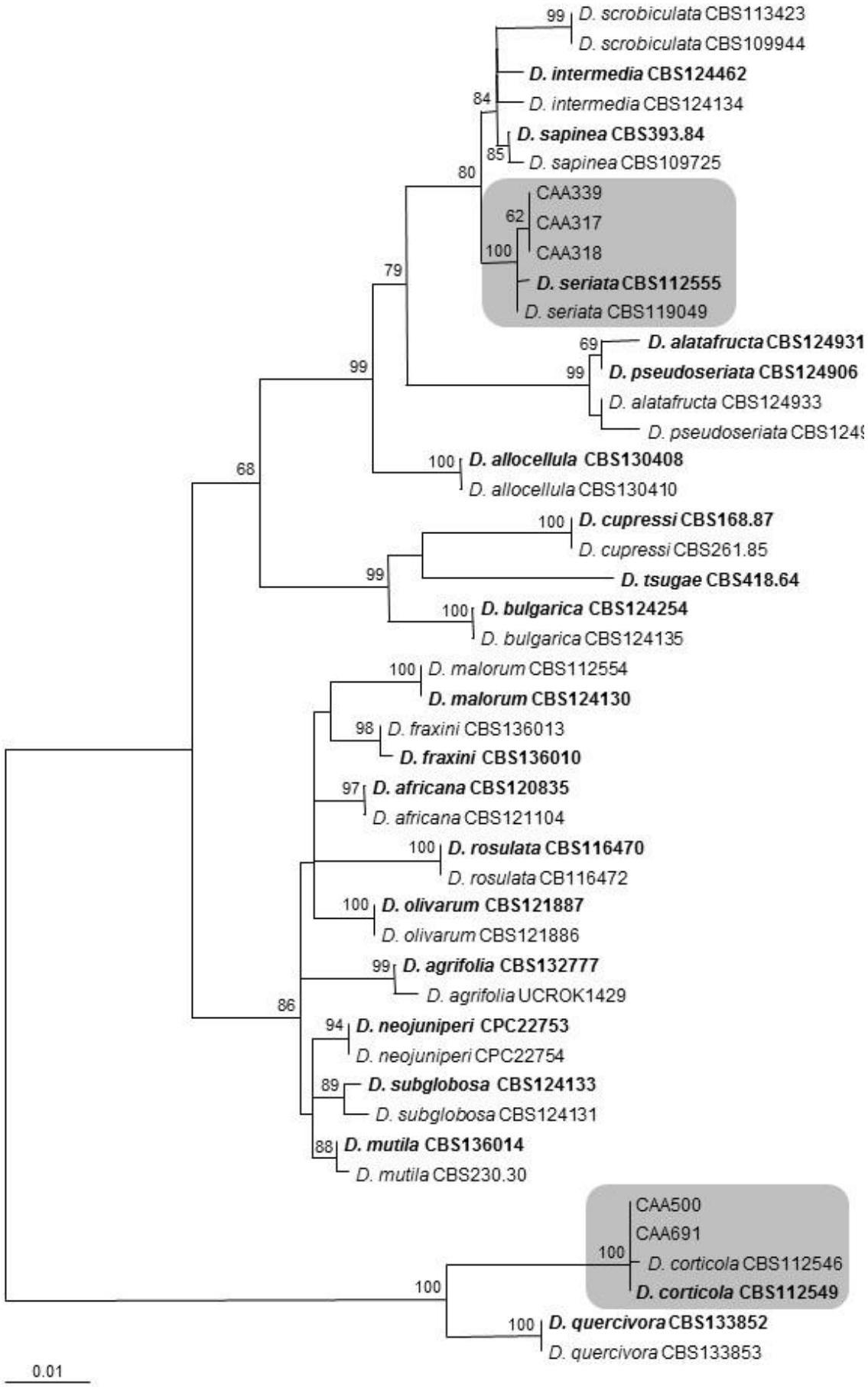


Figure 2.1: Phylogenetic relationships of *Botryosphaeria* species inferred using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-1648.7795) is shown. Bootstrap values (> 50%) are given at the nodes. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.6969)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Ex-type strains are in bold face and clades containing isolates from this study are highlighted.

Figure 2.2: Phylogenetic relationships of *Diplodia* species inferred using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-2593.8187) is shown. Bootstrap values (> 50%) are given at the nodes. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1840)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Ex-type strains are in bold face and clades containing isolates from this study are highlighted (see next page).


99 D. scrobiculata CBS113423
D. scrobiculata CBS109944
D. intermedia CBS124462
84 D. intermedia CBS124134
D. sapinea CBS393.84
85 D. sapinea CBS109725
80
CAA339
62 CAA317
100 CAA318
79 D. seriata CBS112555
D. seriata CBS119049
69 D. alatafructa CBS124931
99 D. pseudoseriata CBS124906
99 D. alatafructa CBS124933
D. pseudoseriata CBS124
68 100 D. allocellula CBS130408
D. allocellula CBS130410
100 D. cupressi CBS168.87
D. cupressi CBS261.85
99 D. tsugae CBS418.64
100 D. bulgarica CBS124254
D. bulgarica CBS124135
100 D. malorum CBS112554
D. malorum CBS124130
98 D. fraxini CBS136013
D. fraxini CBS136010
97 D. africana CBS120835
D. africana CBS121104
100 D. rosulata CBS116470
D. rosulata CB116472
100 D. olivarum CBS121887
D. olivarum CBS121886
99 D. agrifolia CBS132777
D. agrifolia UCROK1429
86
94 D. neojuniperi CPC22753
D. neojuniperi CPC22754
89 D. subglobosa CBS124133
D. subglobosa CBS124131
88 D. mutila CBS136014
D. mutila CBS230.30
CAA500
CAA691
100 D. corticola CBS112546
D. corticola CBS112549
100
100 D. quercivora CBS133852
D. quercivora CBS133853
0.01

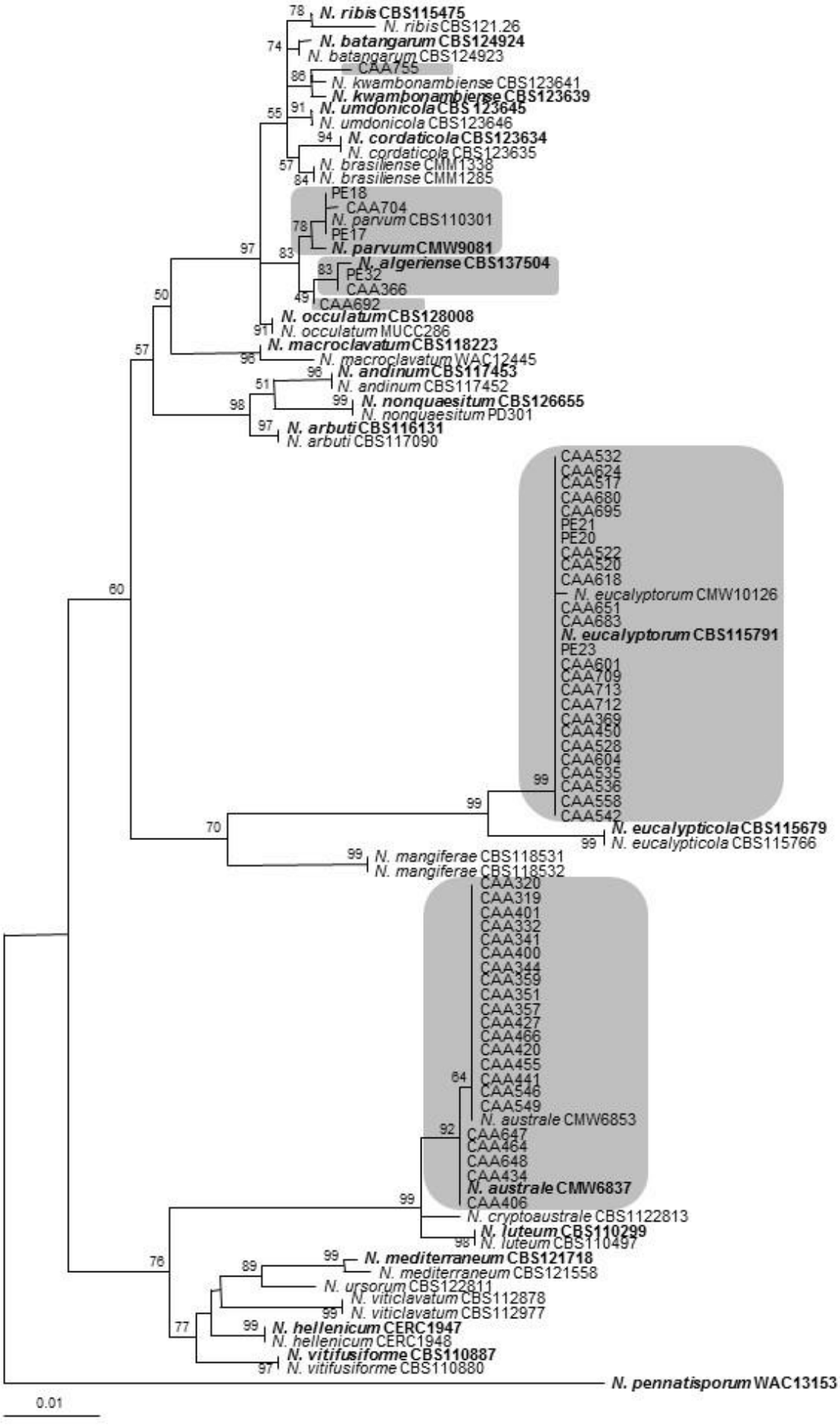

N. ribis CBS115475
N. ribis CBS121.26
N. batangarum CBS124924
N. batangarum CBS124923
CAA755
N. kwambonambiense CBS123641
N. kwambonambiense CBS123639
N. umdonicola CBS 123645
N. umdonicola CBS123646
N. cordaticola CBS123634
N. cordaticola CBS123635
N. brasiliense CMM1338
N. brasiliense CMM1285
PE18
CAA704
N. parvum CBS110301
PE17
N. parvum CMW9081
N. algeriense CBS137504
PE32
CAA366
CAA692
N. occulatum CBS128008
N. occulatum MUCC286
N. macroclavatum CBS118223
N. macroclavatum WAC12445
N. andinum CBS117453
N. andinum CBS117452
N. nonquaesitum CBS126655
N. nonquaesitum PD301
N. arbuti CBS116131
N. arbuti CBS117090
CAA532
CAA624
CAA517
CAA680
CAA695
PE21
PE20
CAA522
CAA520
CAA618
N. eucalyptorum CMW10126
CAA651
CAA683
N. eucalyptorum CBS115791
PE23
CAA601
CAA709
CAA713
CAA712
CAA369
CAA450
CAA528
CAA604
CAA535
CAA536
CAA558
CAA542
N. eucalypticola CBS115679
N. eucalypticola CBS115766
N. mangiferae CBS118531
N. mangiferae CBS118532
CAA320
CAA319
CAA401
CAA332
CAA341
CAA400
CAA344
CAA359
CAA351
CAA357
CAA427
CAA466
CAA420
CAA455
CAA441
CAA546
CAA549
N. australe CMW6853
CAA647
CAA464
CAA648
CAA434
N. australe CMW6837
CAA406
N. cryptoaustrale CBS1122813
N. luteum CBS110299
N. luteum CBS110497
N. mediterraneum CBS121718
N. mediterraneum CBS121558
N. ursorum CBS122811
N. viticlavatum CBS112878
N. viticlavatum CBS112977
N. hellenicum CERC1947
N. hellenicum CERC1948
N. vitifusiforme CBS110887
N. vitifusiforme CBS110880
N. pennatisporum WAC13153
0.01

Figure 2.3: Phylogenetic relationships of *Neofusicoccum* species inferred using the Maximum Likelihood method based on the Tamura 3-parameter model. The tree with the highest log likelihood (-2571.4691) is shown. Bootstrap values (> 50%) are given at the nodes. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1952)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Ex-type strains are in bold face and clades containing isolates from this study are highlighted (see previous page).

Botryosphaeria isolates clustered within the *B. dothidea* (Moug. : Fr.) Ces. & De Not. clade (Figure 2.1) with high bootstrap support (88%). Isolates belonging to Diplodia grouped into two clades (Figure 2.2). One set of isolates fell within the clade corresponding to *D. corticola* A.J.L. Phillips, A. Alves & J. Luque with 100% bootstrap support. The second set of isolates formed a sub-clade within the clade corresponding to *D. seriata* De Not. although with only moderate bootstrap support (65%). Finally, the *Neofusicoccum* isolates clustered within six different clades (Figure 2.3) although not all of them received high (> 70%) bootstrap support. Thus, most of the isolates were placed in two clades corresponding to *N. australe* (Slippers, Crous & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips and *N. eucalyptorum* (Crous, H. Smith & M.J. Wingf.) Crous, Slippers & A.J.L. Phillips. The remaining isolates clustered within the clades corresponding to *N. parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *N. kwambonambiense* Pavlic, Slippers & M.J. Wingf., *N. algeriense* A. Berraf-Tebbal & A.J.L. Phillips and one putative novel species: *Neofusicoccum* sp. closely related to *N. parvum*.

Table 2.1 summarizes the results of identification, source and abundance of each species found in this study. In addition, figure 2.4 resents the distribution of the species within the sampled regions throughout the country. Among the isolates obtained the genus *Neofusicoccum* was clearly dominant and represented 95% of the isolates on both symptomatic and asymptomatic plant tissues. Of the *Neofusicoccum* species identified *N. australe* and *N. eucalyptorum* were the most frequent in both symptomatic and asymptomatic plant tissues and represented, respectively, 49.8% and 41.9% of the isolates obtained. In addition to *N. australe* and *N. eucalyptorum*, the species *N. parvum* and *B. dothidea* were also isolated from both symptomatic and asymptomatic plants. On the other hand, *D. corticola*, *D. seriata*, *N. kwambonambiense*, *N*. *algeriense* and *Neofusicoccum* sp. were isolated only from plants with canker and dieback symptoms. Regarding the type of plant tissue from which endophytic isolates were obtained, *B. dothidea* was retrieved from branches, *N. australe* and *N. eucalyptorum* from branches and leaves equally and *N. parvum* only from roots.

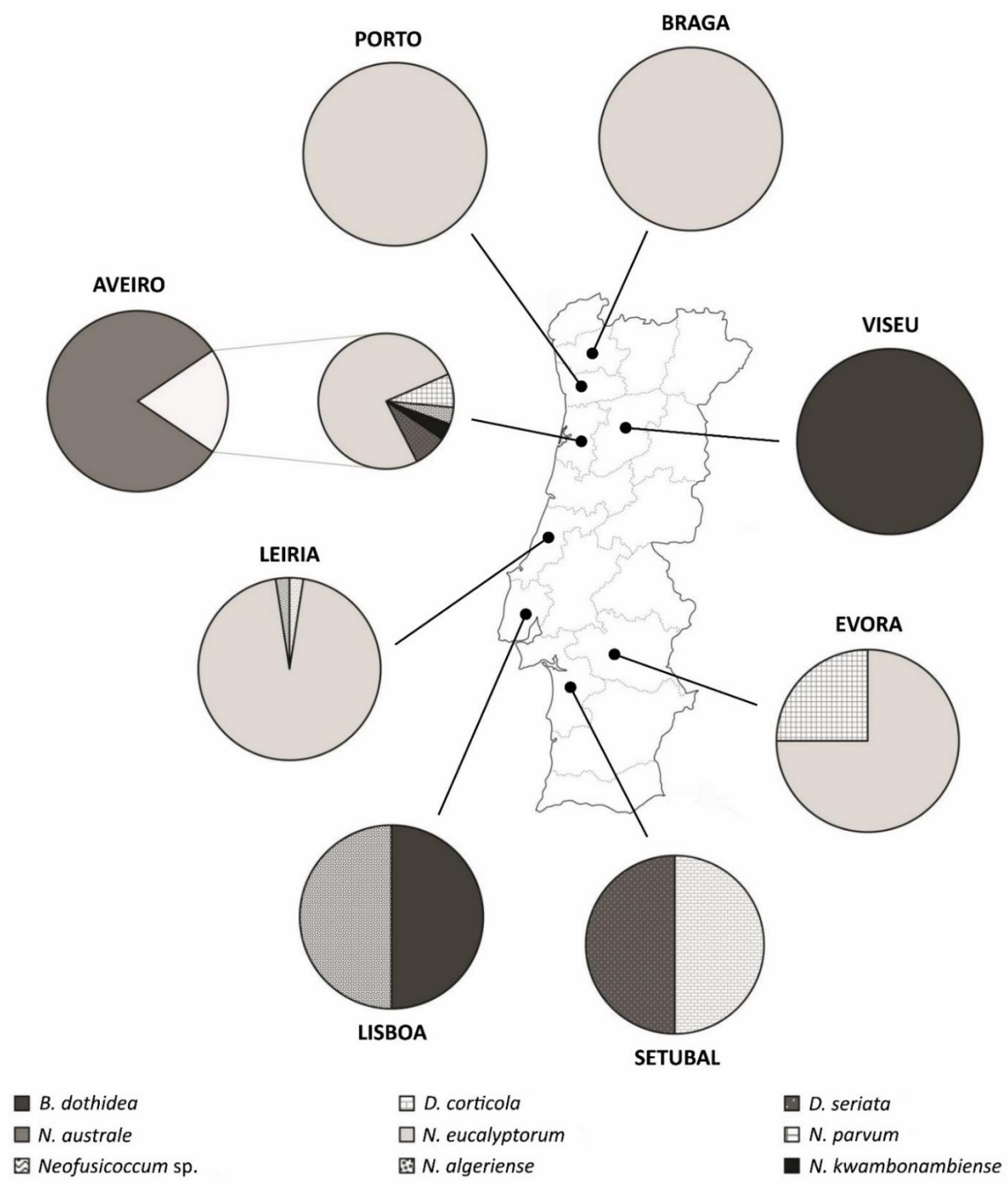


Figure 2.4: Map of Portugal indicating the distribution of Botryosphaeriaceae species within each sampled region.

Table 2.2: List of isolates obtained from *E. globulus* and used in this study.

Species	Isolate number[a,c]	Locality	Collector	GenBank[b]	
				ITS	EF
Botryosphaeira dothidea	PE26	Torres Vedras, Lisboa	H. Bragança & E. Diogo	KT440893	KT440954
B. dothidea	CAA642	Cepões, Viseu	C. Barradas	KT440894	KT440953
Diplodia corticola	CAA500	Alcochete, Sesimbra	L. Neves	KT440895	KT440958
D. corticola	CAA691	Bombarral, Leiria	C. Barradas	KT440896	KT440959
D. seriata	CAA317	G. Encarnação, Aveiro	A. Alves	KT440897	KT440955
D. seriata	CAA318	G. Encarnação, Aveiro	A. Alves	KT440898	KT440956
D. seriata	CAA339	G. Encarnação, Aveiro	A. Alves	KT440899	KT440957
Neofusicoccum australe	CAA319	G. Encarnação, Aveiro	A. Alves	KT440900	KT440960
N. australe	CAA320	G. Encarnação, Aveiro	A. Alves	KT440901	KT440961
N. australe	CAA332	G. Encarnação, Aveiro	A. Alves	KT440902	KT440962
N. australe	CAA341	G. Encarnação, Aveiro	A. Alves	KT440903	KT440963
N. australe	CAA344	G. Encarnação, Aveiro	A. Alves	KT440904	KT440964
N. australe	CAA351	G. Encarnação, Aveiro	A. Alves	KT440905	KT440965
N. australe	CAA357	G. Encarnação, Aveiro	A. Alves	KT440906	KT440966
N. australe	CAA359	Anadia, Aveiro	L. Neves	KT440907	KT440967
N. australe	CAA400	G. Encarnação, Aveiro	A. Alves	KT440908	KT440968
N. australe	CAA401	G. Encarnação, Aveiro	A. Alves	KT440909	KT440969
N. australe	CAA406	G. Encarnação, Aveiro	A. Alves	KT440910	KT440970
N. australe	CAA420	G. Encarnação, Aveiro	A. Alves	KT440911	KT440971
N. australe	CAA427	G. Encarnação, Aveiro	A. Alves	KT440912	KT440972
N. australe	CAA434	G. Encarnação, Aveiro	A. Alves	KT440913	KT440973
N. australe	CAA441	G. Encarnação, Aveiro	A. Alves	KT440914	KT440974
N. australe	CAA455	G. Encarnação, Aveiro	A. Alves	KT440915	KT440975
N. australe	CAA464	G. Encarnação, Aveiro	A. Alves	KT440916	KT440976
N. australe	CAA466	G. Encarnação, Aveiro	A. Alves	KT440917	KT440977

Species	Isolate number[a,c]	Locality	Collector	GenBank[b]	
				ITS	EF
N. australe	CAA546	Aveiro, Aveiro	A. Alves	KT440918	KT440978
N. australe	CAA549	Aveiro, Aveiro	A. Alves	KT440919	KT440979
N. australe	CAA647	Murtosa, Aveiro	A. Alves	KT440920	KT440980
N. australe	CAA648	Murtosa, Aveiro	A. Alves	KT440921	KT440981
N. eucalyptorum	CAA369	Anadia, Aveiro	L. Neves	KT440922	KT440982
N. eucalyptorum	CAA450	G. Encarnação, Aveiro	A. Alves	KT440923	KT440983
N. eucalyptorum	CAA517	Bidoeira de Cima, Leiria	C. Barradas	KT440924	KT440984
N. eucalyptorum	CAA520	Bidoeira de Cima, Leiria	C. Barradas	KT440925	KT440985
N. eucalyptorum	CAA522	Bidoeira de Cima, Leiria	C. Barradas	KT440926	KT440986
N. eucalyptorum	CAA528	Bidoeira de Cima, Leiria	C. Barradas	KT440927	KT440987
N. eucalyptorum	CAA532	Bidoeira de Cima, Leiria	C. Barradas	KT440928	KT440988
N. eucalyptorum	CAA535	Bidoeira de Cima, Leiria	C. Barradas	KT440929	KT440989
N. eucalyptorum	CAA536	Bidoeira de Cima, Leiria	C. Barradas	KT440930	KT440990
N. eucalyptorum	CAA542	Bidoeira de Cima, Leiria	C. Barradas	KT440931	KT440991
N. eucalyptorum	CAA558	Aveiro, Aveiro	A. Alves	KT440932	KT440992
N. eucalyptorum	CAA601	V. N. Gaia, Porto	A. Alves	KT440933	KT440993
N. eucalyptorum	CAA604	Felgueiras, Porto	A. Alves	KT440934	KT440994
N. eucalyptorum	CAA618	Maia, Porto	C. Barradas	KT440935	KT440995
N. eucalyptorum	CAA624	Maia, Porto	C. Barradas	KT440936	KT440996
N. eucalyptorum	CAA651	Murtosa, Aveiro	A. Alves	KT440937	KT440997
N. eucalyptorum	CAA680	Bombarral, Leiria	C. Barradas	KT440938	KT440998
N. eucalyptorum	CAA683	Bombarral, Leiria	C. Barradas	KT440939	KT440999
N. eucalyptorum	CAA695	Bombarral, Leiria	C. Barradas	KT440940	KT441000
N. eucalyptorum	CAA709	Moura, Beja	C. Barradas	KT440941	KT441001
N. eucalyptorum	CAA712	Moura, Beja	C. Barradas	KT440942	KT441002

Species	Isolate number[a,c]	Locality	Collector	GenBank[b]	
				ITS	EF
N. eucalyptorum	CAA713	Fafe, Braga	A. Alves	KT440943	KT441003
N. eucalyptorum	PE20	Azenha Pintada, Alentejo	H. Bragança, E. Diogo	KT440944	KT441004
N. eucalyptorum	PE21	Azenha Pintada, Alentejo	H. Bragança, E. Diogo	KT440945	KT441005
N. kwambonambiense	CAA755	Aveiro, Aveiro	C. Barradas	KT440946	KT441006
N. parvum	CAA704	Aradas, Aveiro	C. Barradas	KT440947	KT441007
N. parvum	PE17	Azenha Pintada, Alentejo	H. Bragança, E. Diogo	KT440948	KT441008
N. parvum	PE18	Azenha Pintada, Alentejo	H. Bragança, E. Diogo	KT440949	KT441009
Neofusicoccum sp.	CAA692	Bombarral, Leiria	C. Barradas	KT440950	KT441010
N. algeriense	CAA366	Anadia, Aveiro	L. Neves	KT440951	KT441011
N. algeriense	PE32	Torres Vedras, Lisboa	H. Bragança, E. Diogo	KT440952	KT441012
B. agaves	MFLUCC 10-0051	Doi Nang Khaw, Thailand	P. Chomnunti	*JX646790*	*JX646855*
B. agaves	**CBS 133992**	Doi Tung, Thailand	R. Phookamsak	*JX646791*	*JX646856*
B. avasmontanum	**CBS 121769**	Windhoek, Namibia	F.J.J. van der Walt, J. Roux	*EU101303*	*EU101348*
B. corticis	ATCC 22927	USA	R.D. Millholland	*DQ299247*	*EU673291*
B. corticis	**CBS 119047**	New Jersey, USA	P.V. Oudemans	*DQ299245*	*EU017539*
B. dothidea	CBS 110302	Montemor-o-Novo, Portugal	A.J.L. Phillips	*AY259092*	*AY573218*
B. dothidea	**CBS 115476**	Crocifisso, Switzerland	B. Slippers	*AY236949*	*AY236898*
B. fabicerciana	CMW 27108	Fujian, China	M.J. Wingfield	*HQ332200*	*HQ332216*
B. fabicerciana	**CBS127193**	Fujian, China	M.J. Wingfield	*HQ332197*	*HQ332213*
B. fusispora	**MFLUCC 10-0098**	Doi Tung, Thailand	S. Boonmee	*JX646789*	*JX646854*
B. fusispora	MFLUCC 11-0507	Chiang Mai, Thailand	R. Cheewangkoon	*JX646788*	*JX646853*
B. ramosa	**CBS 122069**	Bell Gorge, Western Australia	T.I. Burgess, M.J. Wingfield	*EU144055*	*EU144070*
B. scharifii	**CBS 124703**	Tehran, Iran	J. Abdollahzadeh	*JQ772020*	*JQ772057*
B. scharifii	CBS 124702	Iran	J. Abdollahzadeh, A. Javadi	*JQ772019*	*JQ772056*
D. africana	**CBS 120835**	Paarl, South Africa	U. Damm	*EF445344*	*EF445382*

Species	Isolate number[a,c]	Locality	Collector	GenBank[b]	
				ITS	EF
D. africana	CBS 121104	Paarl, South Africa	U. Damm	*EF445344*	*EF445383*
D. agrifolia	**CBS 132777**	California, USA	S. Lynch, A. Eskalen	*JN693507*	*JQ517317*
D. agrifolia	UCROK 1429	California, USA	S. Lynch, A. Eskalen	*JQ411412*	*JQ512121*
D. alatafructa	**CBS 124931**	Mpumalanga, South Africa	J.W.M. Mehl, J. Roux	*FJ888460*	*FJ888444*
D. alatafructa	CBS 124933	Mpumalanga, South Africa	J. Mehl, J. Roux	*FJ888478*	*FJ888446*
D. allocellula	**CBS 130408**	Pretoria, South Africa	F. Jami, M. Gryzenhout	*JQ239397*	*JQ239384*
D. allocellula	CBS 130410	Pretoria, South Africa	F. Jami, M. Gryzenhout	*JQ239399*	*JQ239386*
D. bulgarica	CBS 124135	Plovdiv, Bulgaria	S. G. Bobev	*GQ923852*	*GQ923820*
D. bulgarica	**CBS124254**	Plovdiv, Bulgaria	S. G. Bobev	*GQ923853*	*GQ923821*
D. corticola	CBS 112546	Huelva, Spain	M. E. Sánchez, A. Trapero	*AY259110*	*DQ458872*
D. corticola	**CBS112549**	Aveiro, Portugal	A. Alves	*AY259100*	*AY573227*
D. cupressi	**CBS168.87**	Bet Dagan, Israel	Z. Solel	*DQ458893*	*DQ458878*
D. cupressi	CBS 261.85	California, USA	L.L. Huillier	*DQ458894*	*DQ458879*
D. intermedia	**CBS124462**	Caparica, Portugal	A.J.L Phillips	*GQ923858*	*GQ923826*
D. intermedia	CBS 124134	Torres Vedras, Portugal	S. Santos	*HM036528*	*GQ923851*
D. malorum	CBS 112554	Monte da Caparica, Portugal	A.J.L. Phillips	*AY259095*	*DQ458870*
D. malorum	**CBS124130**	Monte da Caparica, Portugal	A.J.L. Phillips	*GQ923865*	*GQ923833*
D. mutila	**CBS 136014**	Aveiro, Portugal	A.J.L. Phillips	*KJ361837*	*KJ361829*
D. mutila	CBS 230.30	California, USA	L.L. Huillier	*DQ458886*	*DQ458869*
D. neojuniperi	**CBS 138652**	Thailand	T. Trakunyingcharoen	*KM006431*	*KM006462*
D. neojuniperi	CPC22754	Thailand	T. Trakunyingcharoen	*KM006432*	*KM006463*
D. olivarum	CBS 121886	San Pietro Vernotico, Italy	F. Salvatore	*EU392297*	*EU392274*
D. olivarum	**CBS121887**	Scorrano, Italy	C. Lazzizera	*EU392302*	*EU392279*
D. pseudoseriata	**CBS 124906**	Paysandu, Uruguay	C. Perez	*EU080927*	*EU863181*
D. pseudoseriata	CBS 124907	Paysandu, Uruguay	C. Perez	*EU080922*	*EU863179*

Species	Isolate number[a,c]	Locality	Collector	GenBank[b]	
				ITS	EF
D. quercivora	**CBS133852**	Tabarka, Tunisia	B.T. Linaldeddu	*JX894205*	*JX894229*
D. quercivora	CBS 133853	Tabarka, Tunisia	B.T. Linaldeddu	*JX894206*	*JX894230*
D. rosulata	**CBS 116470**	Gambo, Ethiopia	A. Gure	*EU430265*	*EU430267*
D. rosulata	CBS 116472	Gambo, Ethiopia	A. Gure	*EU430266*	*EU430268*
D. sapinea	CBS 109725	Habinsaran, South Africa	M.J. Wingfield	*DQ458896*	*DQ458881*
D. sapinea	**CBS 393.84**	Putten, Netherlands	H.A. van der Aa	*DQ458895*	*DQ458880*
D. scrobiculata	CBS 109944	Mexico	M.J. Wingfield	*DQ458899*	*DQ458884*
D. scrobiculata	CBS 113423	Mexico	M.J. Wingfield	*DQ458900*	*DQ458885*
D. seriata	**CBS 112555**	Montemor-o-Novo, Portugal	A.J.L. Phillipis	*AY259094*	*AY573220*
D. seriata	CBS 119049	Italy	L. Mugnai	*DQ458889*	*DQ458874*
D. tsugae	**CBS 418.64**	British Columbia, Canada	A. Funk	*DQ458888*	*DQ458873*
Lasiodiplodia theobromae	**CBS 164.96**	Madang, Papua New Guinea	A. Aptroot	*AY640255*	*AY640258*
L. gonubiensis	**CBS115812**	Gonubie, South Africa	D. Pavlic	*AY639595*	*DQ103566*
Macrophomina phaseolina	CBS 162.25	Uganda	Unknown	*KF531826*	*KF531803*
M. phaseolina	CBS 227.33	Unknown	S.F. Ashby	*KF531825*	*KF531804*
N. andinum	CBS117452	Mountain Range, Venezuela	S. Mohali	*DQ306263*	*DQ306264*
N. andinum	**CBS117453**	Mountain Range, Venezuela	S. Mohali	*AY693976*	*AY693977*
N. arbuti	**CBS 116131**	Washington, USA	M. Elliott	*AY819720*	*KF531792*
N. arbuti	CBS 117090	California, USA	M. Elliott	*AY819724*	*KF531791*
N. algeriense	**CBS 137504**	Algeria	A. Berraf-Tebbal	*KJ657702*	*KJ657715*
N. algeriense	ALG9	Algeria	A. Berraf-Tebbal	*KJ657704*	*KJ657721*
N. australe	**CMW6837**	Australia	M.J. Wingfield	*AY339262*	*AY339270*
N. australe	CMW 6853	Australia	M.J. Wingfield	*AY339263*	*AY339271*
N. batangarum	CBS 124923	Kribi, Cameroon	D. Begoude, J. Roux	*FJ900608*	*FJ900654*
N. batangarum	**CBS 124924**	Kribi, Cameroon	D. Begoude, J. Roux	*FJ900607*	*FJ900653*

Species	Isolate number[a,c]	Locality	Collector	GenBank[b]	
				ITS	EF
N. brasiliense	CMM1285	Brazil	M.W. Marques	*JX513628*	*JX513608*
N. brasiliense	CMM1338	Brazil	M.W. Marques	*JX513630*	*JX513610*
N. cordaticola	**CBS 123634**	Sodwana Bay, South Africa	D. Pavlic	*EU821898*	*EU821868*
N. cordaticola	CBS 123635	Kosi Bay, South Africa	D. Pavlic	*EU821903*	*EU821873*
N. cryptoaustrale	**CBS 122813**	Pretoria, South Africa	H.M. Maleme	*FJ752742*	*FJ752713*
N. eucalypticola	**CBS 115679**	Victoria, Australia	M.J. Wingfield	*AY615141*	*AY615133*
N. eucalypticola	CBS 115766	Tidbubilla, Australia	M.J. Wingfield	*AY615143*	*AY615135*
N. eucalyptorum	**CBS 115791**	South Africa	H. Smith	*AF283686*	*AY236891*
N. eucalyptorum	CMW 10126	South Africa	H. Smith	*AF283687*	*AY236892*
N. kwambonambiense	**CBS 123639**	Kwambonambi, South Africa	D. Pavlic	*EU821900*	*EU821870*
N. kwambonambiense	CBS 123641	Tzaneen, South Africa	D. Pavlic	*EU821919*	*EU821889*
N. luteum	**CBS 110299**	Oeiras, Portugal	A.J.L. Phillips	*AY259091*	*AY573217*
N. luteum	CBS 110497	Portugal	A.J.L. Phillips	*EU673311*	*EU673277*
N. macroclavatum	**CBS 118223**	Denmark, Western Australia	T. Burgess	*DQ093196*	*DQ093217*
N. macroclavatum	WAC 12445	Australia	T.I. Burguess	*DQ093197*	*DQ093218*
N. mangiferae	CBS 118531	Bowen, Australia	G.I. Johnson	*AY615185*	*DQ093221*
N. mangiferae	CBS 118532	Australia	G.I. Johnson	*AY615186*	*DQ093220*
N. mediterraneum	CBS 121558	Scorrano, Italy	F. Salvatore	*GU799463*	*GU799462*
N. mediterraneum	**CBS 121718**	Greece	P.W. Crous, M.J. Wingfield, A.J.L. Phillips	*GU251176*	*GU251308*
N. nonquaesitum	**CBS 126655**	California, USA	F.P Trouillas	*GU251163*	*GU251295*
N. nonquaesitum	PD 301	Chile	E.X. Briceño, J.G. Espinoza, B.A. Latorre	*GU251164*	*GU251296*
N. occulatum	**CBS 128008**	Queensland, Australia	T.I. Burgess	*EU301030*	*EU339509*
N. occulatum	MUCC 286	Queensland, Australia	T. Burgess, G. Pegg	*EU736947*	*EU339511*
N. parvum	**CMW 9081**	New Zealand	G.J. Samuels	*AY236943*	*AY236917*
N. parvum	CBS 110301	Palmela, Portugal	A.J.L. Phillips	*AY259098*	*AY573221*

Species	Isolate number[a,c]	Locality	Collector	GenBank[b]	
				ITS	EF
N. pennatisporum	**WAC 13153**	Western Australia	K.M. Taylor	*EF591925*	*EF591976*
N. ribis	**CBS 115475**	New York, USA	B. Slippers, G. Hudler	*AY236935*	*AY236877*
N. ribis	CBS 121.26	New York, USA	N.E. Stevens	*AF241177*	*AY236879*
N. umdonicola	**CBS 123645**	Kosi Bay, South Africa	D. Pavlic	*EU821904*	*EU821874*
N. umdonicola	CBS 123646	Kosi Bay, South Africa	D. Pavlic	*EU821905*	*EU821875*
N. ursorum	CBS 122811	Pretoria, South Africa	H. Maleme	*FJ752746*	*FJ752709*
N. viticlavatum	**CBS 112878**	Western Cape, South Africa	F. Halleen	*AY343381*	*AY343342*
N. viticlavatum	CBS 112977	Western Cape, South Africa	F. Halleen	*AY343380*	*AY343341*
N. vitifusiforme	CBS 110880	Western Cape, South Africa	J.M. Van Niekerk	*AY343382*	*AY343344*
N. vitifusiforme	**CBS 110887**	Western Cape, South Africa	J.M. van Niekerk	*AY343383*	*AY343343*

[a]Acronyms of culture collections: ALG, Personal culture collection A. Berraf-Tebbal; ATCC, American Type Culture Collection, Virginia, USA; CAA, A.Alves, Universidade de Aveiro, Portugal; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; CMM, Culture collection of Phytopathogenic Fungi "Prof. Maria Menezes", Universidade Federal Rural de Pernambuco, Recife, Brazil; CMW, M.J. Wingfield, FABI, University of Pretoria, South Africa; CPC, Culture Collection of P.W. Crous, housed at CBS; MFLUCC, Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; MUCC, Murdoch University Culture Collection, Perth, Australia; PD, Department of Plant Pathology, University of California, Davis; UCROK, Department of Plant Pathology and Microbiology, University of California, Riverside; WAC, Department of Agriculture, Western Australia Plant Pathogen Collection, South Perth, Western Australia.

[b] Sequence numbers in italics were retrieved from GenBank. All others were obtained in the present study

[c] Ex-type strains in bold face

Pathogenicity trials

After incubation all inoculated plants developed cankers and necrotic lesions starting from the point of inoculation while control plants did not develop any lesions. Cross-sections of the stems revealed brown discoloration of the wood and wedge-shaped necrotic sectors. Since data did not follow a normal distribution a transformation Ln(X+1) was applied. Analysis of variance revealed significant differences in aggressiveness between the fungal species tested (df = 8; F = 25.80; p < 0.001) (Table 2.3). Thus, *D. corticola* and *N. kwambonambiense* were the most aggressive while *B. dothidea* and *D. seriata* were the least aggressive. All fungal pathogens were consistently re-isolated from all inoculated plants, while no pathogens were isolated from control plants, thus confirming Koch's postulates.

Table 2.3: Back-transformed means ± SD of lesion lengths (cm) caused by each fungal species inoculated on *E. globulus* clone AL-18 and back-transformed LSD values. Means followed by the same letters do not differ significantly according to LSD (P ≤ 0.05).

Species	Isolate	Mean lesion length (cm) ± SD
N. kwambonambiense	CAA755	10.12 ± 0.41 a
D. corticola	CAA500	9.95 ± 0.47 a
N. algeriense	CAA366	4.35 ± 0.47 b
N. eucalyptorum	CAA558	3.15 ± 0.23 bc
N. australe	CAA398	3.07 ± 0.27 bc
N. parvum	CAA704	2.25 ± 0.41 cd
Neofusicoccum sp.	CAA692	1.66 ± 0.91d
B. dothidea	CAA642	0.64 ± 0.20 e
D. seriata	CAA318	0.34 ± 0.26 e
LSD 5%	0.511	
LSD 1%	0.736	
LSD 0.1%	1.059	

Discussion

To our knowledge this is the first detailed study concerning fungi in the family Botryosphaeriaceae associated with *E. globulus* plantations in Portugal. Nine species belonging to three genera (*Botryosphaeria*, *Neofusicoccum* and *Diplodia*) were identified from symptomatic and asymptomatic plant material. The vast majority of the isolates obtained were identified as members of the genus *Neofusicoccum*. A similar trend has been reported in previous studies that revealed this genus to be the most common and diverse on *Eucalyptus* in most areas where this host is grown. A diverse assemblage of *Neofusicoccum* species has been found to occur on *Eucalyptus* spp. as canker and dieback pathogens and as endophytes (Mohali et al. 2007; Pérez et al. 2010; Slippers et al. 2009), but apparently different species dominate in different regions (Gezahgne et al. 2004; Slippers et al. 2004b, 2009).

Neofusicoccum eucalyptorum and *N. australe* were the most abundant species found in this study. Both species occurred in plants showing disease symptoms as well as in healthy plants. *Neofusicoccum eucalyptorum* was first described as causing stem cankers on *E. grandis* and *E. nitens* in South Africa (Smith et al. 2001). Later, Slippers et al. (2004b) showed that *N. eucalyptorum* was the dominant species in *Eucalyptus* native forests and plantations in eastern Australia, representing about 50% of the isolates on this host and area. Considering this abundance and wide distribution these authors suggested that *N. eucalyptorum* was native on *Eucalyptus* in Australia. Interestingly, this species has not yet been found on *Eucalyptus* in Western Australia (Burgess et al. 2005, 2006). Meanwhile, *N. eucalyptorum* has been reported from several *Eucalyptus* species in Chile and Uruguay (Pérez et al. 2010; Slippers et al. 2009). Here we report the occurrence of this species in Portugal and the Northern Hemisphere for the first time. Moreover, until now the only other known reports on *E. globulus* (as endophyte) are from Tasmania (Burgess et al. 2006) and Uruguay (Pérez et al. 2010). It is likely that *N. eucalyptorum* was introduced into Portugal via human-mediated transfer of *E. globulus* germplasm from its native origin. The fact that it is widespread throughout almost all sampled regions in Portugal (Figure 2.4) further strengthens this hypothesis. *Neofusicoccum eucalyptorum* is apparently highly specialized on *Eucalyptus* species, but recently it has been reported on several other genera of Myrtaceae in South Africa and Uruguay (Pavlic et al. 2007; Pérez et al. 2009, 2010; Pillay et al. 2013).

The species *N. australe* has been associated with a very wide range of hosts (Phillips et al. 2013) including several *Eucalyptus* species, such as *E. globulus*, *E. grandis, E. gomphocephala, E. marginata* and *E. diversicolor* in South Africa and Australia (Barber et al. 2005; Burgess et al. 2005, 2006; Slippers et al. 2004b; Taylor et al. 2009). *Neofusicoccum australe* is known to occur in Portugal and has been reported from several coniferous hosts (Alves et al. 2013) but this report on *E. globulus* represents a new host association for the country. It has also been associated with canker of *E. globulus* in Spain (Armengol et al. 2008). This species was the most abundant in current study, but interestingly it was restricted to a single region (Figure 2.4). In Portugal it is possible that *N. australe* moved to eucalypts from other hosts such as conifers where it is common (Alves et al. 2013). A similar situation has been reported in Western Australia, where it is the dominant species associated with introduced *E. globulus* and native eucalypts (Burgess et al. 2006). Moreover, it has not been recorded in Tasmania, the native range of *E. globulus* (Burgess et al. 2006).

Curiously, the sister species *N. luteum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, which is the more frequent species in conifers such as pines in Portugal (Alves et al. 2013) was not found in *E. globulus*. This is even more surprising since eucalypt plantations are frequently located in close proximity to pine plantations and in some situations they even occur in mixed forest stands. In the same way, it has been found on *Syzygium cordatum* (Myrtaceae) in South Africa (Pavlic et al. 2007) but has not been reported on eucalypts in that country. In fact, to our knowledge *N. luteum* has

never been reported from *Eucalyptus* species anywhere in the world, including in their native range where it is also found on other hosts. Nevertheless, isolates of this species from *S. cordatum* in South Africa were highly aggressive to a *Eucalyptus* clone (Pavlic et al. 2007) and therefore *N. luteum* represents a potential threat to *Eucalyptus* plantations.

The remaining *Neofusicoccum* species isolated in this study were poorly represented but are nevertheless noteworthy. *Neofusicoccum parvum* was isolated from both symptomatic and asymptomatic plants, but the most striking feature is that the endophytic isolates were retrieved from roots. This is quite unusual among the Botryosphaeriaceae, which are more frequent in above ground plant tissues (Slippers and Wingfield 2007). This species has an exceptionally large host range (Phillips et al. 2013) being common in *Eucalyptus* and other Myrtaceae and has been reported from these hosts in Australia, South Africa, Uruguay, Venezuela, Zambia and China (Burgess et al. 2005; Chen et al. 2011; Chungu et al. 2010; Mohali et al. 2007; Pavlic et al. 2007; Pérez et al. 2010; Slippers et al. 2004b). In Ethiopia it was the only species associated with stem canker on *Eucalyptus* plantations (Gezahgne et al. 2004). More recently, it has been reported as the main cause of canker and dieback of *E. globulus* in Spain (Iturritxa et al. 2011). In Portugal, *N. parvum* has also been isolated from conifers (Alves et al. 2013) but this is the first time it is reported on *E. globulus* in this country. *Fusicoccum eucalypti* Sousa da Câmara was described from *E. globulus* in Portugal (Sousa da Câmara 1929). Later, Sutton & Dyko (1989) reduced it to synonymy with *Natrassia mangiferae* (Syd. & P. Syd.) B. Sutton & Dyko, but Slippers et al. (2005) rejected this synonymy on the basis of the conidial sizes reported for *F. eucalypti*, which they considered to be more similar to those of *N. parvum*. However, in the absence of authentic cultures and DNA sequence data this possibility cannot be confirmed.

In the past, *N. parvum* (= *B. parva* Pennycook & Samuels), *N. ribis* (= *B. ribis* Grossenb. & Duggar) and *B. dothidea* have been frequently confused (Phillips et al. 2013; Sakalidis et al. 2011, 2013). Using a combination of morphological characters and multiple gene genealogies Slippers et al. (2004a) clearly separated these species. Thus, according to Sakalidis et al. (2013) all disease and host reports that precede this taxonomic revision are unreliable, and for this reason only records collected since 2004 with corresponding DNA sequence data, or isolates collected earlier but for which sequence data are available, can be considered reliable. In recent years the use of multiple gene genealogies to delineate species limits in the *N. parvum*-*N. ribis* species complex resulted in the recognition of five additional cryptic species, namely *N. umdonicola* Pavlic, Slippers & M.J. Wingf., *N. cordaticola* Pavlic, Slippers & M.J. Wingf., *N. kwambonambiense* Pavlic, Slippers & M.J. Wingf. (Pavlic et al. 2009a), *N. batangarum* Begoude, Jol. Roux & Slippers (Begoude et al. 2010) and *N. occulatum* Sakalidis & T.I. Burgess (Sakalidis et al. 2011).

In a study of the *Neofusicoccum* species associated with grapevine trunk diseases in Algeria, (Berraf-Tebbal et al. 2014) introduced *Neofusicoccum algeriense* sp. nov. In

the current work we isolated this species from stems and branches of symptomatic *E. globulus* trees. This represents not only the first report of *N. algeriense* in a host other than grapevine but also the first report of the species in Europe.

A single isolate of *N. kwambonambiense* was obtained from an *E. globulus* plant with a stem canker. *Neofusicoccum kwambonambiense* was originally described from symptomless branches and leaves, dying branches and pulp of ripe fruits of *Syzygium cordatum* (Myrtaceae) in South Africa (Pavlic et al. 2009a,b). Later, Sakalidis et al. (2011) reported it from *E. dunnii* and *Corymbia torelliana* in Australia and more recently (Pillay et al. 2013) isolated it from *E. grandis* in South Africa. By reanalyzing all sequence data available for the *N. parvum-N. ribis* complex (Sakalidis et al. 2013) performed a host and geographical distribution of each cryptic species. Thus, *N. kwambonambiense* was found to occur on four continents, six countries and 14 hosts. However, this work represents not only a new host association for the species but also a new record for Portugal.

One putative novel species *Neofusicoccum* sp. was identified. Despite several attempts the single isolate of this species could not be induced to sporulate in culture thus hindering any morphological analyses. Moreover, there was no strong bootstrap support in phylogenetic analyses for the clade including it. Thus, this isolate will be further characterized in the future including sequence analyses of more loci to clearly resolve its status as a separate species.

Botryosphaeria dothidea was the only representative of the genus found in this study both as endophyte and disease-related. This species was previously thought to be common on *Eucalyptus*, and was reported as an endophyte on *E. grandis* and *E. nitens* in South Africa (Smith et al. 1996) and on *E. grandis* and *E. globulus* in Uruguay (Bettucci and Saravay 1993, 1997). Additionally it was also associated with canker and dieback of *Eucalyptus* in South Africa (Smith et al. 1994). However, as explained above many of these early identifications are unreliable and it is now clear that the species occurs rarely on *Eucalyptus* (e.g. Rodas et al. 2009). Our results confirm this since only 3 isolates in the entire collection were identified as *B. dothidea*. A search of the SMML Fungus-Host Distribution Database (http://nt.ars-grin.gov/fungaldatabases/) retrieved a single report of *B. dothidea* (as *B. berengeriana*) on *E. globulus* in Portugal. Slippers et al. (2004a) regarded *B. berengeriana* De Not. as a synonym of *B. dothidea* because they could not find any morphological differences between the type material of both species. In view of the taxonomic confusions discussed previously we cannot be entirely confident on the reliability of this report in the SMML database. Therefore, we regard the present study as the first confirmed report of *B. dothidea* on *E. globulus* in Portugal.

Diplodia species are known to occur worldwide on a large diversity of hosts (Phillips et al. 2013) but are remarkably absent from *Eucalyptus*, even though they are found on other hosts grown in the same areas. For example, Pérez et al. (2010) described *D. pseudoseriata* C.A. Pérez, R.A. Blanchette, B. Slippers & M.J. Wingfield from eight different species of native Myrtaceae trees in Uruguay but could not find it on non-native *Eucalyptus* plantations in close proximity. A search of the SMML Fungus-Host

Distribution Database and Index Fungorum (http://www.indexfungorum.org) retrieved three *Diplodia* species described on *Eucalyptus*, *D. australiae* Speg., *D. eucalypti* Cooke & Harkn. and *D. tenuis* Cooke & Harkn. However, given the taxonomic confusions surrounding the genus *Diplodia* in the past these reports need to be confirmed. An analysis of type material (if extant and in good conditions) will confirm them as members of this genus but given the constraints of morphological identification (Phillips et al. 2013) it is virtually impossible to accurately determine the exact species identity in modern terms.

Within the collection of isolates analyzed seven of them retrieved from symptomatic plants were identified as *D. corticola* (3 isolates) and *D. seriata* (4 isolates). Both species are reported from *Eucalyptus* for the first time worldwide. *Diplodia corticola* is a canker and dieback pathogen of cork oak and other oak species in Portugal and throughout the Mediterranean region (Alves et al. 2004) from where it is most likely to be native. This species is one of the main pathogens involved in native cork oak and holm oak forests decline (Alves et al. 2004; Linaldeddu et al. 2014). More recently it has been reported on several oak species in different regions of the USA probably as a result of multiple introductions (Lynch et al. 2013). This species seems to be highly specialized on *Quercus* spp. but has also been associated with dieback and canker of grapevines in Spain and the USA (Pintos Varela et al., 2011; Urbez-Torres et al. 2010). Here we report it from *E. globulus* thus widening the species host range. This occurrence on *E. globulus* is not that surprising considering that *Eucalyptus* plantations commonly co-exist alongside native oaks (mostly cork oak) and host jumps may well be common in the Botryosphaeriaceae (Pavlic et al. 2007; Pérez et al. 2009, 2010). *Diplodia seriata* is a cosmopolitan, plurivorous species (Phillips et al. 2013) and the current report on *E. globulus* adds to an already exceedingly large host range. In Portugal so far it has been associated with *Vitis vinifera*, *Malus sylvestris*, *Thuja plicata*, *Chamaecyparis lawsoniana* and *Fraxinus ornus* (Alves et al. 2013, 2014; Phillips et al. 2012) but being a generalist species it is likely to occur on many other hosts.

In pathogenicity trials isolates representative of each species were shown to incite stem cankers on an *E. globulus* clone, but there was an obvious variation in aggressiveness between species. *Diplodia corticola* and *N. kwambonambiense* although being poorly represented in this study were the most aggressive species. Both species seem to be emergent pathogens on this host and represent a potential threat to *Eucalyptus* plantations that should not be overlooked. In fact, the works of Pillay et al. (2013) and Sakalidis et al. (2013) have already shown that *N. kwambonambiense* is more common on *Eucalyptus* than previously realized. For the remaining *Neofusicoccum* species this study further confirms their pathogenic potential towards *Eucalyptus* already revealed previously (e.g. Armengol et al. 2008; Burgess et al. 2005; Chen et al. 2011; Gezahgne et al. 2004; Iturritxa et al. 2011; Mohali et al. 2009; Pavlic et al. 2007). *Neofusicoccum algeriense*, which is here reported for the first time on eucalypts, has been associated with wedge-shaped cankers on grapevines (Berraf-Tebbal et al. 2014).

This species also showed to be pathogenic to *E. globulus* in the inoculation trials and represents yet another *Neofusicoccum* species able to cause disease on this host. However, it was poorly represented in the isolate collection (2 isolates) and thus its relevance as a pathogen of eucalypts is still not clear. On the other hand, *B. dothidea* and *D. seriata* are apparently weakly pathogenic to this host. This also confirms that *B. dothidea* once considered an important pathogen of *Eucalyptus* plantations (Slippers et al. 2009; Smith et al. 1994) is actually not of such relevance.

Previous studies have shown that there is variability in pathogenicity among different isolates of a single species (e.g. Burgess et al. 2005; Gezahgne et al. 2004; Mohali et al. 2009; Pavlic et al. 2007; Pérez et al. 2009; Rodas et al. 2009). In this work only one isolate per species was tested in pathogenicity trials and therefore care must be taken when drawing conclusions about the aggressiveness of each species. Future studies including more isolates should be done in order to gauge the whole spectrum of pathogenic variability within each species found.

Most, if not all, Botryosphaeriaceae species have a cryptic, endophytic stage in their life cycle (Slippers and Wingfield 2007) a feature that is further supported by our results showing that the majority of the species identified were also isolated from healthy plant tissues. Members of this family have frequently been described as opportunistic pathogens that incite disease when plants are under some kind of stress such as drought (most commonly), physical damage, biotic stress caused by other pathogens and pests, among others. Under conditions of changing climate with increased stress on plant communities it is likely that diseases caused by Botryosphaeriaceae species will increase in incidence and impact (Slippers and Wingfield 2007). In such a scenario of climate change some species reported here as rare and/or less aggressive might become relevant pathogens of *Eucalyptus* plantations.

Here we show that a diverse collection of Botryosphaeriaceae species occurs in *E. globulus* in Portugal and that these represent a potential hazard to plantations. Further work should be done to monitor the Botryosphaeriaceae communities to evaluate the introduction of novel pathogens, gene flow between *Eucalyptus* and other hosts, identify host jumps and consequently the emergence of pathogens and the risk they may pose to *Eucalyptus* plantations.

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CHAPTER 3

Mid-infrared spectroscopy (MIR) as a tool to differentiate species in the family Botryosphaeriaceae

Mid-infrared spectroscopy (MIR) as a tool to differentiate species in the family Botryosphaeriaceae

Abstract

Routine identification of species in the family Botryosphaeriaceae can be a challenging task. The identification based on morphological characters has become virtually impossible and is frequently misleading. On the other hand, DNA-based techniques although very powerful in discriminating species can still be time consuming and rather expensive.

Mid-infrared (MIR) spectroscopy has been successfully applied for the identification and characterization of fungi. In this study we evaluated the potential of MIR to discriminate between species in the family Botryosphaeriaceae. For that, a total of 29 well-characterised reference cultures representing 26 species from nine genera were analysed. Although infrared spectra showed an overall similarity there were noticeable differences, which were supported by multivariate statistical analyses.

The analysis of infrared fingerprint profiles allowed the discrimination of the cultures tested from species to strain level. Thus, MIR spectroscopy has shown a great potential as a fast, high-throughput and effective tool to identify fungi in the Botryosphaeriaceae. However, further studies including a larger number of reference cultures are needed in order to develop a comprehensive database of infrared fingerprint profiles to assist species identification.

Keywords: fungi; identification; infrared spectra; multivariate analysis

Introduction

Fungal species of the family Botryosphaeriaceae are well-known pathogens and latent endophytes of a large number of plants with agricultural, forestry and economic importance (Phillips et al. 2013; Slippers and Wingfield 2007). This is a genus and species-rich family comprising at least 19 genera and more than 100 species recognised from culture (Crous et al. 2013; Jami et al. 2014; Liu et al. 2012; Phillips et al. 2013).

The identification of species is based on morphological aspects of the conidia (size, shape, septation, wall thickness and texture) as well as details of conidiogenesis (Denman et al. 2000; Jacobs and Rehner 1998; Slippers et al. 2009) and on DNA sequence of ITS region (internal transcribed spacer region) of the rDNA combined with protein coding genes regions mostly translation elongation factor 1-alpha (tef1) and beta-tubulin (tub2) genes (Alves et al. 2004, 2014; Crous et al. 2015; Phillips et al. 2012). While the identification based on morphological characters can be misleading, DNA-based techniques are very powerful in discriminating species. These recent genotypic

approaches, although widely accepted, present some technical limitations due to reagent costs, protocol complexities and requirement of specific primers for some species, which can be difficult to select. Furthermore, these techniques are based on a small fraction of the genome and do not provide evidence for phenotypic diversification (Mancini et al. 2013; Santos et al. 2010). An ideal method for the rapid identification of fungi should be inexpensive, involve minimum sample preparation and allow direct analyses of the samples (Santos et al. 2010).

Mid-infrared spectroscopy (MIR) is a powerful technique that is fast, effective and low cost, as no reagents are required to perform the analysis. Moreover, there is little or no requirement of sample pre-treatment (Lasch and Naumann 2015). MIR is a vibrational spectroscopic technique in which an infrared beam interacts with the sample and is measured in the spectral region between 4000 and 400 cm^{-1}. Specific frequencies are absorbed corresponding to their molecular modes of vibration that are characteristic of their chemical bonding, structure and composition. This allows determining the molecular groups present in the sample. The obtained infrared spectra are fingerprint-like patterns characteristic of a chemical or biological component (Stuart 2004).

MIR has been successfully used in the identification of various microorganisms, including filamentous fungi. It was used to differentiate between *Aspergillus* and *Penicillium* species by the analysis of conidia (Fischer et al. 2006). Also, the differentiation of closely related *Aspergillus* species was achieved using this technique (Garon et al. 2010; Tralamazza et al. 2013). A spectral library of 486 pure strains was built, using MIR, with 80,79 % of the species correctly assigned by multivariate analysis (Lecellier et al. 2015). *Trametes versicolor* and *Schizoplyllum commune* identification in wood inoculated with pure cultures was also performed, by detection of mycelium, using MIR coupled to a microscope (MIR microspectroscopy) (Naumann et al. 2005). This technique is increasing its importance, since it allows to directly analysing the material, providing a detection of the fungi in situ, as shown by these authors.

As there are no studies related to the identification and characterization of members of Botryosphaeriaceae based on this technique, the objective of this work was to assess the potential of MIR spectroscopy as a tool to discriminate between species of this family.

Materials and Methods

Sample preparation

The fungal strains used in this work are listed in Table 3.1. A total of 29 isolates representing 9 different genera and 26 species of Botryosphaeriaceae were used. These are well-characterized strains whose identification has been performed based on DNA sequence data analysis in previous works.

Fungi were grown in potato dextrose agar (PDA) medium (Merck, Germany) and incubated at 25°C during 7 days under the influence of natural illumination.

Table 3.1: List of species/strains of Botryosphaeriaceae used in this study.

Genus	Species	Isolate number
Botryosphaeria	*B. dothidea*	CAA675
Dothiorella	*Do. vidmadera*	IMI501235a
	Do. iberica	CAA480
	Do. prunicola	CAP187
Diplodia	*D. corticola*	CAA500
	D. intermedia	CAA496
	D. mutila	CAA409
	D. quercivora (1)	CBS133852
	D. quercivora (2)	CBS 133853
	D. seriata	CAA318
Lasiodiplodia	*L. iraniensis*	LASID3
	L. plurivora	CAA012
	L. pseudotheobromae (1)	LASOM2
	L. pseudotheobromae (2)	CBS116460
	L. theobromae	LAMAL1
Macrophomina	*M. phaseolina* (1)	M2CO_B
	M. phaseolina (2)	M40T_B
Neodeightonia	*Neo. phoenicum*	CBS123168
Neofusicoccum	*N. algeriense*	CAA366
	N. australe	CAA398
	N. eucalyptorum	CAA558
	N. kwambonambiense	CAA755
	N. luteum	CAA365
	N. nonquaesitum	PD301
	N. parvum	CAA704
Spencermartinsia	*S. plurivora*	CBS 117006
	S. viticola	CBS 117009
	S. citricola	ICMP 16827
Sphaeropsis	*Sph. citrigena*	ICMP 16812

Mid-infrared spectroscopy

Spectroscopic acquisition was carried out in a MIR (Bruker ALPHA Platinum-ATR FT-IR Spectrometer, Germany) with a resolution of 4 cm^{-1} and 32 scans, in the mid-infrared (region between 4000 and 600 cm^{-1}). Fungal mycelium was collected with a loop and placed directly on the crystal of the horizontal single reflection ATR accessory with a deuterated triglycine sulfate (DTGS) detector. The mycelium was gently air-dried with a cold flow and then measured, in order to decrease the effect of water in the spectra that could mask some important peaks. The sample preparation was standardized and the same procedure was used for all the strains. Five replicate spectra were obtained for each sample. The sampling accessory was cleaned with ethanol (70%) and distilled water between each measurement.

Data analysis

The spectra (obtained in OPUS format) were transferred via JCAMP.DX format to an in-house developed data analysis package (CATS build 97) and each spectral signal was normalized by standard normal variate (SNV). Principal component analysis (PCA) was used to find the major sources of variability in data, to detect outliers and detect the probable presence of clusters. For the hierarchical cluster analysis (HCA), the distance matrix was built upon the Euclidean distance and the agglomerative procedure was based on complete linkage. PCA was performed in CATS build 97 and HCA was performed in Primer v.6.1.6 software.

Results and Discussion

To evaluate the potential of MIR spectroscopy to be used as a tool for the identification of species in the Botryosphaeriaceae we acquired spectra from 29 isolates belonging to 9 different genera and 26 species and performed multivariate analyses.

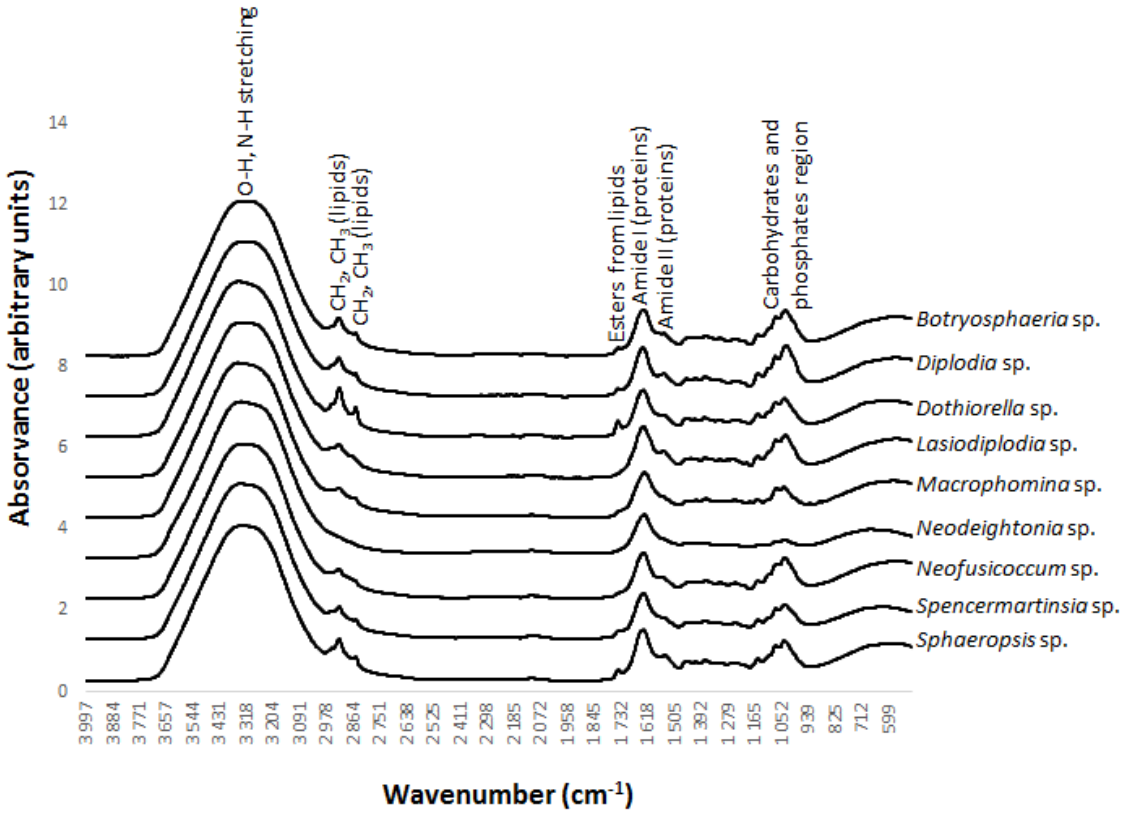

Figure 3.1: MIR spectra representative of each genus studied obtained from mycelium grown on PDA agar in the spectral region between 4000 and 500 cm^{-1}.

Figure 3.1 shows typical spectra for each one of the different genera analysed in this work. Observing the spectra, it is visible that all are apparently very similar, being dominated by O-H and N-H stretching bands, between 3800-3000 cm^{-1}, from biopolymers and remaining water, CH_3 and CH_2 signals from lipids and in some cases a

band in ≈1740 cm^{-1} associated to esters from lipids, signals in protein associated region (1700-1450 cm^{-1}) and carbohydrates and phosphates region (1200-980 cm^{-1}). Contrary to the other species analysed the mycelium from the *Neodeightonia phoenicum* culture was viscous with high water content and it was not possible to completely dry it. This behaviour is mirrored in the spectrum, being the signals of water dominant and that from biopolymers and lipids less pronounced.

Infrared spectroscopy is a surface technique that, in this case, should mainly represent the cellular surface macromolecular composition. A previous study with bacteria showed that infrared spectra from Gram-positive and Gram-negative bacteria are very similar, suggesting that the bulk functional chemical groups of the bacterial surfaces are similar (Jiang et al. 2004). A study using MIR microspectroscopy to analyse fungal isolates reported that, despite of the spectra similarity between different species, the small differences in the structural chemistry of the cell wall or protein/enzyme chemistry may account for the slight differences in the spectra (Oberle et al. 2015). It is known that fungal cell wall contains not only glycoproteins, but also polysaccharides, mainly glucans and chitin, which vary amongst the different species (Bowman and Free 2006; da Silva et al. 2008). In our case, despite the apparent similarity between spectra because of the similar bulk of common functional groups, the differences in specific chemical composition are also reflected in the small variations of the spectra.

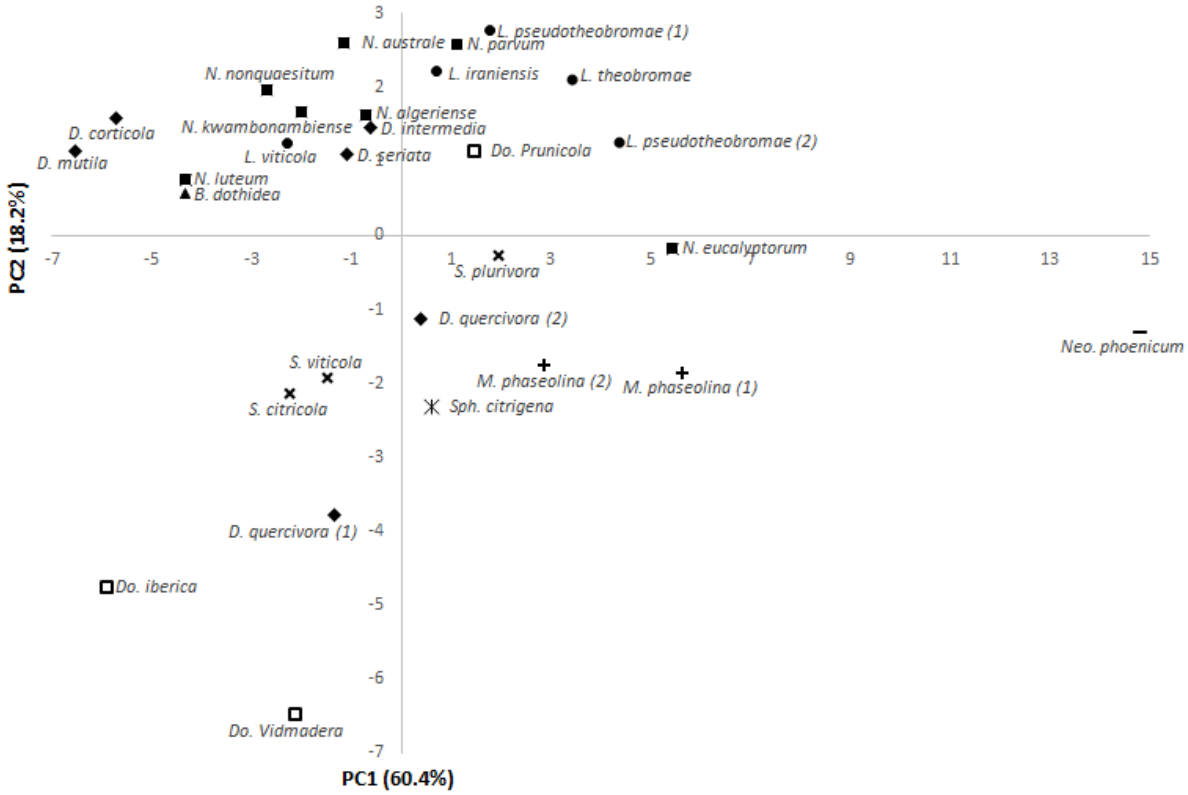

Figure 3.2: Scores scatter plot of the principal component analysis (PC1 vs PC2) of the different fungal strains analysed in the region between 3000-2800 and 1800 and 980 cm^{-1}.

Since the infrared spectra are very complex data, it is necessary to perform multivariate statistical analyses of the data in order to extract more information. Principal component analysis is probably the most widespread chemometric technique and normally the first step in data exploration, as it allows to explore patterns, even in a very complex dataset such as spectra (Brereton 2003). So, in order to assess if spectra of different species from each genus grouped together, a PCA of the spectra was performed (Figure 3.2). As it is reported in other studies on fungal identification with infrared spectroscopy (Fischer et al. 2006; Salman et al. 2010), a clear grouping tendency of the species by their genera is not verified. However, all species are differentiated in the analysis. *Neodeightonia phoenicum,* as it was expected, is separated from the other fungi, as their spectra were more influenced by water signals.

Observing figure 3.3, it is possible to notice that the peaks that most contributed to the distribution observed in figure 3.2 were observed at 2923 cm^{-1} associated to CH_3, 2853 cm^{-1} associated to CH_2 and 1744 cm^{-1} associated to esters from lipids. Peaks from carbohydrates and phosphates region are also important (1200-980 cm^{-1}), as well as peaks from the protein region (1613 cm^{-1}) in some of the cases. This suggests that the various fungi cell walls differ in the composition of these cellular components.

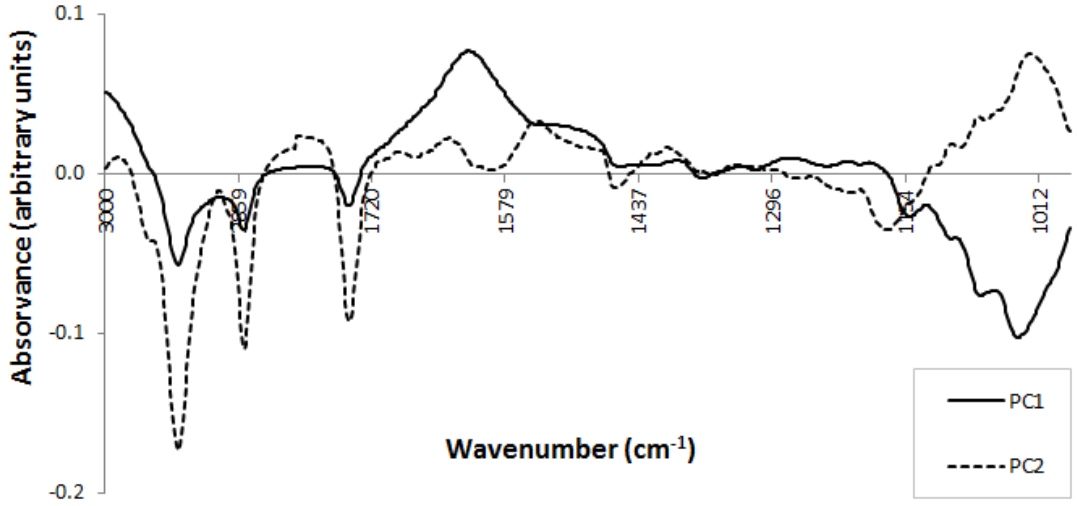

Figure 3.3: Loadings plot profile of the principal component analysis (PC1 vs PC2) of the different fungal strains analysed in the region between 3000-2800 and 1800 and 980 cm^{-1}.

In order to discriminate the different species, a hierarchical cluster analysis (HCA) was performed (Figure 3.4). This analysis aims to classify objects, using different algorithms to calculate the similarities between them, based on a distance matrix, performing their distribution into distinct groups. We observed that species belonging to the same genera were not placed together in the same cluster but it was possible to differentiate species and strains. However, it can be noticed that the two different strains of *Macrophomina phaseolina* were placed together, being impossible, in this case, a discrimination between strains. In a previously reported study, Fischer et al. (2006) found that fungi of different genera were mixed in different clusters, but a

distinction between the different species was successfully accomplished, however, they were unable to discriminate different strains of the same species. Curiously, in our study, the 2 strains of *Diplodia quercivora* and *Lasiodiplodia theobromae* were located in different clusters, thus allowing a distinction at the strain level. This can be due, for example, to the production of secondary metabolites that can differ between strains and influence the cluster distribution (Fischer et al. 2006) or to small differences in the structure of the cell wall (Oberle et al. 2015), as MIR is very sensitive to slight differences in the composition of the samples. Considering that IR spectroscopy is not based on phylogenetic data, as the spectra reflect the macromolecular composition of the fungal surface rather than genetic relatedness, this distribution is not unexpected. A similar behaviour was verified in PCR fingerprinting of Botryosphaeriaceae, also a non-phylogenetic technique, which revealed no relationships at the genus level, being the species of the same genus grouped in different clusters (Alves et al. 2007).

Fungal identification may be a difficult task with frequent revisions of the taxonomic schemes, making it an extensive and complex process. Additionally, it is gradually becoming clearer that fungal identification and authentication requires a polyphasic approach to generate quality data (Santos et al. 2010).

DNA sequencing of the ITS region frequently in combination with other loci is becoming the standard method for fungal identification. For the Botryosphaeriaceae specifically it is currently almost impossible to reliably identify species without the use of multi loci sequence data (Phillips et al. 2013). Other molecular methodologies such as ARDRA (Alves et al. 2005), rep–PCR (Alves et al. 2007), micro satellite loci (de Wet et al. 2003), PCR-RFLPs (Dreaden et al. 2014), species specific primers (Luchi et al. 2005), ISSRs (Zhou et al. 2001) have been successfully used to detect, discriminate and identify Botryosphaeriaceae species. However, these techniques are generally quite expensive, complex and time-consuming. Alternative techniques are needed in order to overcome these limitations, and to quickly, efficiently and accurately identify species thus facilitating diagnosis and disease management.

MIR spectroscopy technique has demonstrated its potential to be applied to identification, characterization and authentication of several filamentous fungi (Fischer et al. 2006; Garon et al. 2010; Lecellier et al. 2014, 2015; Naumann et al. 2005; Tralamazza et al. 2013). The advantages of this approach as a microbial identification method are the rapid and simple sample preparation procedure, the short time of analysis and the reliability of the data. Furthermore, the MIR spectrometer is not expensive and does not require any reagents. From a statistical point of view, the construction of a reference spectrum library is crucial for accurate microbial identification and it should be assembled based on well-characterized strains and species. An unidentified isolate analysed under the same conditions as those of the reference spectra, should be comparable with the reference spectrum library. If the library contains an identical or a very similar spectrum, identification is possible. The success of the method is therefore directly dependent on the size and

comprehensiveness of the reference library spectra (Santos et al. 2010). It is very important to keep in mind that MIR spectra are influenced by variation of plating methods, growth temperature, incubation time and the drying method of the microorganism suspension located on the sample holder. So, the standardization of culture conditions, sample preparation and spectral acquisition parameters (number of scans and spectral resolution) is a critical point for achieving reproducibility of spectral data acquisition, being crucial to avoid misidentifications (Kummerle et al. 1998).

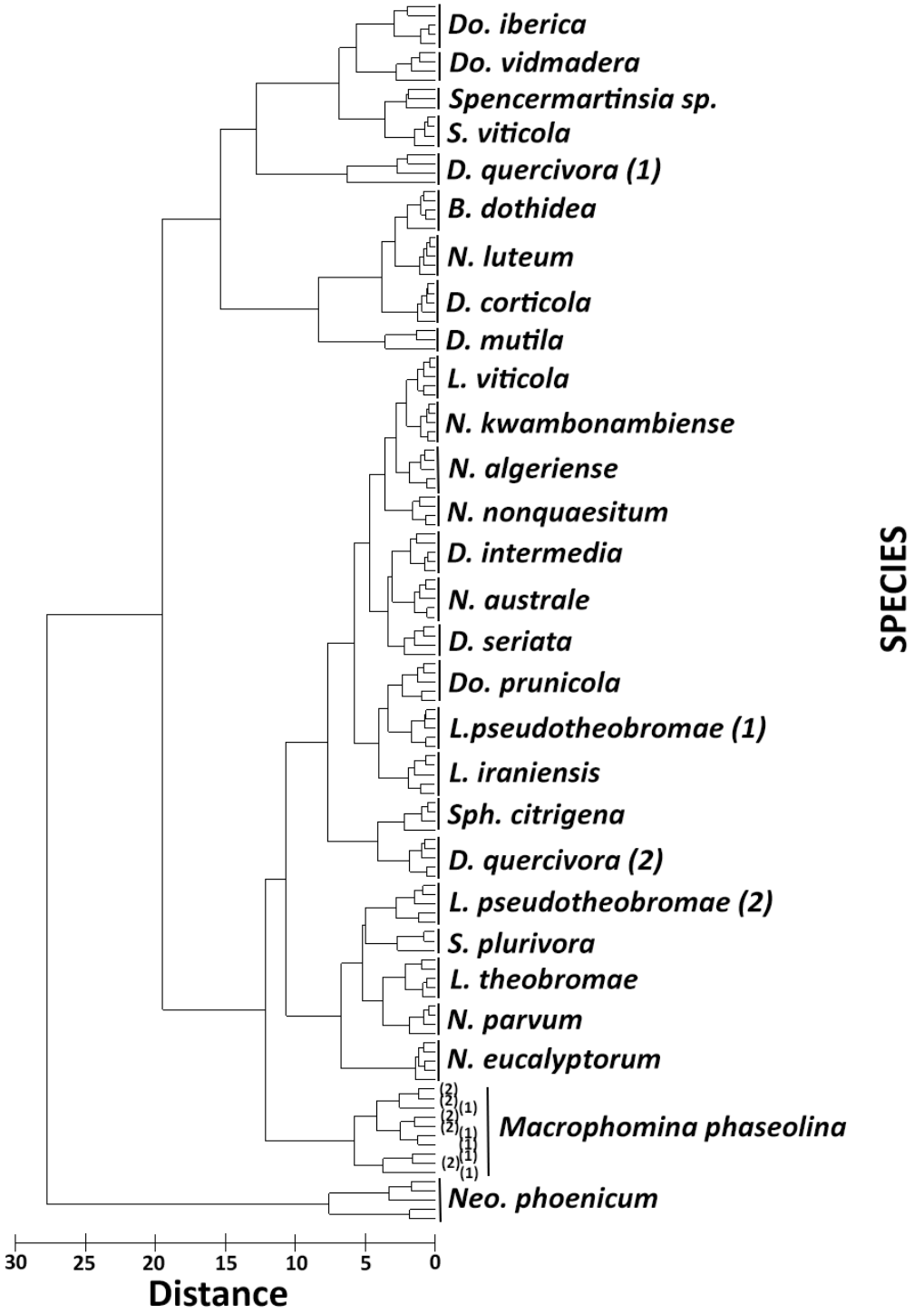

Figure 3.4: HCA of spectra obtained from fungal mycelium in the spectral region between 3000-2800 and 1800 and 980 cm^{-1}.

In the case of previous studies using MIR to analyse fungi, the protocols used tend to be complex and not applicable to all cases. Lecellier et al. (2015) constructed a library with 140 species of filamentous fungi (43 different genera) with 80.8% of the strains correctly assigned. In this work, all the cultures were obtained from spores inoculated in YM broth and then dissociated and washed prior to MIR analysis. This method, however, is time and reagent consuming and depends on the ability of the fungi to sporulate, which is difficult in the case of some Botryosphaeriaceae species. Species in this group tend to not sporulate easily and eventually lose the ability to sporulate when maintained in culture for long periods. In our work, we propose a rapid protocol in which the sample preparation was much simpler than in other previous reported studies, as we directly collected the mycelium from the petri plates and dried it for a few seconds. Additionally, we successfully discriminated all the species and, furthermore, we discriminated different strains of *D. quercivora* and *L. pseudotheobromae*, being these results very promising, taking into account other reports using MIR to identify fungal species, in which complex procedures to prepare the samples were used (Ergin et al. 2013, 2016; Fischer et al. 2006; Lecellier et al. 2015) or a smaller number of species were discriminated (Ergin et al. 2013; Salman et al. 2010).

In our study, MIR spectroscopy showed a great potential to be a powerful, cost-effective, fast and high-throughput alternative technique for identification of species of fungi from the family Botryosphaeriaceae. Taking into account the promising results, future studies including all currently known species and more isolates should be carried out in order to fully validate this technique. In this way, a robust and more detailed library can be constructed, thus increasing the probability to correctly identify an unknown sample. In future studies, it would be also interesting to develop MIR microspectroscopy in order to detect species of Botryosphaeriaceae directly in infected plant material.

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CHAPTER 4

Effects of *Botryosphaeria*, *Diplodia* and *Neofusicoccum* species on two *Eucalyptus* species and one hybrid: from pathogenicity to physiological performance

Effects of *Botryosphaeria*, *Diplodia* and *Neofusicoccum* species on two *Eucalyptus* species and one hybrid: from pathogenicity to physiological performance

Abstract

Several Botryosphaeriaceae species are important endophytes and latent pathogens of *Eucalyptus* spp. in their native and non-native ranges. Recently, nine species of Botryosphaeriaceae were identified on eucalypts plantations in Portugal, however, pathogenicity tests were only carried out with one clone of *E. globulus*. Knowledge about differences in tolerance among different *Eucalyptus* species and hybrids is needed in order to develop suitable control and management strategies for plantations.

The aims of this study were to assess the pathogenicity of nine Botryosphaeriaceae species towards three different eucalypts as well as to evaluate the differences in plant susceptibility to disease.

Obvious differences in fungal aggressiveness were found, in which *N. kwambonambiense* and *D. corticola* were the most aggressive species while *B. dothidea* and *D. seriata* were the less aggressive ones. Further, hybrid plants were the ones with smaller lesions. Nevertheless, no direct relation between fungal aggressiveness and plant physiological responses was found. *Eucalyptus nitens* plants were the unique that did not present modifications on the morpho-physiological profile when infected, however presented severe symptoms diseases. Taking all the results into account, hybrid plants could be a good alternative for Portuguese plantations considering Botryosphaeriaceae diseases control.

Keywords: eucalypts, Botryosphaeriaceae, pathogenicity, aggressiveness, plant physiology

Introduction

Eucalyptus species and hybrid clones are among the most exploited forest species across the world, mainly because of their valuable wood and fibre properties for the pulp and paper industry (Booth 2013; Wingfield et al. 2015). Nevertheless, its cultivation range is limited by climate conditions, particularly water availability and low temperatures. These limitations have led forest breeding managers to explore *Eucalyptus* physiological plasticity in order to enhance adaptation and increase productivity and cultivation range in different regions of the world (Keskitalo 2011; White et al. 2014). *Eucalyptus globulus* Labill. (blue gum tree), which is well adapted to Mediterranean climate (Granda et al. 2014), is the dominant species in plantations in Portugal (Bragança et al. 2015). However, this species is sensitive to low temperatures

and freezing from colder regions can cause serious damage to these plants (Scarascia-Mugnozza et al. 1989). In these areas, species or hybrids that are more tolerant to freezing could be an important alternative to *E. globulus*. It is the case of *E. nitens* (Maiden), a frost-tolerant species (Navarrete-Campos et al. 2012), with the additional benefit of being less susceptible to *Gonipterus platensis*. The use of hybrids in Mediterranean forest plantations is still not as common as in other countries (e.g. Brazil), but it represents a suitable alternative as many hybrid have significant advantages in growth and tolerance to pests and diseases, and also exhibit broad environmental plasticity (Cid et al. 1999).

Botryosphaeriaceae species have been associated with native and non-native *Eucalyptus* plantations (Chen et al. 2011; Mohali et al. 2009; Rodas et al. 2009; Smith et al. 1994). In early studies, *Botryosphaeria dothidea* and *Neofusicoccum ribis* were the most often reported species in eucalypts growing in temperate areas (Chen et al. 2011; Slippers et al. 2009), but it is now known that these species are uncommon in eucalypts. According to recent studies, at least 29 species, belonging to 8 different genera, have been confirmed as associated with these hosts (Barradas et al. 2016; Phillips et al. 2013; Pillay et al. 2013). Surveillance studies and pathogenicity tests have associated Botryosphaeriaceae species with several symptoms observed in eucalypts, mainly cankers on the stems followed by kino exudation, dieback of shoots and branches, coppice failure and, in more severe cases, host death (Gezahgne et al. 2004; Mohali et al. 2009; Pavlic et al. 2007; Pérez et al. 2009, 2010; Rodas et al. 2009; Roux et al. 2000, 2001; Smith et al. 1994). However, these reports focused on fungal aggressiveness and physiological status of the infected plants remains virtually unstudied. The morpho-physiological parameters commonly used to assess plant physiological performance include growth, chlorophyll a fluorescence, leaf gas-exchange, water content, water potential and photosynthetic pigments content, among others (Alves et al. 2011; Granda et al. 2014; White et al. 1996). They represent good indicators of primary metabolism and plant defence mechanisms (Berger et al. 2007; Correia et al. 2014) and their integration in pathogenicity studies is recommended.

Considering pathogenicity interpretation, plant phenotypic and genotypic plasticity is also a very relevant factor that is often ignored. In fact, it is recognized that the host-pathogen interaction depends on genetic variation from both sides (Rowntree et al. 2011). With respect to this, there is a need to investigate the response of different forest reproductive material used in eucalypt plantation to the infection with Botryosphaeriaceae species, as well as putative differences in tolerance/susceptibility (Barradas et al. 2016; Mohali et al. 2009). Therefore, the aim of the present study is to compare the pathogenicity of *Botryosphaeria*, *Diplodia* and *Neofusicoccum* species towards two different *Eucalyptus* species (*E. globulus* and *E. nitens*) and one hybrid (*E. globulus* x *E. cypellocarpa*) in order to assess their role in tolerance/susceptibility to diseases. By including a physiological approach, we aimed to contribute to fulfill the lack of knowledge regarding plant responses under pathogen attack.

Materials and Methods

Plant material

Pathogenicity of fungal isolates was tested towards different *Eucalyptus* plants stock nursery material currently used in Portuguese plantations and known for presenting different field behaviour. *E. globulus* (clone AL-18) was selected from an open pollination family and it was first tested in the early nineties with very good survival results in drought prone areas. It presents a reduced susceptibility to *Mycosphaerella* diseases but it is highly susceptible to *Gonipterus* sp., a major pest problem in eucalypt plantations. The hybrid *E. globulus* x *E. cypellocarpa* (clone YG-15) is putatively drought resistant but field trials are still ongoing. *Eucalyptus nitens* represents plants obtained from seed, and according to field trials, this species is more suitable to high altitude plantations due to their frost-tolerance. Also, these plants are less susceptible to *Gonipterus* sp.. All plants and information were supplied by Altri Florestal SA (Portugal).

Six months-old plants were transplanted to 2 L plastic pots filled with 3:2 (w/w) peat:perlite and acclimated for a month in a greenhouse with daily watering until artificial inoculation.

Fungal culture and plant inoculation

Fungal isolates used in the present work were obtained both from asymptomatic and symptomatic *E. globulus* plants (Table 4.1). The samples were collected between 2012 and 2013 and identified in a previous work (Barradas et al. 2016). The cultures were maintained in the culture collection of Artur Alves (CAA), Universidade de Aveiro, Portugal on Potato Dextrose Agar (PDA, Merck, Germany) at 4°C.

Table 4.1: Fungal isolates used in pathogenicity tests.

Isolate	Local	Colector	Symptom	Tissue	Species
CAA642	Cepões, Viseu	C. Barradas	Asymptomatic	Twigs	*B. dothidea*
CAA500	Alcochete, Sesimbra	L. Neves	Disease	Stem	*D. cortícola*
CAA318	G. Encarnação, Aveiro	A. Alves	Disease	Twigs	*D. seriata*
CAA366	Anadia, Aveiro	L. Neves	Disease	Stem	*N. algeriense*
CAA434	G. Encarnação, Aveiro	A. Alves	Disease	Twigs	*N. australe*
CAA558	Aveiro, Aveiro	A. Alves	Disease	Twigs	*N. eucalyptorum*
CAA755	Aveiro, Aveiro	C. Barradas	Disease	Twigs	*N. kwambonambiense*
CAA704	Aradas, Aveiro	C. Barradas	Asymptomatic	Roots	*N. parvum*
CAA692	Bombarral, Leiria	C. Barradas	Disease	Twigs	*Neofusicoccum* sp.

Plant inoculation was initiated by surface disinfection of the stem to be wounded with 70% ethanol. A shallow wound was made on the stem base (5 cm above soil) using a sterile scalpel in order to remove the bark and expose the cambium. Mycelial plugs (5 mm diameter) from the active margin of 1-week-old pure cultures, on PDA at 25°C, were placed into the wound with the mycelial surface facing the cambium. The inoculation sites were then sealed with Parafilm® (Pechiney Plastic Packaging Company, Chicago,

USA) to prevent desiccation of the plug. Plugs of sterile PDA were similarly applied into stems of control plants.

Trial conditions and monitoring of the infection

The trial was carried out for two months under greenhouse environmental conditions between June and July of 2013. Six plant replicates were randomly assigned to each treatment. Inoculated and control plants were watered every day and fertilized once per week with 100 mL of a solution containing 5ml/L NPK (5-8-10).

Evolution of visual external symptoms (stem lesions, foliar chlorosis and wilting) and presence of fungal reproductive structures were periodically checked.

Length of lesions

At the end of the experiment, two months after inoculation, external lesion was measured, plant bark was then removed to expose internal lesion and length of this lesion was also recorded. Afterwards, fungi were re-isolated on PDA to verify Koch's postulates. Thus, pieces of wood from the edges of the lesions were immersed in sodium hypochlorite for 2 min, then immersed in 70% ethanol for 1 min, and finally rinsed in sterile distilled water and blotted dry on sterile filter paper. Disinfested plant tissue was placed on PDA and incubated at room temperature (~20°C) for a week. Fungal identification was based on colony and conidial morphology.

Evaluation of plant morpho-physiological performance

At the end of the experiment, morphological and physiological parameters were recorded. Homogenous fully developed leaves from the same node were randomly selected from each plant to measure chlorophyll a fluorescence and leaf gas-exchange related parameters. The same and nearby leaves were then harvested (four leaves in total) to determine relative water content (RWC), or immediately frozen in liquid nitrogen and stored at -80ºC to quantify total soluble sugars (TSS) and photosynthetic pigments (total chlorophyll and carotenoids). Water potential and growth were also estimated. Six plants were analysed for each treatment for each stock plant material.

Growth and water status

Growth was determined as the difference between the final and initial height (inoculation day) that were measured in each plant.

For leaf relative water content (RWC), tissue fresh weight (FW) was recorded for 4 leaf discs (diameter = 11 mm) of selected leaves that were transferred to tubes with de-ionized water and maintained overnight in dark at 4°C. After 24 h and carefully removing the excess of water from leaf surface, turgid weight (TW) was registered and leaves were dried at 80°C until constant weight. Dry leaf samples were weighed for dry

weight (DW) obtaining. RWC was calculated using the following equation: RWC (%) = (FW – DW) / (TW – DW)*100.

Midday shoot water potential (ψ_{md}) measurements were carried out in all plants using a Scholander-type pressure chamber (PMS Instrument Co., Corvallis, OR) as described by (Correia et al. 2014).

Leaf gas-exchange measurements

Stomatal conductance (gs, mol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and net CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1}) were measured in all plants using a portable infrared gas analyser (LCpro-SD, ADC BioScientific Ltd., UK) equipped with the broad photosynthesis chamber. The following conditions were maintained inside the chamber during all the measurements: ambient CO_2 concentration: 370-400 µlL^{-1}; air flux: 500 µmol s^{-1}; block temperature: 25°C; relative humidity of the incoming air: 35-50 %. To find out the saturation light intensity A/PPFD (photosynthetic photon flux density; light response curves of CO_2 assimilation) curves were performed with the following PPFD: 2500, 2000, 1500, 1000, 750, 500, 250, 100, 50 and 0 µmol m^{-2} s^{-1}. After A/PPFD data analysis, punctual measurements at saturation light intensity were performed at 1000 µmol m^{-2} s^{-1}. Data were recorded when the measured parameters were stable (2–6 min).

Photosynthetic pigments and chlorophyll *a* fluorescence analysis

Steady-state modulated chlorophyll fluorescence was determined with a portable fluorometer (Mini-PAM; Walz, Effeltrich, Germany) on the same leaves as used for the gas-exchange measurement according to Correia et al. (2014). Light-adapted components of chlorophyll fluorescence were measured: steady-state fluorescence (F), maximal fluorescence (F'm), variable fluorescence F'v (equivalent to F'm – F) and quantum yield of photosystem II (PSII) equivalent to F'v / F'm. After leaves were dark adapted (20 min at least), the following values were obtained: F0 (minimum fluorescence), Fm (maximum fluorescence), Fv (variable fluorescence, equivalent to Fm – F0) and Fv／Fm (maximum quantum yield of PSII).

Total chlorophyll and carotenoid content was determined following the method described by Sims & Gamon (2002). Acetone/Tris (50 mM) buffer at pH 7.8 (80:20) (v/v) was used to extract pigments from leaves. Samples were twice homogenized and centrifuged for 5 min at 10.000×g at 4°C. The supernatants were transferred to a new tube coated by aluminum foil and the absorbances at 663 nm, 537 nm, 647 nm and 470 nm were determined (Thermo Fisher Scientific spectrophotometer, Genesys 10-uv S).

Total soluble sugars (TSS) content

Total soluble sugars (TSS) were determined using the anthrone method as described by Irigoyen et al. (1992). Frozen leaves were ground with 80% (v/v) ethanol and incubated at 80°C for 1 h. Samples were centrifuged for 10 min at 10.000×g at 4°C

and 1.5 ml of anthrone was added to the supernatant before incubation (100°C, 10 min). Absorbance was read at 625 nm and TSS content was calculated against a D-glucose standard curve.

Statistical analyses

Prior to analysis, data was transformed (Table 4.2) to correct non-normality and heteroscedasticity. Two-way ANOVA (analysis of variance) was computed for all results. This analysis enabled us to verify the effect of the fungi and plant group, as well as the interaction between both factors. In cases that plant response was dependent on the plant group, we decided to analyse it separately, and one-way ANOVA was performed to evaluate the fungal effect in each plant group. When applicable, Tukey multiple comparison tests were employed to identify significant differences between fungi. A significance level of 0.05 was considered for all analysis.

Principal components analysis (PCA) was carried out to assess the morpho-physiological profiles of plants (fungi x plant group) by reducing the multivariate data matrix to an interpretable bidimensional plot that explains the highest proportion of variation of the data (ter Braak and Verdonschot 1995). This technique enabled us to get a global overview of the effect of the fungi among different plant group. Data were centred and standardized to reduce scale effects. One-way ANOVA of the principal components (PC1 and PC2) was used to found out which fungi produced significant differences in morpho-physiological profiles when compared with control. Statistical analyses were performed on software Minitab® 17.1.0.

Results

Monitoring of the infection

The evolution of the infection was monitored during the experiment, checking visual external symptoms (stem lesions, foliar chlorosis and wilting) and presence of fungal reproductive structures. Besides chlorosis that appeared occasionally in all plant groups (including control plants) without any relevant patterns, we noticed more severe infection symptoms on *E. nitens* inoculated with *N. kwambonambiense*. In fact, in these plants foliar chlorosis, dead leaves and branches progressed very rapidly and half of the plants died. Moreover, absence of fungal reproductive structures was observed for all fungal species.

At the end of the experiment, all inoculated plants presented lesions around the inoculation sites, while no lesions were observed in control plants. All the fungal species inoculated were successfully re-isolated and none were found in controls, which means Koch's postulates were successfully verified.

Length of lesions

In relation to lengths of lesions, significant differences were found among the 8 fungal isolates, and those differences varied according to the plant group (Table 4.2, Figure 4.1).

Table 4.2: One-way ANOVA summary table for morpho-physiological parameters obtained to three commercial stock material of *Eucalyptus* plants (*E. globulus*, hybrid and *E. nitens*). *F* value *p*-value are shown for source of variation (fungal infection). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, NS, non-significant.

Parameter	*E. globulus*			Hybrid			*E. nitens*		
	F	***p***	**Sig.**	***F***	***p***	**Sig.**	***F***	***p***	**Sig.**
External Lesion ln(x+1)	50.54	0.000	***	11.37	0.000	***	7.55	0.000	***
Internal Lesion ln(x+1)	28.57	0.000	***	19.07	0.000	***	7.65	0.000	***
Growth	3.12	0.004	**	10.23	0.000	***	1.56	0.154	NS
RWC	8.75	0.000	***	2.68	0.016	*	2.60	0.019	*
Ψmd	12.60	0.000	***	4.59	0.000	***	3.10	0.005	**
A	14.15	0.000	***	5.04	0.000	***	0.27	0.980	NS
E	38.36	0.000	***	1.11	0.371	NS	0.86	0.570	NS
gs	23.93	0.000	***	12.36	0.000	***	1.44	0.198	NS
Ci	19.24	0.000	***	25.22	0.000	***	0.53	0.844	NS
ϕ_{PSII}	0.95	0.489	NS	2.43	0.022	*	1.84	0.086	NS
Fv/Fm	4.67	0.000	***	5.27	0.000	***	0.78	0.637	NS
T. chlorophyll	1.96	0.061	NS	18.09	0.000	***	4.04	0.001	NS
Carotenoids	6.52	0.000	***	17.00	0.000	***	6.05	0.000	***
TSS	19.83	0.000	***	6.22	0.000	***	12.54	0.000	***

External lesions were larger in *E. globulus* and smaller in hybrid plants (Table 4.2, Figure 4.1). In *E. globulus* plants, *N. kwambonambiense* produced significantly larger external lesions followed by *N. australe, N. parvum,* and *D. corticola*. The species *N. kwambonambiense* and *D. corticola* also produced significantly larger external lesions in hybrid plants. In *E. nitens* plants, *N. kwambonambiense,* and *N. australe* were the species that caused larger external lesions.

Internal lesions were mostly related to the external lesions (Table 4.2, Figure 4.1). *Eucalyptus globulus* plants showed, in general, larger internal lesions than the hybrid. *Eucalyptus nitens* plants presented a great variability between isolates, as well as the biggest standard deviation (SD) (Table 4.2, Figure 4.1). In *E. globulus*, opposite to the observed in external lesions, *D. corticola* produced large internal lesions, comparable to the effect of *N. kwambonambiense*.

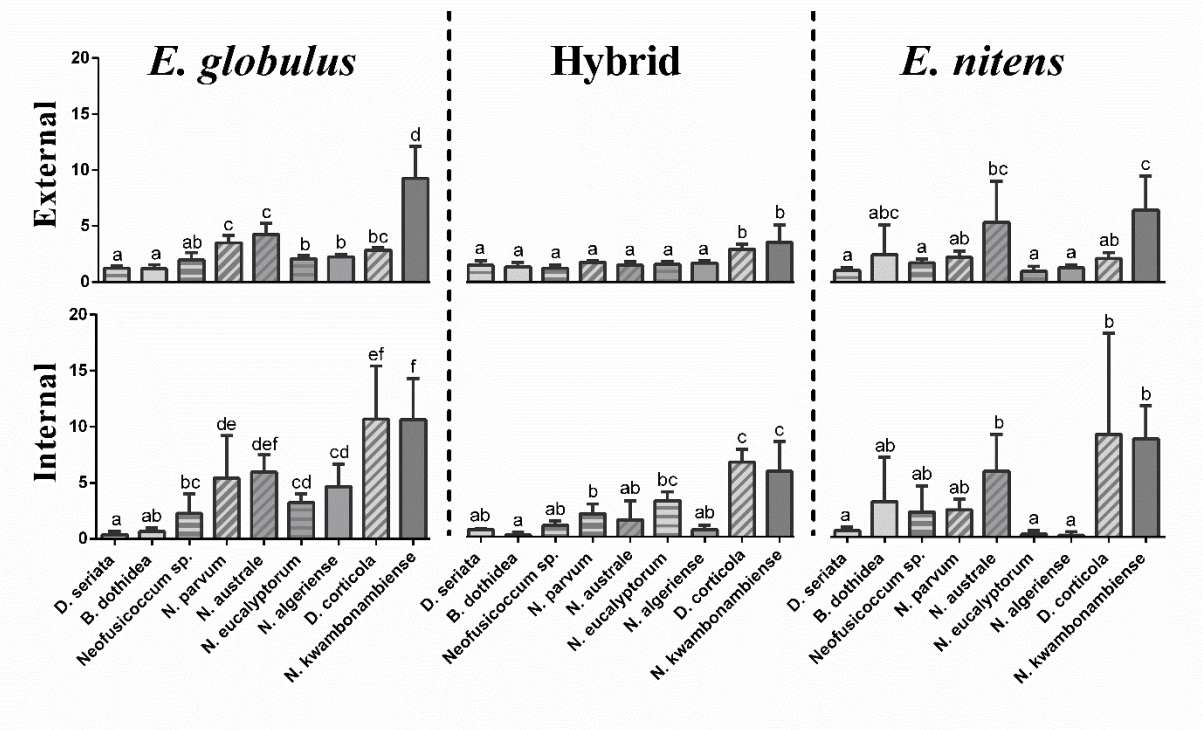

Figure 4.1: Internal and external lesions measured to three commercial stock material of *Eucalyptus* plants (*E. globulus*, hybrid and *E. nitens*) inoculated with different fugal species (*B. dothidea*, *D. corticola*, *D. seriata*, *N. algeriense*, *N. australe*, *N. eucalyptorum*, *N. kwambonambiense*, *N. parvum*, *Neofusicoccum* sp.) after two month of inoculation. Data are presented as mean±SD and different letters indicate significant differences between fungal species ($p \leq 0.05$).

Evaluation of plant morpho-physiological performance

Growth and plant water status

Considering plant growth of *E. globulus*, plants inoculated with *D. seriata* and *N. eucalyptorum* grew significantly more than control plants (Table 4.2, Figure 4.2). Hybrid plants inoculated with *D. corticola*, *D. seriata,* and *N. algeriense* also displayed higher growth. On the other hand, no significant differences were observed in *E. nitens* plants (Table 4.2, Figure 4.2).

Relative water content (RWC) significantly increased in *E. globulus* inoculated with *D. seriata* compared to control (Table 4.2, Figure 4.2); no significant differences were found in inoculated hybrid plants. *Eucalyptus nitens* plants inoculated with *D. corticola* presented lower values for this parameter.

Regarding midday shoot water potential (ψ_{md}), *E. globulus* plants presented significantly lower values when inoculated with *N. kwambonambiense* (Table 4.2, Figure 4.2); while *B. dothidea, D. seriata* and *N. eucalyptorum* showed higher values compared to control. In the hybrid plants, only those inoculated with *N. kwambonambiense* and *Neofusicoccum* sp. showed a significantly higher value. On the other hand, *E. nitens* plants inoculated with *B. dothidea* and *N. parvum* showed higher water potential.

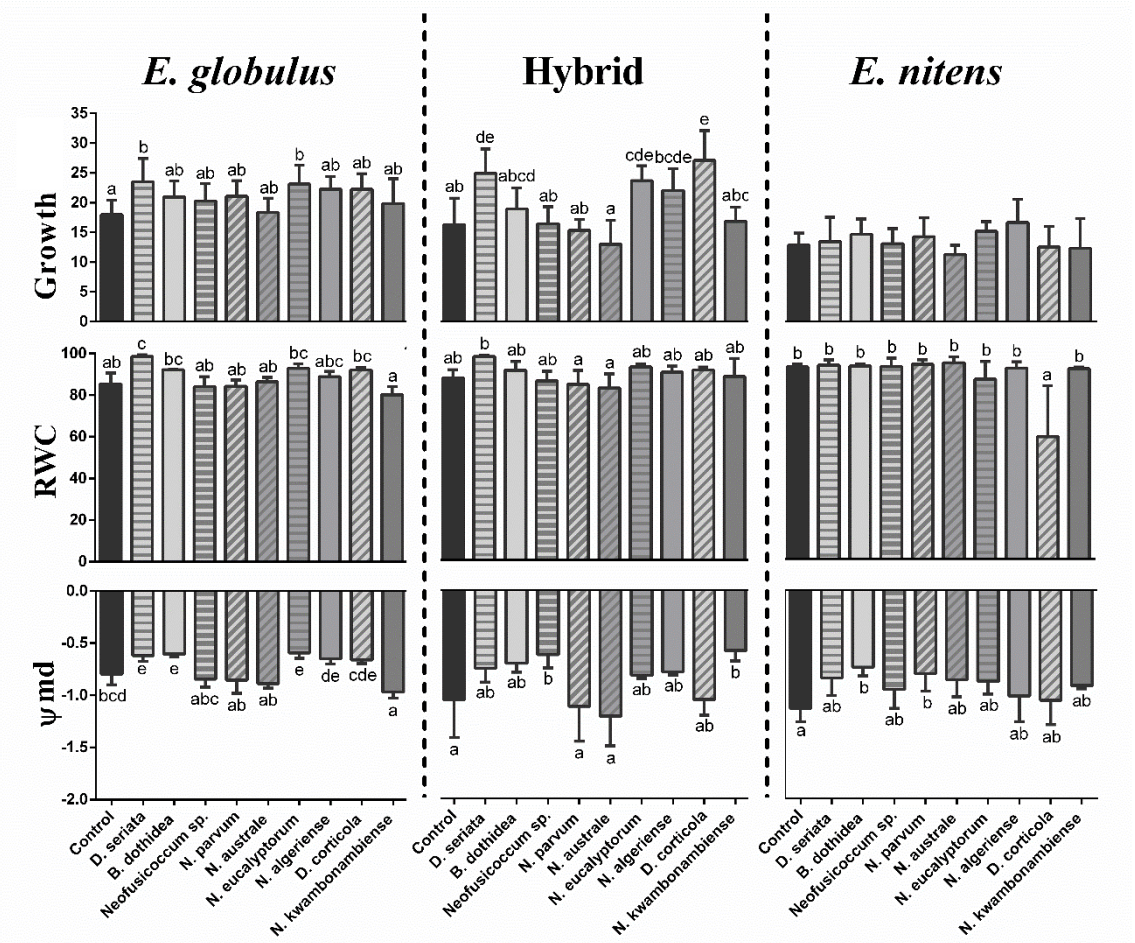

Figure 4.2: Growth (cm), relative water content (RWC, %) and midday shoot water potential (ψmd, MPa) measured to three commercial stock material of *Eucalyptus* plants (*E. globulus*, hybrid and *E. nitens*) inoculated with different fugal species (*B. dothidea*, *D. corticola*, *D. seriata*, *N. algeriense*, *N. australe*, *N. eucalyptorum*, *N. kwambonambiense*, *N. parvum*, *Neofusicoccum* sp.) after two month of inoculation. Data are presented as mean±SD and different letters indicate significant differences between fungal species ($p \leq 0.05$).

Leaf gas-exchange measurements

The leaf gas-exchange measurements showed significant differences in infected *E. globulus* and hybrid plants, while *E. nitens* did not present any significant differences (Table 4.2, Figure 4.3).

Regarding *E. globulus*, plants inoculated with *B. dothidea*, *D. corticola*, *D. seriata*, *N. algeriense* and *N. eucalyptorum* presented a significantly lower net CO_2 assimilation rate (A) compared to control, whereas *D. corticola*, *D. seriata*, *E. algeriense* and *N. eucalyptorum* showed generally higher transpiration rate (E) and stomatal conductance (gs). Hybrid plants inoculated with *B. dothidea, D. corticola, D. seriata*, *N. algeriense* and *Neofusicoccum* sp. also showed significant lower A values than control. Transpiration rate (E) was not affected and only *B. dothidea* and *N. algeriense* showed significantly higher gs values than control.

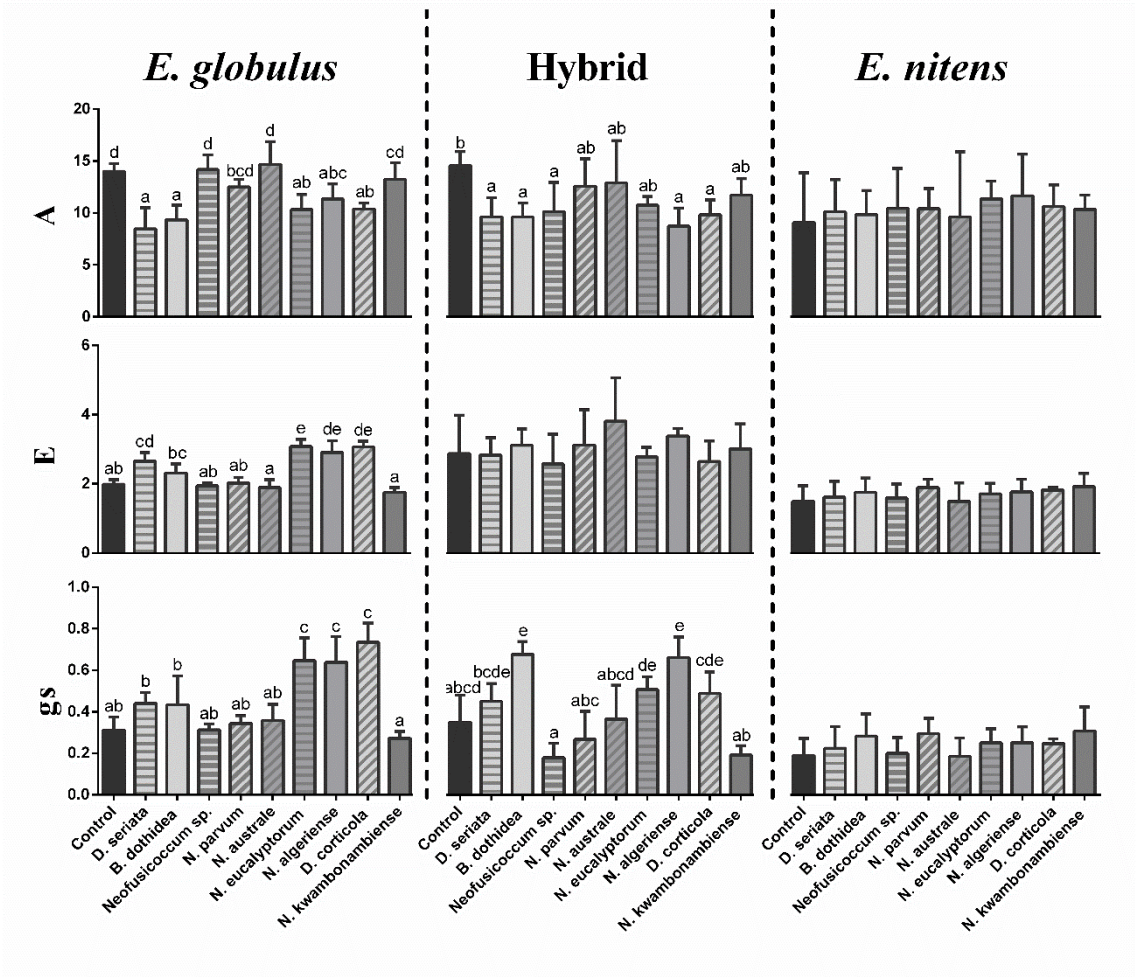

Figure 4.3: Leaf gas-exchange (CO_2 assimilation rate (A, µmol CO_2 m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and Stomatal conductance (gs, mol H_2O m^{-2} s^{-1})) in leaves measured to three commercial stock material of *Eucalyptus* plants (*E. globulus*, hybrid and *E. nitens*) inoculated with different fugal species (*B. dothidea*, *D. corticola*, *D. seriata*, *N. algeriense*, *N. australe*, *N. eucalyptorum*, *N. kwambonambiense*, *N. parvum*, *Neofusicoccum* sp.) after two month of inoculation. Data are presented as mean±SD and different letters indicate significant differences between fungal species ($p \leq 0.05$).

Photosynthetic pigments and chlorophyll *a* fluorescence analysis

Considering photosystem II photochemistry, only small differences were found among the inoculated plants but no differences were detected in relation to control (Table 4.2, Figure 4.4). *Eucalyptus globulus* plants did not present significant differences in the total chlorophylls content, while plants inoculated with *D. corticola, D. seriata, N. algeriense* and *N. eucalyptorum* showed significant lower values in the carotenoid content in relation to control plants. Relatively to hybrid plants inoculated with *B. dothidea*, *D. corticola*, *D. seriata*, *N. algeriense, N. australe and N. eucalyptorum* total chlorophylls content increased in relation to non-inoculated ones (Table 4.2, Figure 4.5). Moreover, the inoculation with *B. dothidea, D. seriata, N. algeriense* and *N. eucalyptorum* also led to lower carotenoids, while *N. australe* induced a greater

accumulation. Finally, *E. nitens* plants inoculated with *N. parvum* and *Neofusicoccum* sp. presented significant higher values in the total chlorophylls and carotenoid analysis compared to control plants.

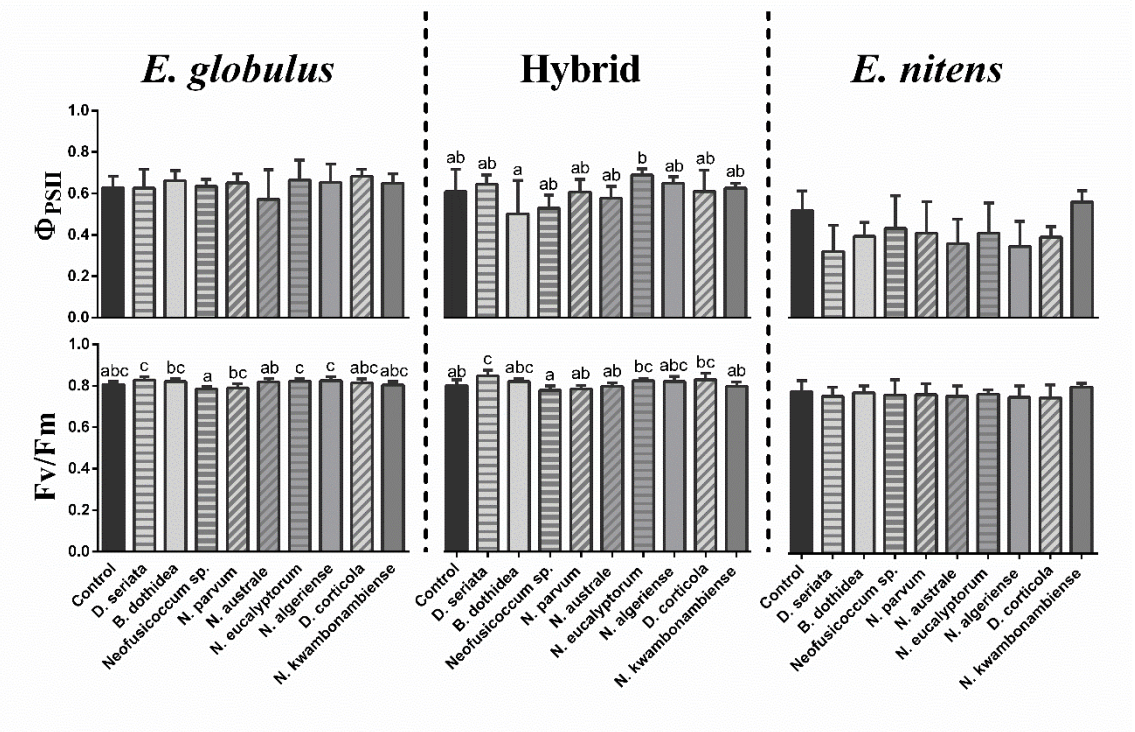

Figure 4.4: ϕ_{PSII} and Fv/Fm in leaves measured to three commercial stock material of *Eucalyptus* plants (*E. globulus*, hybrid and *E. nitens*) inoculated with different fugal species (*B. dothidea*, *D. corticola*, *D. seriata*, *N. algeriense*, *N. australe*, *N. eucalyptorum*, *N. kwambonambiense*, *N. parvum*, *Neofusicoccum* sp.) after two month of inoculation. Data are presented as mean±SD and different letters indicate significant differences between fungal species ($p \leq 0.05$).

Total soluble sugars (TSS) content

Regarding TSS quantification, *E. globulus* plants inoculated with *B. dothidea, D. corticola* and *N. algeriense* significantly increased their value compared to control (Table 4.2, Figure 4.5). Hybrid plants inoculated with *D. seriata* showed lower TSS content. Considering *E. nitens* plants, TSS significantly increased in plants inoculated with *D. corticola* and decreased after *B. dothidea* and *N. kwambonambiense* inoculation.

Multivariate approach of physiological profile

Principal components analysis (PCA) provided a global overview of the morpho-physiological plant status when inoculated with fungi. A clear separation between *E. nitens* plants and the other ones was verified (Figure 4.6). Sample scores of *E. nitens* were all located on the negative PC1 while almost of the sample scores of *E. globulus* and hybrid plants were located on positive PC1. This distribution was related to lower values of growth, gs, Fv/Fm, E and ϕ_{PSII} observed on *E. nitens* plants, compared to *E. globulus* and hybrid.

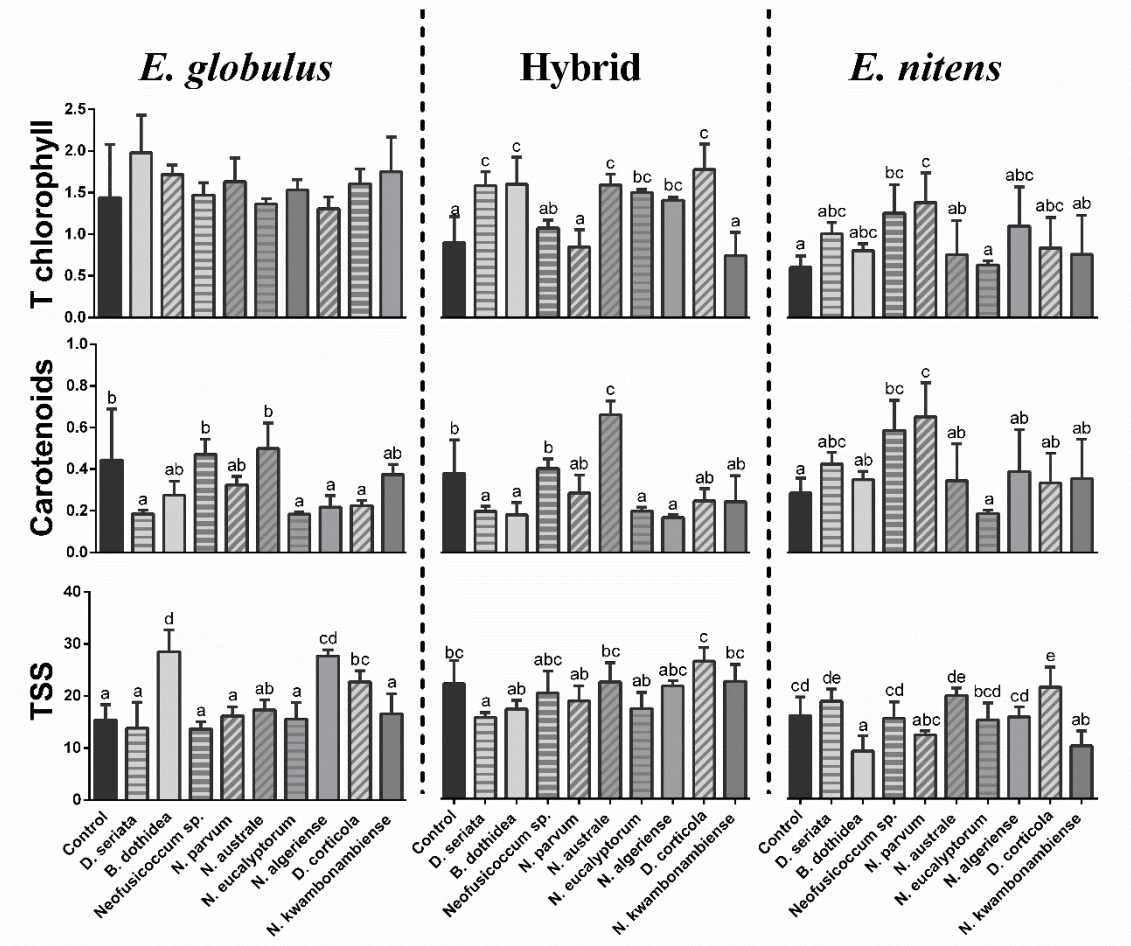

Figure 4.5: Total chlorophyll (µmol g^{-1} FW), carotenoid content (µmol g^{-1} FW) and total soluble sugars (TSS, mg g^{-1} FW) in leaves measured to three commercial stock material of *Eucalyptus* plants (*E. globulus*, hybrid and *E. nitens*) inoculated with different fugal species (*B. dothidea*, *D. corticola, D. seriata, N. algeriense*, *N. australe, N. eucalyptorum*, *N. kwambonambiense*, *N. parvum*, *Neofusicoccum* sp.) after two month of inoculation. Data are presented as mean±SD and different letters indicate significant differences between fungal species ($p \leq 0.05$).

Table 4.3: One-way ANOVA summary table for PC1 and PC2 obtained in principal component analysis (PCA). F value *p*-value are shown for source of variation (fungal infection). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, NS, non-significant.

Parameter	*E. globulus*			Hybrid			*E. nitens*		
	F	***p***	**Sig.**	***F***	***p***	**Sig.**	***F***	***p***	**Sig.**
PC1	17.74	0.000	***	3.35	0.005	***	0.87	0.563	NS
PC2	29.73	0.000	***	12.01	0.000	***	1.88	0.084	NS

Furthermore, One-way ANOVA of PCs revealed that *E. globulus* plants inoculated with *B. dothidea, D. corticola, D. seriata, N. algeriense* and *N. eucalyptorum* showed different morpho-physiological profile when compared with control plants (Table 4.3 and 4.4). In hybrid plants, the same fungi and also *Neofusicoccum* sp. induced alterations in the morpho-physiological profile. Finally, One-way ANOVA of PCs revealed no differences between inoculated and control *E. nitens* plants.

Table 4.4: Dunnett multiple comparison tests. Each fungi was compared with a Control. Fungi labeled with (*) are significantly different from the control.

	E. globulus		**Hybrid**		***E. nitens***	
	PC1	**PC2**	**PC1**	**PC2**	**PC1**	**PC2**
B. dothidea	*	*		*		
D. cortícola	*	*		*		
D. seriata	*	*		*		
N. algeriense	*	*		*		
N. australe						
N. eucalyptorum	*	*		*		
N. kwambonambiense				*		
N. parvum						
Neofusicoccum sp.				*		

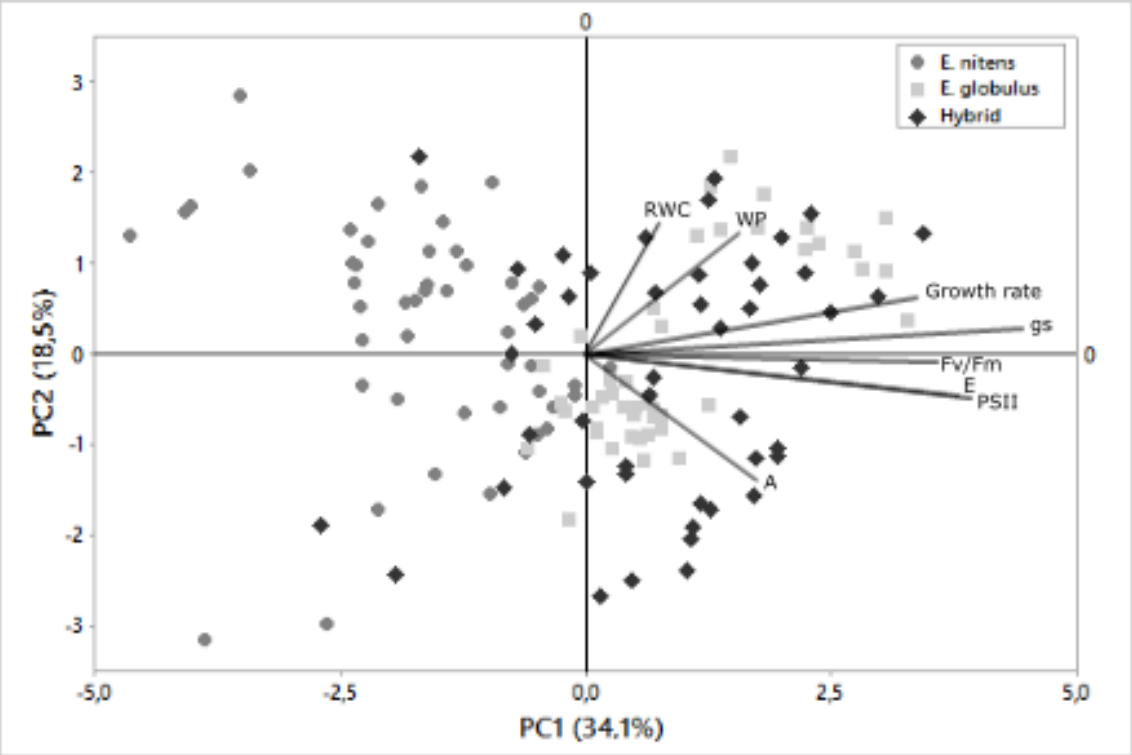

Figure 4.6: PCA biplot of the morpho-physiological data of the three commercial stock material of *Eucalyptus* plants inoculated with different fugal species (*B. dothidea, D. corticola, D. seriata, N. algeriense, N. australe*, *N. eucalyptorum, N. kwambonambiense, N. parvum, Neofusicoccum* sp.).

Discussion

The aim of this study was to evaluate the pathogenicity of *Botryosphaeria*, *Diplodia* and *Neofusicoccum* species (family Botryosphaeriaceae) towards different plant stock nursery material: *E. globulus* (clone), hybrid *E. globulus* x *E. cypellocarpa* (clone) and *E. nitens* (seed germination), resorting to pathogenicity tests and also morpho-physiological indicators.

Botryosphaeriaceae species cause serious diseases on eucalypts as cankers, dieback or even host death with high economic losses over the world (Chen et al. 2011; Slippers et al. 2009). Several studies have been conducted in order to detect these pathogens on eucalypts plantations (Barradas et al. 2016; Burgess et al. 2005; Chen et al. 2011; Gezahgne et al. 2004; Mohali et al. 2007; Rodas et al. 2009; Slippers et al. 2004) and pathogenicity tests have also been performed both in greenhouses and field trials in order to understand which species are more aggressive to these plants (Gezahgne et al. 2004; Mohali et al. 2009; Pavlic et al. 2007; Pérez et al. 2010). In a previously greenhouse study (Barradas et al. 2016), we found that *D. corticola* and *N. kwambonambiense* were the most aggressive species to a clone of *E. globulus* while *B. dothidea* and *D. seriata* were the least aggressive ones. Considering the lesions and the symptoms produced by the different Botryosphaeriaceae species in the present work, the results are in accordance with those findings and, furthermore, this tendency was verified in all the *Eucalyptus* plants in study.

Neofusicoccum kwambonambiense (Barradas et al. 2016; Sakalidis et al. 2011) and *D. corticola* (Barradas et al. 2016) were found to be the most aggressive species to *E. globulus* in previous studies. Our results reinforce the idea that these species may become a potential threat to eucalypt plantations in the near future since they were the most aggressive to *E. globulus* and also to the other studied plant groups. Earlier studies also reported *N. parvum* and *N. eucalyptorum* as the most aggressive of the studied species (Gezahgne et al. 2004; Mohali et al. 2009; Pavlic et al. 2007; Pérez et al. 2009, 2010). In our study, we confirmed the aggressiveness of both species but they were not the most aggressive ones, corroborating previous reported results of *E. globulus* (Barradas et al. 2016). The species *B. dothidea* and *D. seriata* were the least aggressive in our study. In early studies, *B. dothidea* was usually reported as endophyte and pathogenic to eucalypts (Bettucci and Alonso 1997; Smith et al. 1994, 1996), but it is now clear that this species is uncommon and weakly pathogenic or non-pathogenic to these plants (Barradas et al. 2016; Pavlic et al. 2007; Rodas et al. 2009). *Diplodia seriata* is a plurivorous and cosmopolitan species, however, only recently was found on eucalypts and pathogenicity tests reported it as weakly pathogenic (Barradas et al. 2016). In the current study, this species also reveals little aggressiveness to all the plant groups.

Even though our results indicate a clear differentiation concerning species pathogenicity, only one isolate per species was tested. Since it is recognized that different isolates from the same species may present different aggressiveness behaviour, and it has already been described to Botryosphaeriaceae species (Mohali et al. 2009; Piškur et al. 2011), studies including more isolates per species should be done in order to clarify this issue.

In spite of the efforts to assess Botryosphaeriaceae species aggressiveness to eucalypts, little is known about differences in tolerance/susceptibility among different forest reproductive material of *Eucalyptus*. Mohali et al. (2009), for instance, tested four

commercial *Eucalyptus* clones and found differences in their tolerance when infected by *N. ribis* and *N. parvum*. The three eucalypt groups in the present study also exhibited differences in their susceptibility considering lesions and symptoms developed. In our case, in general, *E. globulus* presented larger lesions while hybrid plants were the less affected ones. On the other hand, *E. nitens* presented a higher intra-specific variability most likely because these plants were obtained from seed while the other tested groups were clonal. The differences displayed by the three plant groups regarding plant susceptibility may arise from the fact that they represent different genotypes, consequently they exhibit different behaviour when in the presence of fungi, as expected and previously report (Rowntree et al. 2011).

Under pathogen attack the most common plant response is the downregulation of photosynthesis and other processes associated with primary metabolism that is essential for plant growth (Rojas et al., 2014). Several studies were carried out in order to assess the mechanisms involved (Berger et al. 2007; Linaldeddu et al. 2009; Sghaier-Hammami et al. 2013), but only few used eucalypts as a model (Alves et al. 2011; Dempsey et al. 2012; Pinkard and Mohammed 2006) and mainly addressed leaf and root diseases. For instance, Alves et al. (2011) tested two *E. urophylla* clones, one resistant and one susceptible to the rust fungus *Puccinia psidii*. They found no modifications in the resistant clone, while gas exchange and chlorophyll a fluorescence were negatively affected in the susceptible one. A significant decrease in photosynthesis rate was also been obtained for *E. globulus* leaves infected with *Mycosphaerella* spp. (Pinkard and Mohammed 2006). In another study, the root disease by *Phytophthora cinnamomi* induced early decrease in photosynthesis (A), stomatal conductance (gs) and quantum efficiency of PS II (Φ_{PSII}) in the susceptible *E. sieberi* while in the resistant *E. sideroxylon* these responses started later (Dempsey et al. 2012).

As far as we know, this is the first study conducted in order to understand the interaction between Botryosphaeriaceae species and eucalypts in terms of morpho-physiological response. Considering the global overview of the plant morpho-physiological status, in which the parameters were analysed together in a PCA, a clear distinction between *E. nitens* and the other two plant groups (*E. globulus* and hybrid) was found. In general, *E. nitens* plants presented growth, gs, Fv/Fm, E and ϕ_{PSII} low values in relations to *E. globulus* and the hybrid. Additionally, we verified that all infected *E. nitens* plants conserved their morpho-physiological profile when compared with control plants. On the contrary, *Botryosphaeria dothidea, D. corticola, D. seriata, N. algeriense*, *N. eucalyptorum*, *N. kwambonambiense* and *Neofusicoccum* sp. (hybrid only) induced modifications in the morpho-physiological profile of *E. globulus* (PC1 and PC2) and hybrid (PC2 only) plants. Taking into account that sample scores of *E. nitens* were all located together and isolated from the other two plant groups and inoculated fungi had no effect on morpho-physiological status of these plants, we hypothesised that the differences observed among the plant groups on PCA were related to differences on forest reproductive material and not to fungal infection. This is not unexpected since it

is recognize that plant with different genotypes could present different defence mechanisms (Berger et al. 2007). Besides, Botryosphaeriaceae species have been reported as latent pathogens, meaning they can persist for long periods without induce damage or diseases in their hosts until some stress triggers the infection (Burgess et al. 2005; Chen et al. 2011; Smith et al. 1996). Under these experimental conditions, *E. nitens* seems to present lower primary metabolism since growth and photosynthetic activity related parameters are reduced (low gs, Fv/Fm, E and ϕ_{PSII}) compared to the other plant groups.

Variation in the morpho-physiological parameters did not allow us to find a relation between morpho-physiological modifications and fungal aggressiveness. Furthermore, Fv/Fm values were within the ranges reported for healthy plants (Correia et al. 2014; Mielke et al. 2000; Schreiber et al. 1994). This emphasizes that no impact on primary photochemistry of PSII was achieved by fungal attack. Taking all data together these plants were able to preserve its homeostasis under the conditions tested, an ability already suggested by other authors (Atkinson and Urwin 2012; Bostock et al. 2014; Ramegowda and Senthil-Kumar 2015).

Pathogen-host interaction is a complex and dynamic process (Mundy and Manning 2011; Rai and Agarkar 2014). Plants are naturally hosts of infesting fungi that live inside of them without causing any disease symptoms. In this case, the plant defence responses and endophyte nutrient requirement are balanced (Kogel et al. 2006). This balanced stage is considered the most common case while diseases (caused by imbalance between plant defence and fungi attack) may occur occasionally (Kogel et al. 2006; Mundy and Manning 2011). Our results suggest that the reported plant-pathogen interactions correspond to balanced stages since no patterns nor a relation between plant status vs. fungal aggressiveness were found, further the plants primary metabolism was little affected. In fact, Botryosphaeriaceae species are considered opportunistic pathogens because they occur often in a latent stage as endophytes until some stress triggers the disease development (Burgess et al. 2005; Chen et al. 2011). Also, we hypothesize that the plants were able to form barrier zones in tissues around the inoculation point and confine the fungi to the necrotic tissues maintaining a healthy status. The formation of barrier zones or reaction zones as a non-specific response to wounding or infection was previously proposed for eucalypts (Naidoo et al. 2014; Tippett and Shigo 1981), and it has been reported on *E. nitens* (Barry et al. 2000) after inoculation and *E. globulus* after wounding (Eyles et al. 2003). The development of tyloses can lead to vessels occlusion preventing the fungal spread (Barry et al. 2000, 2001; Pouzoulet et al. 2014) a phenomenon already described for eucalypts, including *E. nitens* (Barry et al. 2000, 2001), which is another possible explanation. In addition, the three plant groups in study exhibit different tolerance/susceptibility behaviour. A recent study suggests that xylem diameter is an important factor regarding plants susceptibility to fungal vascular disease (Pouzoulet et al. 2014). The authors suggest that plant genotypes with small vessel diameter seem to be more resistant to diseases.

Considering *Eucalyptus* plants, February et al. (1995) proposed that vessel morphology is more related to environmental factors such as available water. Even so, they found some differences in plasticity of anatomical response to water availability between the *Eucalyptus* hybrids studied. Furthermore, it was reported that vessel dimensions of *E. globulus* and *E. nitens* increased from pith to bark (Hudson et al. 1998; Leal et al. 2003). We suggest that the plant groups in our study may present different xylem diameter vessels, however further investigations should be done to validate our assumptions. In the present study, we verified that *N. kwambonambiense* and *D. corticola* were the most aggressive fungal species; however, no patterns in the plant-pathogen interaction were verified. In other words, the highest aggressiveness of these fungi was not directly related to particular morpho-physiological modifications in plants. Despite of the differences observed related to fungal aggressiveness, the plants generally presented a healthy appearance without major morpho-physiological injuries. This finding corroborates the idea that Botryosphaeriaceae species are latent pathogens that can live in plants without causing any symptoms until some external factor or alteration in plant-pathogen interaction induce their switch of lifestyle (Slippers and Wingfield, 2007). This plant response could possibly be altered if the experiment time was extended or a second stress (such as drought) was imposed. In fact, drought stress has been associated to development of Botryosphaeriaceae diseases (Chen et al. 2011; Mohali et al. 2007; Slippers and Wingfield, 2007) and Botryosphaeriaceae species took 8 months to induce symptoms in a study carried out with *Vitis vinifera* (van Niekerk et al. 2011).

Understanding which fungi are more aggressive to eucalypts and which plants are more tolerant/susceptible is essential to develop suitable control and management strategies for plantations. *Eucalyptus nitens*, in spite of being the plant group with small morpho-physiological modifications, it presented larger lesions and more severe symptoms (a half of the plants died) than other plant groups. The hybrid, on the other hand, presented smaller lesions and intermediate susceptibility to pathogens. Considering all this new knowledge, we consider that hybrid plants could be a good alternative to *E. globulus* in the case of Botryosphaeriaceae species switch their lifestyle in response to new environmental conditions. Nevertheless, additional studies should be done, as the field behaviour of the plants is not completely understood.

The morpho-physiological parameters studied, in spite of being widely used to assess modifications on plant defence mechanisms (Berger et al., 2007), in our case did not enable an understanding of Botryosphaeriaceae-eucalypts interaction. Other signaling compounds also reported as involved in plant defence responses should be included and explored in future works. For instance, assessing phytohormones (jasmonic acid (JA), ethylene (ET) and salicylic acid (SA)), phenolic compounds, reactive oxygen species (ROS) (Atkinson and Urwin 2012; Kovalchuk et al. 2013; Naidoo et al. 2014; Torres 2010), and phytotoxic metabolites already described to

Botryosphaeriaceae species (Bertsch et al. 2013; Fernandes et al. 2014) among others, could provide knowledge to decipher these plant-pathogen interactions.

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CHAPTER 5

Drought-Disease interaction on *Eucalyptus globulus* under *Neofusicoccum eucalyptorum* infection

Drought-Disease interaction on *Eucalyptus globulus* under *Neofusicoccum eucalyptorum* infection

Abstract

Eucalyptus species are widely spread over the world being extensively planted and exploited by industry. Drought and pathogens are known to affect the establishment and productivity of *Eucalyptus* plantations worldwide. The aim of this work was to evaluate the pathogenicity of *Neofusicoccum eucalyptorum* towards *E. globulus*, both in drought stressed and well-watered plants. The effect of a previous drought-priming step, and the role of water status at the time of inoculation were also evaluated. Lesion length, plant growth and physiological parameters (relative water content, water potential, photosynthetic pigments and lipid peroxidation) were determined. Our results indicate that water stressed plants are more susceptible to *N. eucalyptorum* than non-stressed ones. However, this response was particularly relevant when the plant was inoculated while water limitation was already occurring. Moreover, drought primed plants presented a slightly increased resistance to fungal infection. This study reinforces the importance of exploring the interaction between biotic and abiotic stress responses in *Eucalyptus* susceptibility and the role of physiological performance involved.

Keywords: *Botryosphaeriaceae*, eucalypts, abiotic and biotic stress, stress interaction, plant physiological performance

Introduction

Eucalyptus species are widely spread over the world being one of the most important forest genera commercially exploited (Booth 2013; Mohali et al. 2009) and covering a global area of approximately 20 million ha (Booth 2013; Du et al. 2015). The outstanding diversity of the genus *Eucalyptus*, adaptability and fast-growth has made it a global renewable resource of fibre (Booth 2013) and energy, offering enormous scope as an alternative to meet the growing wood demands of the world as well as to save natural forests from deforestation (Booth 2013; Rodas et al. 2009; Wingfield et al. 2015). *Eucalyptus globulus* (Labill.) is a suitable species for temperate areas and it currently occupies approximately 800 000 ha (ICNF 2013) and 760 000 ha (cited by Gominho et al., 2014) of forest trees in Portugal and Spain, respectively. These areas are characterized by Mediterranean-like climate where hot summers and low water availability are becoming more frequent (Allen et al. 2010). Although eucalypts plantations have a high adaptive capacity to changes in climatic conditions (Booth 2013) major plantation problems are expected, affecting plantation establishment and forest

productivity (Wingfield et al. 2015) being the potential of overcoming these limitations dependent of genotype plasticity (Correia et al. 2014a,b).

Plants have to cope with a plethora of biotic stresses such as pathogen attacks throughout their life cycle. In a scenario of extreme events, frequency of plant diseases should be favoured even in well-established plantations (Lindner et al. 2010; Sturrocka et al. 2011). Moreover, abiotic stresses could also influence the physiological status of the plant changing their predisposition to disease and favouring the attack by pathogens (Bostock et al. 2014). Although the concept of stress predisposition to disease in plants is not new (Ma and Michailides 2001; Wargo 1996), the threat of climate change adds urgency for a better understanding of abiotic and biotic interaction (Lindner et al. 2010; Sturrocka et al. 2011). Besides, this is an increasingly important research topic where remarkable scientific output has been accomplished (Pautasso et al. 2015) but for which there is still much to explore. The prediction of global changes effects on plant health is complicated by interactions with climate changes stress drivers such as drought (Desprez-Loustau et al. 2006; Jactel et al. 2012). Even so, the majority of experiments are being done addressing each stress factor in an individual manner (Suzuki et al. 2014) in contrast to what happens in the field (Atkinson and Urwin 2012). Plant responses to drought are well known and described as complex and dependent on the time and the intensity of the stress (Ramegowda and Senthil-Kumar 2015). The water shortage in plants triggers diverse physiological, biochemical and molecular responses which intend to: a) reduce water loss by stomatal closure and reducing light absorbance; b) maximize water uptake by increasing root size and depth and c) protect the plants against the damaging effects of dehydration (Chaves et al. 2003; Rennenberg et al. 2006; Xu et al. 2010). Due to the impact of water limitation in plant yield these mechanisms have been extensively studied in *Eucalyptus* (e.g. Correia et al. 2014a,b; Costa e Silva et al. 2004). On the other hand, the pathogen attack also leads to changes in plant physiological processes such as primary and secondary metabolism related to the induction of defence programmes, which affect growth and development of the plant (Berger et al. 2007).

In addition, the abiotic stress history that plant undergoes during its life cycle can alter the outcome of plant-pathogen interaction. Pre-exposure to stress (invading pathogens or abiotic stress) may alter plant´s subsequent responses forcing a switch to a primed state of enhanced defense (Conrath et al. 2015; Wang et al. 2014). Primed plants show more rapid and robust activation of defense responses comparing to non-primed ones. For instance, Ding et al. (2012) noticed that drought-priming *Arabidopsis* plants (pre-exposure of plants to multiple short drought stress) led to higher expression of stress responsive genes and confer tolerance to later occurring of water deficit. However, little is known about enhanced defense capacity of drought stress primed plants to fungal pathogens, being the generation of ROS a suggested mechanism of defense response in plants that subsequently caused the tolerance (Ramegowda et al.

2013). In fact, there is still a knowledge gap related to the effect of stress interactions on woody plants growth, and *Eucalyptus* is not an exception.

In the present report this interaction was addressed using a fungal species of the family Botryosphaeriaceae. These fungi are well known as opportunistic pathogens that cause diseases on stressed or wounded *Eucalyptus* (Chen et al. 2011; Gezahgne et al. 2004; Mohali et al. 2007). Many species of the family Botryosphaeriaceae have been associated with symptoms observed in eucalypt plantations over the world, namely canker, dieback and kino exudation (Chen et al. 2011; Slippers et al. 2009) that may culminate in death with high economic losses. Many studies were conducted regarding the occurrence and interaction of Botryosphaeriaceae species associated with eucalypts, for example in Australia (Barber et al. 2005; Burgess et al. 2005, 2006; Mohali et al. 2009), and South Africa (Slippers et al. 2004; Smith et al. 1994, 1996, 2001). Smith et al. (2001) reported for the first time *Neofusicoccum eucalyptorum* in *Eucalyptus* trees in South Africa. This fungus was described as pathogenic and endophytic on several *Eucalyptus* spp. (Pérez et al. 2010; Slippers et al. 2004; Smith et al. 2001), and recently it has been confirmed as a pathogen of *Eucalyptus globulus* in Portugal (Barradas et al. 2016). In addition it represents one of the most frequent Botryosphaeriaceae species associated with this forest tree in Portugal (Barradas et al. 2016).

Few reports have explored the interaction of biotic and abiotic stresses on *Eucalyptus* susceptibility to disease (Lucas, 2003; Old et al., 1990; Swart et al., 1992). Considering both the economic importance of *E. globulus* and the urgent need to investigate tree response to multiple stresses as highlighted above, the research reported here aimed to evaluate the role of drought stress, including both the role of drought predisposition and drought-priming, in *E. globulus* interaction with *N. eucalyptorum* by assessing plant infection and physiological performance.

Materials and Methods

Plant material

In this study *E. globulus* clonal plants (AL-13) supplied by Altri Florestal SA (Portugal) were used. Rooted, 6-month old cuttings were transplanted to 2 L plastic pots filled with 3:2 (w/w) peat:perlite and left to grow under greenhouse conditions under a well-watered regime (80% field capacity, FC), during 1 month before the experimental set up.

Fungal culture and plant inoculation

The isolate of *Neofusicoccum eucalyptorum* (CAA369) used in the present study was obtained from *E. globulus* in Portugal and identified in a previous work (Barradas et al. 2016). The fungus was isolated in January 2012 and maintained on Potato Dextrose Agar (PDA, Merck, Germany) at 4°C. For plant inoculation the region of the stem to be wounded was surface disinfested with 70% ethanol and a shallow wound was made on

the stem base (5 cm above soil) of each plant using a sterile scalpel in order to remove the bark and expose the cambium. Mycelial plugs (5 mm diameter) from the active margin of 1-week-old fungal cultures, on PDA at 25°C, were placed into the wound with the mycelial surface facing the cambium. The inoculation sites were then sealed with Parafilm® (Pechiney Plastic Packaging Company, Chicago, USA) to prevent desiccation. Plugs of sterile PDA were inoculated into stems of control plants.

The fungus was re-isolated from each plant to confirm that it was consistently associated with the disease symptoms. For that, pieces of wood from the edges of the lesions were immersed in sodium hypochlorite for 3 min, then immersed in 70% ethanol for 2 min, and finally rinsed in sterile distilled water and blotted dry on sterile filter paper. Surface disinfested plant tissue was placed on PDA and incubated at room temperature. Fungal identification was based on conidial morphology.

Experimental design

The experiments began on May 2012 and lasted for 2 months. There were 6 plant replicates per treatment combination. Plants were randomly assigned to each treatment (supplementary data).

Experiment A:

This experiment was designed to evaluate the interaction drought-pathogen as well as the effect of drought-priming. The trial was conducted as a completely randomized full-factorial design with two levels of drought-priming (primed or non-primed) crossed with two water treatment levels (well-watered: WW or water-stressed: WS) as well as two levels of inoculum type (fungus: F or PDA as a control inoculum: F0), resulting in eight treatment combinations.

Experiment B:

This experiment was designed to evaluate the effect of drought and pathogen acting simultaneously on the plant (multiple stress concept) vs drought predisposition of the plant to pathogen attack (predisposition concept). The trial used non-primed plants and was conducted as a completely randomized full-factorial design with two different treatments: inoculation simultaneous to water stress imposition (WS_F) and inoculation after water stress imposition (WS_{F1}).

Plant treatments

Non-primed plants were maintained in a well-watered (WW) regime, being watered every day until reaching 80% field capacity (FC). Primed plants were subjected to one cycle of water stress (WS) followed by a recover. Briefly, watering was stopped until the moisture level reached 18% of FC (WS) and maintained in this condition for 10 days. Then they were rewatered to 80% FC (WW). The plants were maintained at 80% FC for 1 month and then assigned to the different experiments. The field capacity was

determined by the gravimetric method, meaning the set composed by pot, plant and soil were weighed after what plants were watered until reaching the desired FC.

Monitoring of infection

Throughout the experiment, the evolution of visual external symptoms such as stem lesions, foliar chlorosis and wilting were recorded periodically. After two months, the length of stem lesions caused by the pathogen was measured.

Morphological parameters

The plants height was measured on the first (inoculation time) and last day of the experiment. The plants growth was determined as the difference between the final and initial height of the plants.

Physiological parameters

At the end of the experiment, physiological parameters were recorded. Relative water content, water potential and chlorophyll *a* fluorescence were determined *in vivo* in fully developed leaves from the third node of each plant. Two leaves of a close location in the plant were harvested, frozen in liquid nitrogen and stored at -80°C to further determine pigment content (total chlorophylls and carotenoids), and lipid peroxidation. Six plants per treatment were analysed.

Water status

The relative water content (RWC) was determined as suggested by Weatherley (1950), using one leaf per plant. Tissue fresh weight (FW) was recorded and leaf samples were transferred to tubes with de-ionized water and maintained overnight in dark at 4°C. On the second day, after carefully removing excess water from leaf surface, turgid weight (TW) was registered and leaves were dried at 80°C for 72 h. After drying, leaf samples were reweighed and dry weight (DW) was recorded. RWC was calculated using the following equation: RWC (%) = (FW – DW) / (TW – DW)*100. Water potential (ψ_{md}) was measured using a Scholander-type pressure chamber (PMS Instrument Co., Corvallis, OR) at midday (solar time) using the apical stem.

Photosynthetic pigments

Total chlorophylls and carotenoid content was determined by the method described by Sims and Gamon (2002). In short, acetone/Tris (50 mM) buffer at pH 7.8 (80:20) (v/v) was used to extract pigments from the leaves. Samples were homogenized and centrifuged for 5 min at 10 000 ×g at 4°C twice. The supernatants were transferred to a new tube and the absorbance at 663 nm, 537 nm, 647 nm and 470 nm was

determined (Thermo Fisher Scientific spectrophotometer, Genesys 10-uv S). The calculations were done according the same authors (Sims and Gamon 2002).

Lipid peroxidation

The content of malondialdehyde (MDA) was used to assess the extent of lipid peroxidation in leaves, commonly taken as an indicator of oxidative stress. MDA was quantified using the method described by Hodges et al. (1999). Briefly, samples were extracted with TCA (trichloroacetic acid) 0.1% and vortexed. After centrifugation, an aliquot of the supernatants was added to a test tube with an equal volume of either: (1) positive (+) TBA (thiobarbituric acid) solution 0.5% (w/v) containing 20% (w/v) TCA; or (2) negative (−) TBA solution consisting in TCA 20%. Samples were heated at 95°C for 30 min and, after cooling and centrifuging, and absorbance was read at 440, 532 and 600 nm. MDA equivalents (nmol.ml^{-1}) were calculated as (A−B/157 000) × 10^6, where A = [(Abs 532_{+TBA})−(Abs 600_{+TBA})−(Abs 532_{-TBA} −Abs 600_{-TBA})], and B = [(Abs 440_{+TBA} −Abs 600_{+TBA}) × 0.0571].

Statistical analyses

Statistical analyses were performed using SigmaPlot 11.0 software. Since plant responses of experiment A mostly depended on interaction between three factors in analysis, we decided to analyse non-primed and primed plants separately, and two-way analysis of variance (ANOVA) was used to assess the effects of water treatment and inoculum type, as well as their interaction, on growth, RWC, water potential, F_v/F_m, pigments, and MDA content. When applicable, Holm-Sidak post-hoc tests were employed to identify significant differences within the same group (WW *vs.* WS, and F *vs.* F0).

Lesion data and experiment B were analysed by one-way ANOVA. Lesion statistical differences were found by analysing the effect of water regime in inoculated plants. In F_1 plants, one-way ANOVA tested the difference between plants inoculated simultaneous to water stress imposition or inoculated after water stress imposition. Prior to analysis, data were either ln or square -transformed to correct non-normality and heteroscedasticity. A significance level of 0.05 was considered for all analysis.

Results

Monitoring of infection

During the experiment, the external symptoms, such as stem lesions, foliar chlorosis and wilting, were monitored and it was found that the appearance of these symptoms were not equal to all the plant groups in study (Figure 5.1). As expected all plants subjected to water deficit regime wilted. The more evident symptoms (wilting, foliar chlorosis, dead leaves) were found only in experiment B in which 33% of WS_{F1} plants died. At the end of the experiment, lesions were visible around the inoculation

site of all inoculated plants while no lesions were found in control plants (Figure 5.1). *Neofusicoccum eucalyptorum* was re-isolated from the lesions and no *Botryosphaeriaceae* species were found in the controls, thus confirming Koch's postulates.

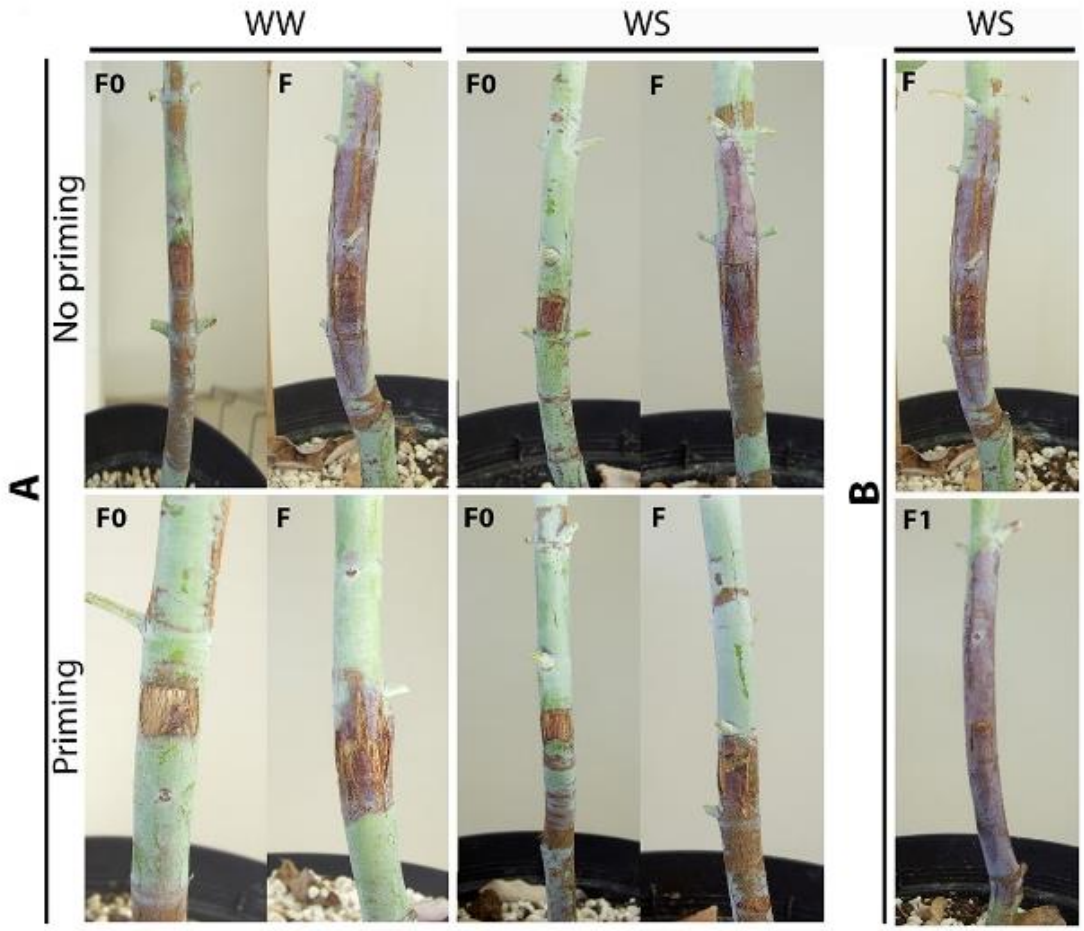

Figure 5.1: Representative pictures of lesions and symptoms produced by *N. eucalyptorum* on *E. globulus* plants infected (F), infected one week after water deficit started (F_1) and no-infected (F0) with *N. eucalyptorum* under well-watered (WW) and water deficit (WS) conditions. A – none previously stressed plants, and B – previously stressed plants.

Table 5.1: One-way ANOVA summary table for lesion length caused by *N. eucalyptorum* on *E. globulus*, for experiment A. *F* value, degrees of freedom (d.f.) and *p*-value are shown for the effect of fungal infection. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, NS, non-significant.

	No priming				Priming			
Parameter	**d.f.**	***F***	***p***	**Sig.**	**d.f.**	***F***	***p***	**Sig.**
Lesion	11	3.025	0.157	NS	11	0.0612	0.814	NS

Concerning stem lesions (Figure 5.1) caused by *N. eucalyptorum*, in experiment A, lesion lengths were not significantly affected by water regime neither in primed plants ($F(11) = 0.0612$, $p < 0.05$) nor in non-primed ones ($F(11) = 3.025$, $p < 0.05$) (Table 5.1, Figure 5.2). Further, the lesions found on non-primed plants were bigger than primed ones. On the other hand, in experiment B, the stem lesions were significantly bigger

($F(11) = 11.5$, $p < 0.05$) in plants that were inoculated after water limitation imposition (WS_{F1}) (Table 5.1, Figure 5.2).

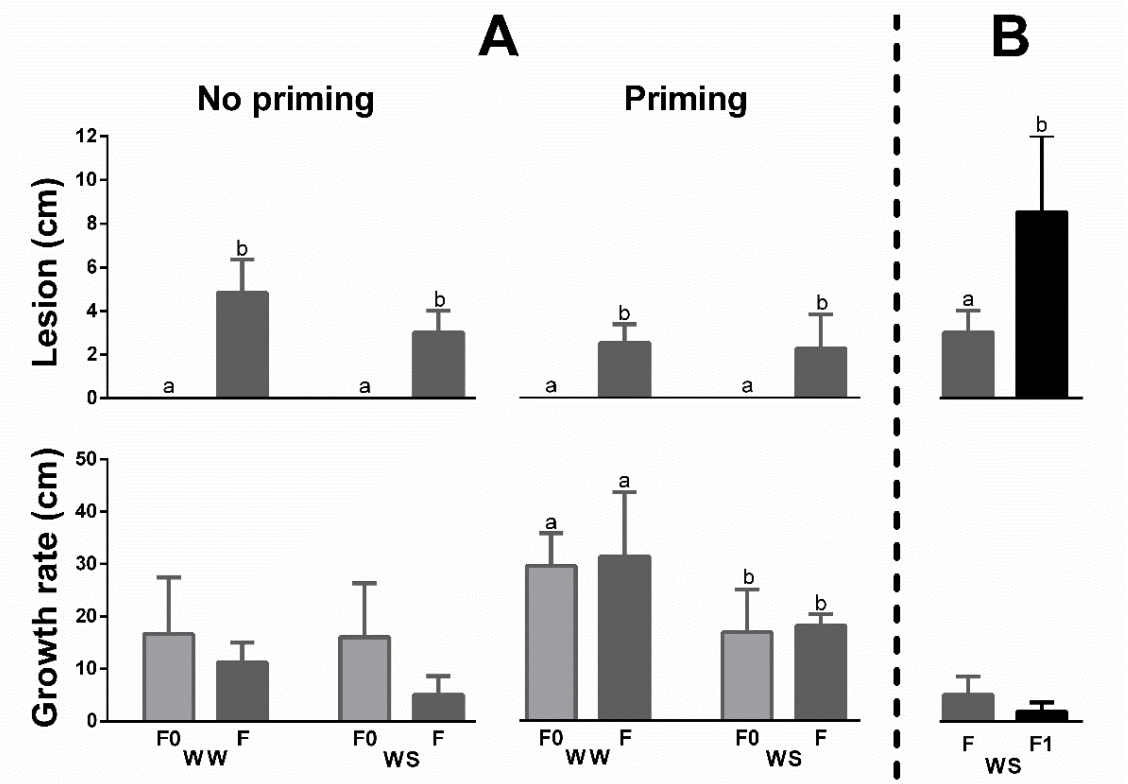

Figure 5.2: Lesion lengths (cm) and growth (cm) in *E. globulus* plants inoculated with *N. eucalyptorum* simultaneous to water stress imposition (F), after water stress imposition (F_1) and with PDA as a control inoculum (F0) under well-watered (WW) and water-stress (WS) conditions. A - Experiment A and B - Experiment B. Different letters express significant differences ($p \leq 0.05$).

Morphological parameters

Plants growth of experiment A showed a different response depending on the existence or not of drought-priming. Non-primed plants did not present any effect of water regime or fungal inoculation while primed ones showed significantly lower growth in WS comparing with WW treatment. Besides, primed plants grew more than non-primed ones (Table 5.3, Figure 5.2).

Table 5.2: One-way ANOVA summary table for lesion length, morphological and physiological traits on *E. globulus* infected with *N. eucalyptorum* for experiment B. *F* value, degrees of freedom (d.f.) and *p*-value are shown for the effect of the inoculation moment. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, NS, non-significant.

Parameter	d.f.	*F*	*p*	Sig.
Lesion (ln)	11	11.5	0.028	*
Growth	11	1.870	0.243	NS
RWC (x^2)	11	1.449	0.295	NS
ψ_{md}	11	44.263	0.003	**
Chlorophyll	11	9.397	0.015	*
Carotenoids	11	6.322	0.036	*
MDA	11	32.040	0.002	**

In relation to experiment B, no differences were observed between plants inoculated simultaneously (WS_F) and after (WS_{F1}) water stress imposition, further plants of this experiment presented the lowest growth (Table 5.2, Figure 5.2).

Table 5.3: Two-way ANOVA summary table for lesion length, morphological and physiological traits on *E. globulus*, for experiment A. *F* value, degrees of freedom (d.f.) and *p*-value are shown for each source of variation (Fungi = fungal infection; Stress = water deficit or interaction). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, NS, non-significant.

Parameter	Source of variation	No priming				Priming			
		d.f.	F	P	Sig.	d.f.	F	P	Sig.
Growth (ln)	Fungi	1	2.894	0.127	NS	1	0.200	0.665	NS
	Stress	1	1.604	0.241	NS	1	7.863	0.021	*
	Interaction	1	1.114	0.322	NS	1	0.136	0.721	NS
	Total	23				23			
RWC (x^2)	Fungi	1	1.094	0.326	NS	1	1.049	0.326	NS
	Stress	1	27.577	<0.001	***	1	2.357	0.163	NS
	Total	23				23			
	Interaction	1	1.304	0.287	NS	1	0.0194	0.893	NS
ψ_{md}	Fungi	1	18.150	0.003	**	1	4.112	0.077	NS
	Stress	1	3.834	0.079	NS	1	2519.133	<0.001	***
	Interaction	1	0.763	0.403	NS	1	4.112	0.077	NS
	Total	23				23			
Chlorophyll	Fungi	1	3.280	0.089	NS	1	0.293	0.595	NS
	Stress	1	57.667	<0.001	***	1	29.969	<0.001	***
	Interaction	1	14.213	0.002	**	1	1.240	0.281	NS
	Total	23				23			
Carotenoids	Fungi	1	2.665	0.119	NS	1	0.815	0.378	NS
	Stress	1	29.161	≤0.001	***	1	40.752	<0.001	***
	Interaction	1	1.583	0.224	NS	1	11.032	0.004	**
	Total	23				23			
MDA	Fungi	1	3.417	0.094	NS	1	0.839	0.381	NS
	Stress	1	3.834	0.079	NS	1	7.219	0.023	*
	Interaction	1	0.763	0.403	NS	1	0.002	0.962	NS
	Total	23				23			

Physiological parameters

Water status

Water regime affected relative water content (RWC) only in non-primed plants of experiment A, which presented WS plants with a reduced RWC compared to WW ones (Table 5.3, Figure 5.3). In this case the fungal inoculation did not have an effect in the RWC (Table 5.3, Figure 5.3) similarly to what happened in experiment B (Table 5.2, Figure 5.3). However, water potential (ψ_{md}) measurements exhibited a different response. In plants of experiment A, ψ_{md} was negatively affected by water regime and fungal inoculation in non-primed plants (Table 5.2, Figure 5.3). On the other hand, in primed plants, we only found an effect of the water regime in which WS presented lower

ψ_{md} values compared to WW (Table 5.3, Figure 5.3). Concerning experiment B, plants inoculated after water stress imposition (WS_{F1}) showed significantly higher values of ψ_{md} (Table 5.2, Figure 5.3).

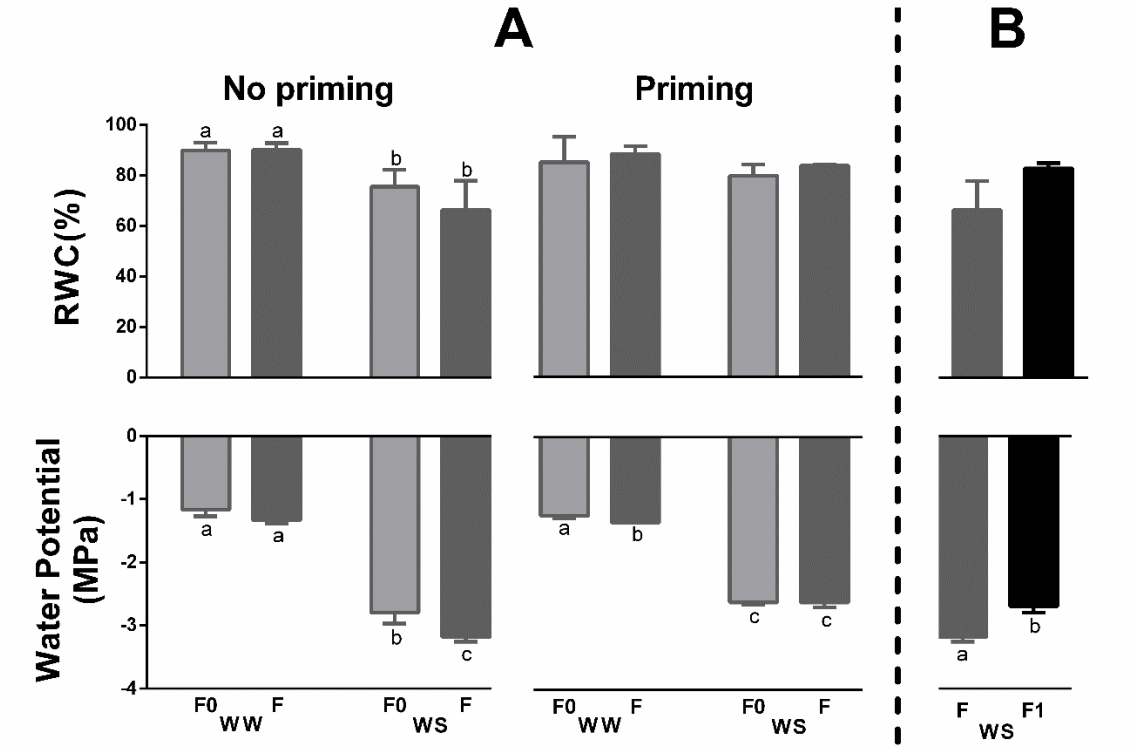

Figure 5.3: Relative water content (RWC, %) and water potential (MPa) in *E. globulus* plants inoculated with *N. eucalyptorum* simultaneous to water stress imposition (F), after water stress imposition (F_1) and with PDA as a control inoculum (F0) under well-watered (WW) and water-stress (WS) conditions. A - Experiment A and B - Experiment B.. Different letters express significant differences ($p \leq 0.05$).

Photosynthetic pigments

Pigment content greatly varied according to drought-priming treatment (experiment A) and in relation to water status at inoculation moment (experiment B).

In the experiment A, total chlorophylls were significantly affected by water regime, and an interaction between WS and fungal infection was also found for non-primed plants (Table 5.3). Total chlorophylls increased in WS plants in relation to WW; also, in WS plants the fungal infection still enhanced this content (Table 5.3, Figure 5.4). In primed plants, WS plants also exhibited higher chlorophyll content, but no effect of fungal infection was evident (Table 5.3, Figure 5.4). In contrast, for plants of experiment B, chlorophylls decreased in WS_{F1} (inoculation after WS imposition) when compared with plants inoculated simultaneous to WS exposure (Table 5.2, Figure 5.4).

In relation to carotenoids, non-primed plants of experiment A showed an effect of water regime (WS plants reaching higher content compared to WW), and no effect of fungal inoculation was found (Table 5.3, Figure 5.4). In primed plants, carotenoids were significantly affected by water regime, and an interaction between water treatment and fungal infection was found (Table 5.3). Water stress increased this pigment in relation

to WW; besides, regarding WS plants, the fungal infection also raised carotenoid content (Table 5.3, Figure 5.4). In plants of experiment B, carotenoids showed the same pattern as the one found in chlorophylls, meaning that plants inoculated after WS imposition presented a lower content (Table 5.2, Figure 5.4).

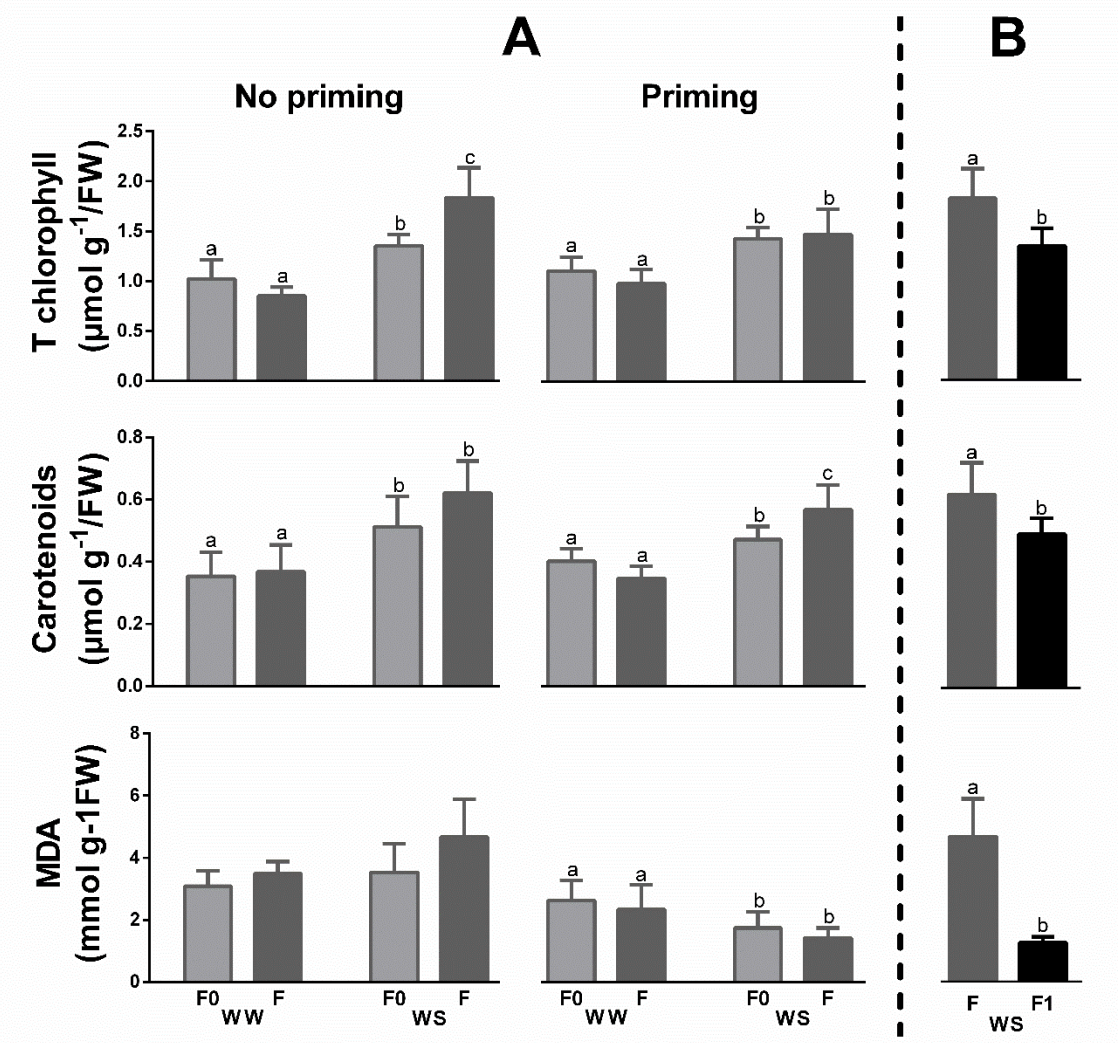

Figure 5.4: Pigments (total chlorophylls and carotenoids, µmol g^{-1} fresh weight) and lipid peroxidation (MDA, mmol g^{-1} fresh weight) in *E. globulus* plants inoculated with *N. eucalyptorum* simultaneous to water stress imposition (F), after water stress imposition (F_1) and with PDA as a control inoculum (F0) under well-watered (WW) and water-stress (WS) conditions. A - Experiment A and B - Experiment B.. Different letters express significant differences ($p \leq 0.05$).

Lipid peroxidation

Relative to lipid peroxidation, non-primed plants of experiment A showed no significant effect of water treatment or fungal inoculation (Table 5.3, Figure 5.4). On the other hand, primed plants were significantly affected by water regime: WS plants presented lower MDA concentration than WW ones (Table 5.3, Figure 5.4). Finally, plants of experiment B that were inoculated after water stress imposition (WS_{F1}) exhibited a highly reduced content in relation to plants which biotic and abiotic stress induction was parallel (WS_F) (Table 5.2, Figure 5.4).

Discussion

Diseases caused by *N. eucalyptorum* have been reported as a threat for eucalypts plantations worldwide (Burgess et al. 2005; Mohali et al. 2009; Pérez et al. 2010; Rodas et al. 2009). Previous studies conducted with 4-month-old plants of *E. grandis* demonstrated that this fungus produced lesions and necrosis after 1 week and induced cankers development after 3 weeks of inoculation (Pérez et al. 2009). Lesions on secondary phloem were also reported 1 month after inoculation of 6-month-old *E. grandis* plants (Smith et al. 2001). Barradas, et al. (2016) inoculated 6-month-old *E. globulus* with this fungus and after 2 month cankers and necrotic lesions were present. The current study confirmed the pathogenicity of *N. eucalyptorum* to *E. globulus*.

In the case of Botryosphaeriaceae-drought interaction on *Eucalyptus* very little is known. Old et al. (1990) found no effect of drought stress on the development of canker in eucalyptus plants infected by *N. ribis*. To our knowledge this is the first study that combines drought-disease interaction for *N. eucalyptorum* on eucalypts plants to explore the role of abiotic stress on plant susceptibility. Although we are far from thinking that we are simulating all environmental conditions occurring in the field, our experimental setup was designed to highlight the importance of drought-infection interaction studies. Taking into account that most of experimental investigations carried out on pathogen-abiotic stress limited their focus on disease-related variables (extent of colonisation, lesion length) (Desprez-Loustau et al. 2006) we decided to go further and include a physiological approach to fulfil this gap. The later decision is particularly important in view that abiotic stresses can influence the plant predisposition to diseases by altering the physiological status of the plant (Bostock et al. 2014). In the case of Botryosphaeriaceae species it has been recognized that abiotic stresses, such as drought, increase the susceptibility of woody plants to these fungi (Mohali et al. 2009; van Niekerk et al. 2011; Sherwood et al. 2015; Slippers and Wingfield 2007). For instance, it was found that the aggressiveness of a latent pathogen *Diplodia sapinea* increased when red pines were under drought (Blodgett et al. 1997; Stanosz et al. 2001). Water stress was also related with the increase in spread and incidence of *D. mutila* on *Quercus robur* (Ragazzi et al. 1999) and van Niekerk et al. (2011) found that Botryosphaeriaceae diseases are much more severe in grapevines when exposed to water stress. Further, Ma and Michailides (2001) associated the development of Botryosphaeria blight (*B. dothidea*) in pistachio with effects of water stress on spore germination, germ tube elongation and mycelial growth of this fungus. Our results reinforce this concept of plant predisposition (Blodgett et al. 1997; Bostock et al. 2014) since lesions length produced by the pathogenic fungus (*N. eucalyptorum)* on *E. globulus* were higher in plants where inoculation occurred after water stress imposition (WS_{F1} plants of experiment B). However, no effects in aggressiveness were observed when inoculation occurs simultaneously with water stress imposition (WS_F plants of experiment B).

Several authors suggest that pre-exposure of plants to a stress driver, known as priming, results in the activation of cellular defence responses increasing plant tolerance in a later stress episode (Bruce et al. 2007; Walter et al. 2013). We addressed this idea by using *Eucalyptus* plants that had been previously subjected to a first drought cycle followed by rewetting. Considering lesions length data, although plants presented just a slightly increased resistance to fungal infection, our results suggest the tendency for induction of a form of "stress memory". This hypothesis is becoming an important component of stress physiology research (Kinoshita and Seki 2014). This is a strategy that deserves more attention due to its potential application as mitigation measure to manage forest health resilience under global change context.

Moving further to morpho-physiological level, we found that water stress was the factor which most affected plant performance. This is neither surprisingly or new as it has already been reported by several authors on *E. globulus* plants regarding the impact of water limitation in physiological parameters such as water potential, RWC, growth, pigments, MDA content among others (Correia et al. 2014b; Costa e Silva et al. 2004; Granda et al. 2014). In our experiment, water stress had an impacting effect in the above-mentioned parameters with different degrees depending on the treatment. In experiment A we noticed a decrease of water potential together with an increase of photosynthetic pigments. The growth was only negatively affected on primed plants and RWC on non-primed ones. Lipid peroxidation, quantified in terms of MDA content, decreased under WS in primed plants too. Together with the highest growth observed, this physiological response is explained by the occurrence of one cycle of water stress during the priming period, which led to a more efficient physiological performance under a later water deficit event due to changes at the basal level of plant defence status than plants that have never been stressed (non-primed plants). The lower lipid peroxidation can be the reflection of a new basal defence threshold (primed sate) triggered by drought primed plants that led to a higher capacity of reactive oxygen species (ROS) scavenging and higher antioxidant enzymes activity as reported by other authors (Aranega-Bou et al. 2014; Wang et al. 2014a,b). Besides, priming caused potentiation of plant defence responses that frequently have been associated with enhanced resistance to various biotic or abiotic stresses, our data suggest that the type of elicitor used for priming may constrain the later stress response. Drought-primed plants triggered a positive response in a second reoccurring drought stress not so obvious in the biotic one, which suggests a degree of compatibility and specific mechanism underlying the different signalling pathways. This and other questions need more research to clarify how and how long plants are able to conserve this form of 'memory' of a previous stress episode (Wang et al. 2014b) particularly for trees.

A major variation on morpho-physiological status was found for experiment B when comparing plants that were inoculated after water stress imposition (WS_{F1}) in relation to plants simultaneously exposed to both stresses (WS_F). The highest aggressiveness level exhibited by *N. eucalyptorum* was confirmed by larger lesions and

senescence signs, together with the extremely low growth, indicating the importance of water stress time and *Eucalyptus* physiological status on susceptibility to Botryosphaeriaceae infection. This is in agreement with several reports (Mohali et al., 2009; van Niekerk et al., 2011; Sherwood et al., 2015; Slippers and Wingfield, 2007) which defend that damage caused by Botryosphaeriaceae species is more pronounced in stress-weakened plants. Under optimal conditions, plants direct their energy to growth, but under pathogen attack they need to balance a trade-off strategy between energy production and plant defence in order to survive (Atkinson and Urwin 2012). Our results support this commitment, considering the visual symptoms and lower survival rates (66%) associated with low growth and the decrease of the photosynthetic pigments that would reflected the photosynthetic efficacy and subsequently growth of plant as reports as a general consequence of pathogen attack (Bilgin et al. 2010). The higher values of water potential could be a consequence of the decrease of photosynthesis due to decrease of stomatal conductance and transpiration to avoid water losses or due to changes in physical properties of the plant cell wall components achieved by pathogens (Atkinson and Urwin 2012). Cell wall degrading enzymes produced by this class of fungi (Esteves et al. 2014; Fernandes et al. 2014) may change cell wall elasticity to maintain cell turgor decreasing water potential. The lower values of water potential at the inoculation time was an advantage for fungal colonization in comparison with plants inoculated in well-watered conditions as shown by lesion length. *Botryosphaeria dothidea* (Botryosphaeriaceae) is able to develop in stems of various host species only after plant water potentials have reached lower values than the threshold level of –1.2 to –1.3 MPa, with colonisation extent increasing with decreasing water potential (Crist and Schoeneweiss 1975). The study of plant disease interaction is an intricate process were two players are involved in a battle to survive: the host and the pathogen. Plants are capable of perceiving and activate the defence signaling pathways (Bellincampi et al. 2014) even at a long distance from the inoculation point (Eastburna et al. 2011). One of the first defence responses in plants is a rapid production of reactive oxygen species (ROS) leading to redox imbalance and oxidative stress that may affect normal cellular functions (del Rio 2015; Heller and Tudzynski 2011). Thus, ROS generation and regulation that occur in response to both biotic and abiotic stress must be tightly controlled by plants (Dempsey et al. 2012). Likewise, fungal pathogens have evolved antioxidant defence against ROS produced during their own infection as a part of host innate immunity (Mir et al. 2015; Sherwood et al. 2015) that is part of their pathogenicity mechanisms. Besides being toxic for the attacker, ROS may be involved in defensive signalling pathways (Heller and Tudzynski 2011; Sherwood et al. 2015). Recent studies proposed antioxidant enzymes such as catalase and peroxidases as components of antioxidant defence system (Heller and Tudzynski 2011; Liu et al. 2011). Recently, Sherwood and co-workers (2015) reported that drought induces accumulation of hydrogen peroxide (H_2O_2) in Austrian pine (*Pinus nigra* Arnold) shoots, but that shoot infection by the blight and canker pathogen *Diplodia sapinea* leads to

large reductions in H_2O_2 levels in droughted plants. Moreover, during in vitro application of H_2O_2, the fungus responded to this oxidative stress by increasing catalase and peroxidase activities, resulting in substantial H_2O_2 degradation (Sherwood et al. 2015). The level of lipid peroxidation has been widely used as an indicator of ROS mediated damage to cell membranes under stressful conditions. Our results showed that lipid peroxidation decreased in drought stressed plants after fungi inoculation (WS_{F1}), not as a result of an increased plant defence strategy but probably due to the production antioxidant enzymes by the fungus to scavenge ROS, succeeding in its colonization purposes as mentioned above.

Taken all together, the results reported here corroborate the importance of exploring the interaction between biotic and abiotic stress responses in *Eucalyptus*, particularly under global change prediction that forecast the increase of drought and pathogen attack. We found a positive effect of drought on fungal diseases development, but only in the case of plants that were infected while drought stress occurs (WS_{F1}). Our results reveal that plant susceptibility to the fungus increases when inoculated in water stress conditions, indicating that pre-established water deficit weakens basal defence, and facilitates successful pathogen infection (predisposition). Considering the morpho-physiological parameters analysed, we observed that water limitation affected more the plant physiological status than fungi. In addition, primed plants exhibit a more efficient and rapid response to the second stress cycle. According to this complex drought-disease interaction, our report highlights the need for integrative approaches to investigate multiple stress, biotic and abiotic, in order to successfully understand and validate what happens in the field.

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CHAPTER 6

General discussion

General discussion

Eucalyptus species, although mostly natives to Australia, are currently widespread and one of the most important forest trees exploited (Du et al. 2015; Wingfield et al. 2015). Nowadays, there are more than ten well established species and hybrids in commercial plantations (Wingfield et al. 2015), whose distribution is mostly related with environmental conditions. For instance, *E. grandis* W. Hill is more common in tropical/subtropical areas while *E. globulus* Labill. is exploited in temperate zones (Carocha et al. 2015), including Mediterranean climates (Granda et al. 2014), as Portugal.

The increased interest to exploit eucalypts is mainly due to their proprieties that are appreciated by the pulp and paper industry (Booth 2013; Correia et al. 2014; Paine et al. 2011; Wingfield et al. 2015), namely fast-growth, excellent pulp properties, short rotation, easy vegetative propagation and wide adaptability to soils and climates (Brondani et al. 2012; Old et al. 2003). *Eucalyptus* species present high adaptive capacity even though their cultivation range is limited by climate conditions as water availability and low temperatures (Correia et al. 2014; Dutkowski and Potts 2012; Granda et al. 2011).

Eucalypts are naturally hosts of pests and pathogens (Naidoo et al. 2014; Old et al. 2003), however, the incidence and severity of diseases has been intensified in the recent years (Naidoo et al. 2014; Paine et al. 2011; Roux et al. 2005). Relatively to pathogens, Botryosphaeriaceae species have been considered a threat for eucalypts plantations productivity (Chen et al. 2011; Slippers et al. 2009) since they can be found both on native and non-native ranges (Mohali et al. 2009; Rodas et al. 2009; Smith et al. 1994). In fact, these fungi cause severe diseases symptoms in eucalypts, of which dieback of shoots and branches, stems cankers followed by kino exudation and coppice failure are the most frequent and can culminate on host death (Chen et al. 2011; Slippers et al. 2009). As far as it is known, at least 29 species (8 genera) of Botryosphaeriaceae have been recognised as able to successfully colonized eucalyptus plants (Phillips et al. 2013; Pillay et al. 2013; Chapter 2). Recently, in Portugal, it was studied the occurrence of Botryosphaeriaceae species associated both with diseased (stem canker and dieback) and healthy *E. globulus* (Chapter 2). These isolates obtained belong to 9 species within 3 different genera, namely *Botryosphaeria* (*B. dothidea*), *Diplodia* (*D. corticola* and *D. seriata*) and *Neofusicoccum* (*N. algeriense*, *N. australe*, *N. eucalyptorum*, *N. kwambonambiense*, *N. parvum* and *Neofusicoccum* sp.). *Neofusicoccum* was clearly the dominant genus (95% of the isolates) in this area, being *N. australe* and *N. eucalyptorum* the most abundant species. These results were not surprising as a similar tendency of genus abundance and diversity has been reported in most of the areas where this host is established (Gezahgne et al. 2004; Mohali et al. 2007; Pérez et al. 2010; Slippers et al. 2009; Slippers et al. 2004). Interestingly, *N. algeriense*, *D. corticola* and *D. seriata* were associated with *E. globulus* for the first time worldwide, and *N. algeriense*, *N.*

eucalyptorum and *N. kwambonambiense* represent a new report of these species in Portugal.

The early detection and identification of pathogenic fungi is very important to prevent disease outbreaks that may result in important economic losses. The identification and characterization of Botryosphaeriaceae species are, nowadays, based on molecular tools in combination with morphological characters (Crous et al. 2006; Phillips et al. 2013; Slippers et al. 2009). In opposition to morphological characters, DNA-based techniques are very powerful in discriminating species (Alves et al. 2007), however, these techniques may involve complex and time-consume protocols, require expensive reagents, and do not reflect the phenotypic diversification (Mancini et al. 2013; Santos et al. 2010). Mid-infrared spectroscopy (MIR) has demonstrated to be a powerful alternative technique applied to detection, identification, characterization and authentication of several filamentous fungi (Erukhimovitch et al. 2005; Fischer et al. 2006; Lecellier et al. 2015; Naumann et al. 2005; Santos et al. 2010). Concerning the family Botryosphaeriaceae, this technique has also shown great potential for discriminating species using a rapid, inexpensive and easy approach (chapter 3).

Globally, the diversity of Botryosphaeriaceae species associated to eucalypt plantations is well known but knowledge on their pathogenicity/aggressiveness is often underestimated. For instance, pathogenicity tests confirmed the ability of *N. parvum* to cause stem cankers on *E. grandis* and *E. citriodora* (Gezahgne et al. 2004) and *N. eucalyptorum* on *E. grandis* (Pérez et al. 2009). Pavlic et al. (2007) inoculated *E. grandis × E. camaldulensis* clones and found that *N. ribis*, *N. parvum* and *L. theobromae*, were the most aggressive species while *B. dothidea* was the less aggressive one. The same aggressiveness patterns of *N. ribis* and *B. dothidea* were found in *E. grandis* plants (Rodas et al. 2009). The species *N. parvum* and *N. ribis* were the most aggressive to eucalypts and differences in clones tolerance were verified (Mohali et al. 2009). Pérez et al. (2010) inoculated *E. grandis* plants with species isolated both from introduced *Eucalyptus* and native Myrtaceae trees and verified that *L. pseudotheobromae*, *N. eucalyptorum* and the *N. parvum-N.ribis* complex are the most aggressive to these plants. We carried out a greenhouse trial to study the aggressiveness of the species identified in Portuguese plantations (Chapter 2) in which *D. corticola* and *N. kwambonambiense* were found as the most aggressive species to *E. globulus* while *B. dothidea* and *D. seriata* were the least aggressive ones.

At this point, it is very important to bear in mind that the fungal aggressiveness is not only dependent on fungal species but is a result of the host-pathogen interaction that is influenced by genetic variation from both sides (Rowntree et al. 2011) manifested under certain environmental conditions. Thus, there is a need to investigate the response to Botryosphaeriaceae infection among the different forest reproductive material currently exploited in eucalypt plantations. In fact, Mohali et al. (2009) found differences in susceptibility among different clones of *E. urophylla* x *E. grandis* hybrids to *N. parvum* and *N. ribis*. Variation in plants susceptibility was also observed when *E.*

globulus and *E. nitens* species and a hybrid *E. globulus x E. cypellocarpa* were investigated (Chapter 4). Despite these few studies, little is known about the occurrence of differences in susceptibility relating to plant responses. Morpho-physiological parameters represent good indicators of primary metabolism and plant defence mechanisms (Berger et al. 2007; Correia et al. 2014) and their investigation appear to add value in pathogenicity studies. In fact, morpho-physiological responses to fungal infections have been reported in other plant-pathogen models (Berger et al. 2007; Mundy and Manning 2011; Pinkard and Mohammed 2006). However, the interaction between eucalypts and Botryosphaeriaceae species seems to not respond in the same way since modification in morpho-physiological parameters had no logical correlation with fungal species aggressiveness (Chapter 4). Possibly, *Eucalyptus* plants are able to prevent the spreading of Botryosphaeriaceae species with no effect on plant morpho-physiological status. Actually, the formation of barrier or reaction zones (Barry et al. 2000; Eyles et al. 2003; Naidoo et al. 2014; Tippett and Shigo 1981) and the development of tyloses (Barry et al. 2000, 2001) are two phenomena already described as eucalypts defense strategies. Moreover, the differences related to plants susceptibility can be related with variations in xylem diameter, since it was recognised that plants genotypes with small vessel diameter seem to be more resistant to diseases (Pouzoulet et al. 2014), but further studies should be performed to confirm these assumptions.

The recent findings reinforce the idea that Botryosphaeriaceae species are latent pathogens which can live inside their hosts for long periods of time without apparent damage or disease symptoms (Burgess et al. 2005; Chen et al. 2011; Smith et al. 1996) until some stresses trigger the infection and then cause serious diseases on their hosts (Old et al. 1990). In fact, recent reports highlighted the need for better understand multiple and simultaneous stress on plants susceptibility/tolerance (Atkinson and Urwin 2012; Ramegowda and Senthil-Kumar 2015). The incidence and severity of Botryosphaeriaceae diseases are frequently associated with drought (Chen et al. 2011; Mohali et al. 2007; Old et al. 1990; Phillips et al. 2013) being recently found that it is especially important when the infection occurs during water deprivation (Chapter 5). Moreover, eucalypts increased their resistance to fungal infection when subjected to a previously drought stress cycle. This phenomena, known as priming, lead to a more efficient defense response after a previously stress episode (Bruce et al. 2007; Walter et al. 2013, Chapter 5).

During the 21st century, an increase in global temperature and frequency of extreme events is expected according to the report elaborated by the Intergovernmental Panel on Climate Change (IPCC 2013). These climate changes will potentially intensify the occurrence and severity of plant diseases both directly (act on the distribution patterns and aggressiveness of pests and pathogens) and indirectly (affecting host physiology and, consequently, the host-parasite interaction). In fact, the enhancing of fungal diseases during drought periods and hot summers has previously been reported (Desprez-Loustau et al. 2006) and some reports have notice that

Botryosphaeriaceae species benefit of weakened of plants healthy (Pérez et al. 2010; Slippers and Wingfield 2007). In this respect, a better understanding about the relation between these fungi and eucalypts, as well as how the climate conditions influence their interaction is crucial in order to develop suitable control and management strategies for plantations productivity.

Future work

- An interesting future work would be to explore the dynamics of Botryosphaeriaceae communities under different environmental conditions, using next-generation sequencing (NGS) and culture-independent methodologies. In this respect, more areas not included in the present work should be sampled. This approach may allow a better understanding of the endophytic role of these fungal species.
- MIR spectroscopy showed a great potential as an alternative technique for Botryosphaeriaceae species identification. Hence, more isolates and new species should be included in order to fully validate this technique and construct a robust and more detailed library.
- The aggressiveness of one representative isolate per fungal species was evaluated towards two *Eucalyptus* species and one hybrid in an artificial trial. More artificial trials should be done including more than one isolate per species in order assess if there are differences in aggressiveness among different strains of the same species. Further, these trials should also include more *Eucalyptus* species and hybrids.
- Concerning the physiological plant status, in general, plants were not affected by the fungal infection. Different approaches, as hormones quantification, studies in epigenetic, SEM, etc, may give us a better idea about the plant responses facing fungal infections.
- In the field, plants are simultaneously exposed to a broad range of environmental conditions, however we only evaluated drought stress. Artificial trials combining more than one stress drivers should be performed. For example, drought-temperature-disease interaction should provide interesting results and is more approximated to the field conditions.
- In the present thesis, we have found that eucalypt plants previously expose to water deprivation, exhibit an increased defence response to a second stress cycle including fungal infection, a concept known as priming. It would be interesting to apply a first water stress on plants in controlled conditions before transfer them to the field. The performance of these pre-stress plants allowing to prove our assumptions that priming-plants present an increase defence to Botryosphaeriaceae species.

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