UNIVERSIDADE DE ÉVORA

MESTRADO EM BIOLOGIA DE PRAGAS E DOENÇAS DE PLANTAS

Species identification of *Mycosphaerella* leaf blotch disease (MLD) on *Eucalyptus globulus* in Portugal

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Orientadores:
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e
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Dissertação apresentada para obtenção do
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Esta dissertação não inclui as críticas e sugestões feitas pelo júri

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M. SILVA, C. VALENTE, L. NEVES, H. MACHADO (2007) Avaliação do impacto de *Mycosphaerella* em plantações de eucalipto em Portugal. 5º Congresso da Sociedade Portuguesa de Fitopatologia, COIMBRA, PORTUGAL, 22 A 23 NOV..

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TABLE OF CONTENTS

Identificação de espécies responsáveis pela doença "*Mycosphaerella* leaf blotch disease" (MLD) de *Eucalyptus globulus* em Portugal

RESUMO

"*Mycosphaerella* leaf disease" é causada por espécies de *Mycosphaerella* e vários anamorfos. Em Portugal, esta doença foi largamente ignorada até 1998 quando foram verificados sérios estragos em plantações comerciais de *Eucalyptus globulus*.

O objectivo deste trabalho foi dar uma contribuição ao levantamento inicial de "*Mycosphaerella* leaf disease", clarificando a composição do complexo *Mycosphaerella*. É apresentada (Capítulo 1) uma panorâmica da situação munidal e uma lista detalhada das espécies detectadas em Portugal. As espécies foram identificadas com base em métodos moleculares (Capítulo 2) e na sua morfologia (Capítulo 3). Adicionalmente, ao objectivo inicial desta tese, foi estabelecida uma primeira avaliação da susceptibilidade de clones e famílias de eucaliptos em condições experimentais.

Species identification of *Mycosphaerella* leaf blotch disease (MLD) on *Eucalyptus globulus* in Portugal

ABSTRACT

Mycosphaerella leaf disease is caused by species of *Mycosphaerella* and several anamorphic form *genera* that have been associated to *Mycosphaerella*. In Portugal, this disease was largely ignored until 1998 when serious damage on commercial plantations of *Eucalyptus globulus* was reported.

The aim of this work was to give a contribution to initial survey of *Mycosphaerella* leaf disease, clarifying the composition of *Mycosphaerella* complex. An overview of worldwide situation and a detail list of species reported in Portugal is presented (Chapter 1). Species were identified based on molecular methods (Chapter 2) and on their morphology (Chapter 3). Additionally, of the initial aims of this thesis, a first evaluation of clones and families eucalypts susceptibility in experimental conditions was established.

CHAPTER 1

1.1 INTRODUCTION

Commercial plantations of *Eucalyptus* in Portugal (continental) are almost exclusively of *E. globulus* covering 646 700 ha, which represents 20.6% of the total forested area (DGRF, 2007). Superior pulping qualities and fast growth rate are valuable properties of this species.

Mycosphaerella leaf disease (MLD) is one of the most important leaf diseases of eucalypt plantations worldwide but it remains poorly understood, mainly because it is caused by a complex of species. Frequently 4 to 5 different species inhabit the same lesion and the lesions often overlap (Crous *et al.*, 2004). Morphological identification of *Mycosphaerella* species and their anamorphs is difficult, with approximately 62 species of *Mycosphaerella*, and about 34 anamorph species not linked to a *Mycosphaerella* state (Carnegie & Keane, 1998; Crous, 1998; Dick & Dobbie, 2001; Maxwell, *et al.*, 2003; Crous *et al.*, 2004, 2006; Hunter *et al.*, 2004; Burgess, *et al.*, 2007; Carnegie, *et al.*, 2007).

In 1881 *Mycosphaerella molleriana* (Thüm.) Lindau was described as a new species in Portugal (Crous & Wingfield, 1997). During the 1990's further species were reported, including *Mycosphaerella walkeri* R. F. Park & Keane (Crous, 1998) and *Mycosphaerella africana* Crous & M. J. Wingf. (Crous, 1998). In 2004, *Mycosphaerella madeirae* Crous & Denman was described as a new species from Madeira, (Crous *et al.*, 2004). In 2006, other species were added but also poor in information (e.g. Crous *et al.*, 2006).

Repeated and serious defoliation of young *E. globulus* trees was reported recently in Portugal. The increased severity of MLD prompted collaboration between Instituto Superior de Agronomia, (ISA), Estação Florestal Nacional (EFN), Institute RAIZ and Silvicaima, and Professor M. J. Wingfield (Forestry and Agricultural Biotechnology Institute, FABI) in a demonstration project AGRO 550 (2004-2007).

During 2005 RAIZ started an evaluation of MLD disease in 170 industrial plantations throughout Portugal. Initial data indicated that during the period of the surveys, MLD was restricted to the central-north coastal regions (unpublished data). During 2005 a team from Estação Florestal Nacional (EFN) used morphological and molecular characters (Crous *et al.*, 2006) to identify *M. africana*, *Mycosphaerella communis* Crous & J.P. Mansilla, *Mycosphaerella lateralis* Crous & M.J. Wingf., *Mycosphaerella marksii* Carnegie & Keane, *Mycosphaerella nubilosa* (Cooke) Hansf., *Mycosphaerella parva* R. F. Park & Keane (Silva *et al.*, 2007; Silva *et al.*, *submited*) and *Mycosphaerella grandis* Carnegie & Keane *and Mycosphaerella vespa* Carnegie & Keane as new records for Portugal (Silva *et al.*, *submited*).

M. nubilosa and *Mycosphaerella cryptica* (Cooke) Hansf.cause serious defoliation of *E. globulus* in Australia leading to various studies on pathogenicity and epidemiology (Park & Kean, 1984, 1982a, 1982b). Recently, genetic variation in susceptibility of *E. globulus* to *M. nubilosa* was studied in Tasmania (Milgate *et al.*, 2005). Since the highly virulent *M. nubilosa* has been also detected in Portugal, it is imperative that rapid screening tools are developed and tolerant host genotypes selected to reduce the impact of the disease. A first approach on detection of genetic variation in susceptibility to MLD was obtained during a trial established during project AGRO 550 where different families and clones of *E. globulus* were planted at two sites.

The aim of this study was to give a contribution to initial survey of MLD in industrial plantations of *Eucalyptus globulus* in Portugal clarifying the role of each *Mycosphaerella* species present in plantations with severe defoliation.

1.2 LITERATURE REVIEW

1.2.1 The genus *Eucalyptus*

Eucalypts take their name from a Greek root *eu* (well) and *calyptos* (covered). Eucalypts are species which belong mainly to southern hemisphere. An angiosperm, family *Myrtaceae* with 702 species, which belongs to 13 main evolutionary lineages (subgenera) - *Angophora*, *Blakella*, *Corymbia*, *Eudesmia*, *Acerosa*, *Cuboidea*, *Idiogenes*, *Primitiva*, *Eucalyptus*, *Cruciformes*, *Alveolata*, *Symphyomyrtus* and *Minutifructus* (Brooker, 2000; Potts, 2004)

Most species fit in the subgenus *Symphyomyrtus*, which provide the species mostly used for forest stands, such as *Eucalyptus camaldulensis* Dehnh., *Eucalyptus globulus* Labill., *Eucalyptus grandis* Maiden and *Eucalyptus nitens* Deane & Maiden (Maiden) (Potts, 2004).

Eucalypts are native to Australia but since the late 18th century they have been planted worlwide. Eucalypts were first introduced into countries such as India (1790), France (1804), Chile (1823), Brazil (1825), South Africa (1828) and Portugal (1829) (Potts, 2004).

Since then, eucalypts became well-known for their fast growth, straight form, adaptability to soils and a large range of climates like high rainfall, semi-arid, sea level and alpine tree line zones (Potts, 2004). In the latest years they become appreciated for their special wood properties and pulp production.

In 1999 there were about 9.48 million hectares of worldwide industrial plantations through Africa (1.08 M.ha), Asia (2.43 M.ha), Europe (1.32 M.ha), North and Central America (0.08 M.ha), South America (4.09 M.ha) and 0.48 ha in Oceania (Potts, 2004). In 2010, the total planted area is predicted to get to 11.60 million hectares. Split into Africa (1.00 M.ha), Asia (3.02 M.ha), Europe (1.40 M.ha), North and Central America (0.10 M.ha), South America (5.00 M.ha) and Oceania (0.90 M.ha), resulting in a global rise of 22 % (Raga, 2001; Potts, 2004).

Almost 80% of worldwide area is planted with *E. grandis*, *E. globulus* and *E. camaldulensis* and their hybrids. *E. globulus* is mainly found in temperate regions for pulp production and it is one of the most important pulpwood plantation species in the world (Potts, 2004).

The common name of *E. globulus* is Tasmanian blue gum which is endemic to south-eastern Australia. *E. globulus* has four subspecies: *Eucalyptus globulus* subsp. *bicostata* (Maiden, Blakely & J.Simm.) J.B. Kirkp.; *Eucalyptus globulus* subsp. *globulus* Labill.; *Eucalyptus globulus* subsp. *maidenii* (F. Muell.) J.B. Kirkpatr. and *Eucalyptus globulus* subsp. *pseudoglobulus* (Naudin ex Maiden) Kirkpatr. Of these, subspecies *globulus* is the most usually planted in south-eastern and south-western Australia (Carnegie & Ades, 2005) U.S.A. and in Portugal.

Commercial plantations of *Eucalyptus* in Portugal (continental) are almost exclusively of *E. globulus* covering an area of 646 700 ha, which represents 20.6% of the total forested area (DGRF, 2007). In Portugal the eucalypts industrial plantations are mainly concentrated on the coastal area, where it is more productive (Figure 1).

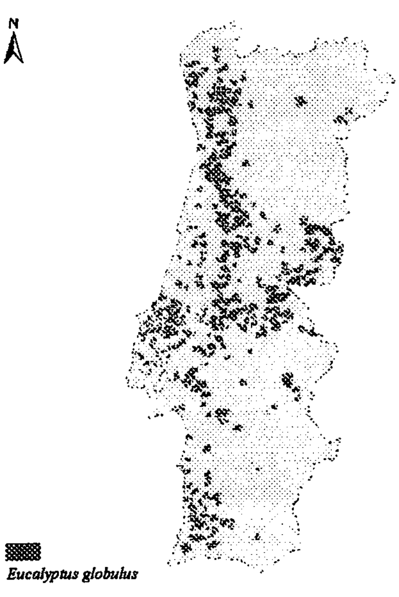

Figure 1- *Eucalyptus globulus* area in Portugal (adapted from Godinho-Ferreira *et al.*, 2005).

1.2.2 Main pathogens of eucalypts

The main pathogens of eucalypts leaves described over the world are: *Aulographina eucalypti* (Cooke & Masse) Arx & E. Mül., *Puccinia psidii* Winter; *Phaeophleospora epicoccoides* (Cooke & Massee) Crous, F.A. Ferreira & Sutton, *Phaeophleospora eucalypti* (Cooke & Massee) Crous, F.A. Ferreira & Sutton, *Phaeophleospora destructans* (M. J. Wingfield & Crous) Crous, F.A. Ferreira & Sutton, *Cryptosporiospsis eucalypti* Sankaran & B. Sutton e *Cylindrocladium* spp.. Many of these pathogens like *Puccinia psidii,* do not occur in Australia (Park *et al.,* 2000).

Carnegie, 2007 recorded several foliar fungi from eucalypt plantations in New South Wales during a forest health survey between 1996-2005, these are: ***Aulographina eucalypti*** (*Thyrinula eucalypti*); *Botryosphaeria eucalyptorum* (*Neofusicoccum eucalyptorum*); *Colletotrichum gloeosporioides*; *Coniothyrium kallangurense*; ***Cryptosporiopsis eucalypti***; *Fairmeniella leprosa*; *Harknesia fumaginea*; *Harknessia eucalypti*; *Kirramyces corymbiae*; ***Kirramyces epicoccoides***; *Kirramyces eucalypti*; *Lembosina corymbiae*; *Lembosina eucalypticola*; *Microsphaeropsis conielloides*; *Mycosphaerella associate*; ***M. cryptica***; *Mycosphaerella 'eucalypti'*; *Mycosphaerella excentricum*; ***M. marksii***; ***M. nubilosa***; ***M. parva***; *Mycosphaerella suberosa*; *Mycosphaerella tumulosa*; *Pseudocercospora* sp.; ***M. walkeri*** (*Sonderhenia eucalyptorum*); *Pestalotiopsis* sp.; *Phaeothyriolum microthyrioides*; ***Pilidiella eucalyptorum***; *Pseudocercospora subulata*; *Pseudocercospora* sp.; ***Readeriella eucalypti***; *Readeriella mirabilis*; *Quambalaria pitereka*; *Quambalaria eucalypti*; *Schizothyrium* sp.; *Stagonospora* sp.; *Stigmina eucalypti*; *Trimmatostroma excentricum* and *Vermisporium falcatum* (the ones in bold occur in *E. globulus*). However, *Mycosphaerella* spp. is considered to be the main responsible of foliar fungal diseases of eucalypt plantations in Australia (Park, 1984; Park, 1988a; Park *et al.,* 2000; Carnegie, 2007).

1.2.3 The genus *Mycosphaerella*

Mycosphaerella Johanson is a large genus which belongs to the class *Ascomycotina*, subclass *Dothideomycetidae*, and order *Mycosphaerellales* (Nannf.) P. F. Cannon and family *Mycosphaerellaceae* (Kirk *et al.*, 2001). More than 3000 species are included on *Mycosphaerella* (Aptroot, 2006)

The first *Mycosphaerella* species was described by Persoon in 1794 on dead leaves of *Corylus* as *Sphaeria corylea* Pers.. Persoon (1801) found more species on other plants and afterwards changed to *Sphaeria maculiformis* Pers. (Aptroot, 2006).

In the 20th century it was decided that the genus name *Sphaerella* had been inopportune, for the reason that it was also used for some green algae and it was changed to *Mycosphaerella*, although some mycologists refused to accept this change (Aptroot, 2006).

1.2.3.1 Morphology

The ascospores contain the most useful morphological characters in the group and are used for taxonomic classification (Table 1) (Aptroot, 2006).

1.2.3.2 Symptoms and climatic conditions

Mycosphaerella spp. and its anamorphs have turned into one of the main diseases affecting eucalypts plantations worldwide (Crous, 1998). It is considered the most significant virulent pathogens of *Eucalyptus* genus and is expected to become gradually more important in the future (Crous *et al.*, 2004).

Table 1 – Morphology of the *Mycosphaerella* spp. (adapted from Aptroot, 2006).

Asci	Always contains 8 ascospores, Arranged irregularly uniseriate to irregularly biseriate or multiseriate (in one bundle). The clavate asci hold an intermediate position. Occur in 3 main types: - viz. pyriform; - cylindrical; - cylindrico-clavate.
Ascomata	Invariably carbonized; Uniformly rather large-cellular with parenchymatous cells; Can be regularly dispersed or aggregated. 13 ascomata simple
Ascospores	Always hyaline; Remain uncoloured after discharge (in most species) Uniseptate; In most of the groups, ascospores are: - biseriate when asci are pyriform, - uniseriate when asci are cylindrical. The septum: - median - conspicuously supramedian - slightly submedian. 3-Septate ascospores (a few taxa). Shape – pyriform The upper and lower ends of the ascospores: - upper end rounder, lower end pointed The wall is thin;
Ascus type	Is always constant within a specimen Size depends on the shape and size of the ascospores it contains.
Ascus wall	The thickness is dependent on the size of the ascospores; (in addition not very constant within even one ascus).
Anamorphs	Variable in the group
Clypens	To not formation.
Hamathecium filaments (periphyses, paraphyses, pseudoparaphyses, paraphysoids)	Always absent
Ostiole	Always apical; The cells are regularly arranged around it.
The wall	Extends also below the hamathecium.

Species identification in this genus is not easy because frequently it is found 4 to 5 species in the same lesion (Crous *et al.*, 2004). Besides, they are host-specific pathogens which grow very weakly in culture, generate small fruiting structures with very conserved morphology (Hunter *et al.*, 2006b).

Mycosphaerella spp. causes loss in photosynthetic area and can lead to defoliation. Other symptoms include cankers (branch lesions), premature branch death, shoot dieback and in some few cases, tree death. These symptoms can result in wood volume and growth rate decrease. The disease affects mainly young trees of eucalypts with juvenile-phase foliage and is relatively easy to assess (Carnegie & Ades, 2005).

The climatic conditions to increase the chances of successful infection are warm wet weather. The effect of temperature during infection on the percentage of leaf area of eucalyptus that was infected, was measured with ascospores and conidia in *M. cryptica* and *M. nubilosa* and the best temperatures for ascospores and conidia infection varied between 15°C and 20°C (Park, 1988a). In Australia it was reported that the major infection disease peak in some species occurs in the summer, especially during warm weather (Park, 1988b; Carnegie & Ades, 2005).

Mycosphaerella spp. which is found on eucalypts seems to be specific and considered to be carried by seeds (Burgess, *et al.*, 2007). Many *Mycosphaerella* species which infect eucalyptus worldwide have not been recorded in Australia (Crous, 1998; Crous *et al.*, 2004, 2006) like *M. molleriana* and may appear on eucalypts outside Australia (Crous, 1998; Crous *et al.*, 2004, 2006).

1.2.3.3 Species of *Mycosphaerella* discovered on *Eucalyptus* spp. worldwide

There are approximately 62 species of *Mycosphaerella* and about 34 anamorphs in *Eucalyptus* spp. worldwide (Park & Keane, 1982a; Park & Keane 1984; Crous *et al.*1993a; Crous *et al.* 1993b; Carnegie & Keane 1994; Crous & Alfenas 1995; Crous & Swart 1995; Crous *et al.*, 1995; Crous & Wingfield 1996; Carnegie & Keane 1997; Crous & Wingfield, 1997a; Crous & Wingfield 1997b; Carnegie & Keane, 1998; Crous 1998; Crous *et al.*,1998; Dick & Dobbie 2001;

Maxwell *et al.*, 2003; Crous *et al.*, 2004; Hunter *et al.*, 2004; Crous *et al.*, 2006; Burgess, *et al.*, 2007; Carnegie *et al.*, 2007).

Mycosphaerella spp. was recorded outside Australia for the first time in 1881 in Portugal and classified as *Sphaerella molleriana* Thüm. in *Eucalyptus globules* (Crous & Wingfield, 1997a; Crous, 1998), and is nowadays known as *M. molleriana*. Until the 20th century there were only 4 species recorded, *M. molleriana*; *M. cryptica*; *Mycosphaerella suttoniae* Crous & M.J. Wingf. and *M. nubilosa* (Crous *et al.*, 1995; Crous & Wingfield, 1997a; Crous & Wingfield 1997b; Crous 1998; Aptroot, 2006). Later, in the 20th century more 27 species were recorded (Park & Keane, 1982a; Park & Keane 1984; Crous *et al.*1993a; Crous *et al.* 1993b; Carnegie & Keane 1994; Crous & Alfenas 1995; Crous & Swart 1995; Crous & Wingfield 1996; Carnegie & Keane 1997; Crous & Wingfield 1997b; Carnegie & Keane 1998; Crous 1998; Crous *et al.*,1998) and in the last seven years approximately 31 species were recorded as new species in *Eucalyptus* spp. (Dick & Dobbie 2001; Maxwell *et al.*, 2003; Crous *et al.*, 2004; Hunter *et al.*, 2004; Crous *et al.*, 2006; Burgess, *et al.*, 2007; Carnegie *et al.*, 2007). A review of all species of *Mycosphaerella* in *Eucalyptus* spp. and its anamorphs was showed with the respective identification year which were recorded and their respective bibliographic references are showed in Table 2. Also, with the countries where species were recorded and their respective bibliographic references in Table 3.

Table 2 - Review of all species of *Mycosphaerella* in *Eucalyptus* spp. worldwide and its anamorphs with the respective identification year which were recorded.

Identification Year	Species	Reference
1881 ≡ *Sphaerella molleriana* Thüm.	***M. molleriana*** (Thüm.) Lindau (anamorph *Colletogloeopsis molleriana*)	Aptroot, 2006; Crous & Wingfield, 1997; Crous *et al.*, 1995;
1891 ≡ *Sphaerella cryptica* Cooke	***M. cryptica*** (Cooke) Hansf. (anamorph *Colletogloeopsis nubilosum*)	Crous *et al.*, 1995
≡ Cercospora epicoccoides Cooke & Massee apud Cooke	***M. suttoniae*** Crous & M.J. Wingf. (anamorph *Phaeophleospora epicoccoides*)	Crous 1998; Crous & Wingfield 1997b
1892 ≡ *Sphaerella nubilosa* Cooke	***M. nubilosa*** (Cooke) Hansf. (anamorph *Uwebraunia juvenis*)	Crous 1998
1918	***M. aggregata*** Carnegie & Keane (anamorph unknown)	Crous 1998; Carnegie & Keane 1994
1982	***M. parva*** R.F. Park & Keane (anamorph unknown)	Park & Keane, 1982a
1984	***M. delegatensis*** Park & Keane (anamorph *Phaeophleospora delegatensis*) ***M. swartii*** Park & Keane (anamorph *Sonderhenia eucalyptorum*) ***M. walkeri*** Park & Keane (anamorph *Sonderhenia eucalypticola*)	Park & Keane 1984
1993	***M. suberosa*** Crous, F.A.Ferreira, Alfenas & M.J.Wingf. (anamorph unknown)	Crous *et al.* 1993a
	M. parkii Crous, M.J.Wingf., F.A.Ferreira & Alfenas (anamorph *Stenella parkii*)	Crous *et al.* 1993b
1994	***M. grandis*** Carnegie & Keane (anamorph unknown) ***M. marksii*** Carnegie & Keane (anamorph *Pseudocercospora epispermogonia*)	Carnegie & Keane 1994
1995	***M. gracilis*** Crous & Alfenas (anamorph *Pseudocercospora gracilis*)	Crous & Alfenas 1995
	M. heimii Bouriquet ex Crous (anamorph *Pseudocercospora heimii*)	Crous & Swart 1995

Table 2 - Review of all species of *Mycosphaerella* in *Eucalyptus* spp. worldwide and its anamorphs with the respective identification year which were recorded (continuation).

Described Year	Species	Reference
1996	***M. africana*** Crous & M.J. Wingf. (anamorph unknown) ***M. crystallina*** Crous & M.J. Wingf. (anamorph *Pseudocercospora crystallina*) ***M. ellipsoidea*** Crous & M.J. Wingf. (anamorph *Uwebraunia ellipsoidea*) ***M. juvenis*** Crous & M.J. Wingf. (anamorph *Uwebraunia juvenis*) ***M. lateralis*** Crous & M.J. Wingf. (anamorph *Dissoconium dekkeri*)	Crous & Wingfield 1996
1997	***M. gregaria*** Carnegie & Keane (anamorph unknown)	Carnegie & Keane 1997
	M. irregularimosa Crous & M.J. Wingf. (anamorph *Pseudocercospora irregularimosa*) ***M. heimioides*** Crous & M.J. Wingf. (anamorph *Pseudocercospora heimioides*)	Crous & Wingfield 1997b
1998	***M. colombiensis*** Crous & M.J. Wingf. (anamorph *Pseudocercospora colombiensis*) ***M. endophytica*** Crous & H. Smith (anamorph *Pseudocercosporella endophytica*) ***M. flexuosa*** Crous & M.J. Wingf. (anamorph unknown) ***M. keniensis*** Crous & T. Coutinho (anamorph unknown) ***M. longibasilis*** Crous & M.J. Wingf. (anamorph unknown) ***M. mexicana*** Crous (anamorph unknown)	Crous 1998
	M. tasmaniensis Crous & M.J. Wingf. (anamorph *Mycovellosiella tasmaniensis*)	Crous *et al.*,1998
	M. vespa Carnegie & Keane (anamorph unknown)	Carnegie & Keane, 1998
2001	***M. intermedia*** M.A. Dick & Dobbiey (anamorph unknown)	Dick & Dobbie 2001
2003	***M. ambiphylla*** A. Maxwell (anamorph *Phaeophleospora sp.*) ***M. aurantia*** A. Maxwell (anamorph unkown)	Maxwell *et al.*, 2003

Table 2 - Review of all species of *Mycosphaerella* in *Eucalyptus* spp. worldwide and its anamorphs with the respective identification year which were recorded (continuation).

Described Year	Species	Reference
2004	***M. communis*** Crous & J.P. Mansilla (*Dissoconium commune*) ***M. madeirae*** Crous & Denman (*Pseudocercospora fide*) ***M. ohnowa*** Crous & M. J. Wingf. (anamorph unknown) ***M. readeriellophora*** Crous & J.P. Mansilla (*Readeriella readeriellophora*) ***M. toledana*** Crous & G. Bills (*Phaeophleospora toledana*)	Crous *et al.*, 2004a; Aptroot, 2006
	M. fori G. C. Hunter, Crous & M. J. Wingf. (*Pseudocercospora* sp.)	Hunter *et al.*, 2004
2006	***M. davisoniellae*** Crous (anamorph *Davisoniella eucalypti*) ***M. eucalyptorum*** Crous & M.J. Wingf. (anamorph unkown) ***M. gamsii*** Crous (anamorph unkown) ***M. perpendicularis*** Crous & M.J. Wingf. (anamorph unkown) ***M. pluritubularis*** Crous & J.P. Mansilla (anamorph unkown) ***M. pseudoafricana*** Crous & T. Coutinho (anamorph unkown) ***M. pseudocryptica*** Crous (anamorph *Colletogloeopsis* sp.) ***M. pseudoendophytica*** Crous & G. Hunter (anamorph *Pseudocercosporella* sp.) ***M. pseudosuberosa*** Crous & M.J. Wingf. (anamorph *Trimmatostroma* sp.) ***M. quasicercospora*** Crous & T. Coutinho (anamorph unkown) ***M. scytalidii*** Crous & M.J. Wingf. (anamorph *Stenella* sp., synanamorph, Scytalidium-like.) ***M. secundaria*** Crous & A.C. Alfenas (anamorph unkown) ***M. stramenti*** Crous & A.C. Alfenas (anamorph unkown) ***M. stramenticola*** Crous & A.C. Alfenas (anamorph unkown) ***M. sumatrensis*** Crous & M.J. Wingf. (anamorph unkown) ***M. verrucosiafricana*** Crous & M.J. Wingf. (anamorph unkown)	Crous *et al.*, 2006

Table 2 - Review of all species of *Mycosphaerella* in *Eucalyptus* spp. worldwide and its anamorphs with the respective identification year which were recorded (continuation).

Described Year	Species	Reference
2007	***M. multiseptata*** Carnegie (anamorph unknown) ***M. pseudovespa*** Carnegie (anamorph unknown) ***M. tumulosa*** Carnegie & Beilharz (anamorph *Pseudocercospora* sp.)	Carnegie *et al.*, 2007
	M. obscuris Barber & T.I. Burgess (anamorph *Pseudocercospora* sp.) ***M. vietnamensis*** Barber & T.I. Burgess (anamorph *Pseudocercospora* sp.) ***M. yunnanensis*** Barber, Dell & T.I. Burgess (anamorph unknown)	Burgess, *et al.*, 2007

Table 3 - Review of all species of *Mycosphaerella* in *Eucalyptus* spp. worldwide and its anamorphs with the countries where species were recorded.

Mycosphaerella species	Anamorph	Host	Occurrence	Reference
M. africana Crous & M.J. Wingf.	unknown	*E. deanii* *E.globulus* *E.grandis* *E.radiata* *E.viminalis*	Colombia **Portugal** South Africa Zambia	Crous & Wingfield 1996; Crous, 1998
M. aggregata Carnegie & Keane	unknown	*E.grandis*	Australia	Carnegie & Keane 1994
M. ambiphylla A. Maxwell	*Phaeophleospora sp.*	*E.globulus*	Australia	Maxwell *et al.*, 2003
M. aurantia A. Maxwell	unknown	*E.globulus*	Australia	Maxwell *et al.*, 2003
M. colombiensis Crous & M.J. Wingf.	*Pseudocercospora colombiensis*	*E. urophylla*	Colombia	Crous, 1998
M. communis Crous & J.P. Mansilla	*Dissoconium commune*	*Eucalyptus sp.* *E.globulus*	**Portugal** Spain	Crous *et al.*, 2004, 2006
M. cryptica (Cooke) Hansf.	*Colletogloeopsis nubilosum*	Approximately 50 species of Eucalypts	Australia Chile New Zealand	Ganapathi & Corbin, 1979; Crous *et al.*, 1995; Wingfield *et al.* 1995; Crous & Wingfield 1997a; Crous 1998
M. crystallina Crous & M.J. Wingf.	*Pseudocercospora crystallina*	*E. bicostata* & *E. grandis* x *camaldulensis*	South Africa	Crous & Wingfield 1996; Crous 1998
M. davisoniellae Crous	*Davisoniella eucalypti*	*E. marginata*	Australia	Crous *et al.*, 2006
M. delegatensis Park & Keane	*Phaeophleospora delegatensis*	*E. delegatensis* *E. obliqua*	Australia	Park & Keane 1984; Crous 1998
M. ellipsoidea Crous & M.J. Wingf.	*Uwebraunia ellipsoidea*	*E. cladocalyx*	South Africa	Crous & Wingfield 1996; Crous 1998
M. endophytica Crous & H. Smith	*Pseudocercosporella endophytica*	*Eucalyptus* sp. *E. grandis*	South Africa	Crous 1998
M. eucalyptorum Crous & M.J. Wingf.	*Unknown*	*Eucalyptus* sp.	Indonesia	Crous *et al.*, 2006
M. flexuosa Crous & M.J. Wingf.	*Unknown*	*E. globulus*	Colombia	Crous 1998
M. fori G. C. Hunter, Crous & M. J. Wingf.	*Pseudocercospora* sp.	*E. grandis*	South Africa	Hunter *et al.*, 2004
M. gamsii Crous	*Unknown*	*Eucalyptus* sp.	India	Crous *et al.*, 2006
M. gracilis Crous & Alfenas	*Pseudocercospora gracilis*	*E. globulus* *E. urophylla*	Indonesia	Crous & Alfenas 1995; Crous 1998

Table 3 - Review of all species of *Mycosphaerella* in *Eucalyptus* spp. worldwide and its anamorphs with the countries where species were recorded (continuation).

Mycosphaerella species	Anamorph	Host	Occurrence	Reference
M. grandis Carnegie & Keane	*Unknown*	*E. grandis* *E. nitens* *E. globulus*	Australia **Portugal**	Carnegie & Keane 1994
M. gregaria Carnegie & Keane	*Unknown*	*E. botryoides,* *E. grandis* *C. maculata* *E. saligna*	Australia	Carnegie & Keane 1997; Crous 1998
M. heimii Bouriquet ex Crous	*Pseudocercospora heimii*	*E. obliqua* *E.urophylla* *Eucalyptus* sp.	Brazil Indonesia, Madagascar **Portugal**	Crous & Swart 1995; Crous 1998; Crous *et al.*, 2006
M. heimioides Crous & M.J. Wingf.	*Pseudocercospora heimioides*	*Eucalyptus* sp.	Indonesia	Crous & Wingfield 1997b; Crous 1998
M. intermedia M.A. Dick & Dobbie	*Unknown*	*E. muelleriana*	New Zealand	Dick & Dobbie 2001
M. irregularimosa Crous & M.J. Wingf.	*Pseudocercospora irregularimosa*	*E. saligna*	Indonésia South Africa	Crous & Wingfield 1997b; Crous 1998
M. juvenis Crous & M.J. Wingf. = *M. nubilosa*	*Uwebraunia juvenis*	*E. globulus* *E. grandis* *E. nitens*	Kenya South Africa Tanzania Zambia	Crous & Wingfield 1996; Crous 1998; Crous *et al.*, 2004
M. keniensis Crous & T. Coutinho	*Unknown*	*E. grandis*	Kenya	Crous 1998
M. lateralis Crous & M.J. Wingf.	*Dissoconium dekkeri*	*E. globulus* *E. grandis x saligna* *E. saligna* *E. nitens* *E.grandis* *E.maidenii* *Eucalyptus sp.*	Australia, **Portugal** South Africa Zambia	Crous & Wingfield 1996; Crous 1998; Maxwell *et al.* 2000; Crous *et al.*, 2006
M. longibasilis Crous & M.J. Wingf.	*Unknown*	*E. grandis*	Colombia	Crous 1998
M. madeirae Crous & Denman,	*Pseudocercospora fide*	*E. globulus*	**Portugal** (Madeira)	Crous *et al.*, 2004; Aptroot, 2006
M. marksii Carnegie & Keane = *M. intermedia*	*Pseudocercospora epispermogonia*	*E. botryoides* *E. fraxinoides* *E. globulus* *E. grandis* *E. nitens* *E. quadrangulata* *E. saligna*	Australia Indonesia **Portugal** (Madeira) South Africa	Carnegie & Keane 1994; Crous & Wingfield 1996; Crous 1998; Crous *et al.*, 2006; Hunter, *et al.*, 2006
M. mexicana Crous	*Unknown*	*Eucalyptus* sp. *E.globulus*	Mexico, Australia	Crous 1998; Maxwell *et al.*, 2003

Table 3 - Review of all species of *Mycosphaerella* in *Eucalyptus* spp. worldwide and its anamorphs with the countries where species were recorded (continuation).

Mycosphaerella species	Anamorph	Host	Occurrence	Reference
M. molleriana (Thūm.) Lindau = *M. ambiphylla* = *M. vespa*	*Colletogloeopsis molleriana*	*E. globulus*	**Portugal** Spain U.S.A.	Crous & Wingfield 1997a; Crous 1998; Crous *et al.*, 2006; Hunter, *et al.*, 2006
M. multiseptata Carnegie	unknown	*E. subvelutina*	Australia	Carnegie *et al.*, 2007
M. nubilosa (Cooke) Hansf. = *M. juvenis*	*Uwebraunia juvenis*	*E. botryoides* *E. bridgesiana* *E. cypellocarpa* *E.globulus* *E. grandis* *E.nitens* *E. uadrangulata* *E. viminalis*	Australia **Portugal** Spain New Zealand	Crous, 1998; Crous *et al.*, 2004, 2006
M. obscuris Barber & T.I. Burgess	*Pseudocercospora* sp.	*E.pellita*, *Eucalyptus* spp.	Vietnam, Indonesia	Burgess, *et al.*, 2007
M. ohnowa Crous & M. J. Wingf.,	*Unknown*	*E. grandis* *E. smithii*	South Africa	(Crous *et al.*, 2004)
M. parkii Crous, M.J.Wingf., F.A.Ferreira & Alfenas	*Stenella parkii*	*E. grandis* *E. saligna* *E. globulus*	Brazil Colombia Indonesia	Crous *et al.* 1993b; Crous & Alfenas, 1995; Crous, 1998
M. parva R.F. Park & Keane	*Unknown*	*E. globulus* *E. grandis*	Australia **Portugal** Spain	Park & Keane, 1982a; Crous 1998; Crous *et al.*, 2006
M. perpendicularis Crous & M.J. Wingf.	*Unknown*	*E. eurograndis*	Colombia	Crous *et al.*, 2006
M. pluritubularis Crous & J.P. Mansilla	*Unknown*	*E. globulus*	Spain	Crous *et al.*, 2006
M. pseudoafricana Crous & T. Coutinho	*Unknown*	*E. globulus*	Zambia	Crous *et al.*, 2006
M. pseudocryptica Crous	*Colletogloeopsis* sp.	*Eucalyptus* sp.	New Zealand	Crous *et al.*, 2006
M. pseudoendophytica Crous & G. Hunter	*Pseudocercosporella* sp.	*E. nitens*	South Africa	Crous *et al.*, 2006
M. pseudosuberosa Crous & M.J. Wingf.	*Trimmatostroma* sp.	*Eucalyptus* sp.	Uruguay	Crous *et al.*, 2006
M. pseudovespa Carnegie	unknown	*E. biturbinata*	Australia	Carnegie *et al.*, 2007
M. quasicercospora Crous & T. Coutinho	*Unknown*	*E. maidenii*	Tanzania	Crous *et al.*, 2006
M.readeriellophora Crous & J.P. Mansilla,	*Readeriella readeriellophora*	*E. globulus*	Spain	Crous *et al.*, 2004

Table 3 - Review of all species of *Mycosphaerella* in *Eucalyptus* spp. worldwide and its anamorphs with the countries where species were recorded (continuation).

Mycosphaerella species	Anamorph	Host	Occurrence	Reference
M. scytalidii Crous & M.J. Wingf.	*Stenella* sp., (synanamorph, Scytalidium-like)	*E. urophylla*	Brazil Colombia	Crous *et al.*, 2006
M. secundaria Crous & A.C. Alfenas	*Unknown*	*Eucalyptus* spp.	Brazil Colombia	Crous *et al.*, 2006
M. stramenti Crous & A.C. Alfenas	*Unknown*	*Eucalyptus* sp.	Brazil	Crous *et al.*, 2006
M. stramenticola Crous & A.C. Alfenas	*Unknown*	*Eucalyptus* sp.	Brazil	Crous *et al.*, 2006
M. suberosa Crous, F.A.Ferreira, Alfenas & M.J.Wingf.	*Unknown*	*E. dunnii* *E. globulus* *E. grandis* *E. muelleriana* *E. molluccana* *E. viminalis*	Australia Brazil Colombia Indonesia Spain New Zealand	Crous *et al.*1993a; Carnegie *et al.* 1997; Crous & Wingfield, 1997a; Crous 1998; Dick & Dobbie 2001
M. sumatrensis Crous & M.J. Wingf.	*Unknown*	*Eucalyptus* sp.	Indonesia	Crous *et al.*, 2006
M. suttonii Crous & M.J. Wingf. (as the feminine "*suttoniae*")	*Phaeophleospora epicoccoides* *Kirramyces epicoccoides*	Approximately 30 species of Eucalypts and *Eucalyptus* sp.	Argentina Australia Bhutan Brazil, Ethiopia Hong Kong Indonesia Italy Madagascar Malawi New Zealand	Sankaran *et al.*, 1995; Crous & Wingfield 1997b; Crous 1998; Aptroot, 2006; Crous *et al.*, 2006
M. swartii Park & Keane	*Sonderhenia eucalyptorum*	*E. coccifera* *E. delegatensis,* *E. dives* *E. elata* *E. fastigata* *E. globoidea* *E. leucoxylon* *E. nitens* *E. obliqua*	Australia New Zealand	Park & Keane 1984; Crous 1998
M. tasmaniensis Crous & M.J. Wingf.	*Mycovellosiella tasmaniensis*	*E. nitens*	Australia	Crous 1998; Crous *et al.*, 1998
M. toledana Crous & G. Bills,	*Phaeophleospora toledana*	*Eucaliptus* sp.	Spain	Crous *et al.*, 2004
M. tumulosa Carnegie & Beilharz	*Pseudocercospora* sp.	*E. moluccana*	Australia	Carnegie *et al.*, 2007
M. verrucosiafricana Crous & M.J. Wingf.	*Unknown*	*Eucalyptus* sp.	Indonesia	Crous *et al.*, 2006

Table 3 - Review of all species of *Mycosphaerella* in *Eucalyptus* spp. worldwide and its anamorphs with the countries where species were recorded (continuation).

Mycosphaerella species	Anamorph	Host	Occurrence	Reference
M. vespa Carnegie & Keane = *M. ambiphylla* = *M. molleriana*	*Coniothryium ovatum*	*E. globulus* *E. viminalis* *Eucalyptus* sp.	Australia **Portugal**	Carnegie & Keane, 1998; Milgate *et al.* 2001; Hunter, *et al.*, 2006
M. vietnamensis Barber & T.I. Burgess	*Pseudocercospora* sp.	*E. camaldulensis* *E. grandis*	Vietnam	Burgess, *et al.*, 2007
M. walkeri Park & Keane	*Sonderhenia eucalypticola*	*E. cladocalyx, E. globulus* *E. maidenii* *E. nitens* *E. polyanthemos* *Eucalyptus* sp.	Australia Chile Colombia Ecuador **Portugal** Spain New Zealand	Park & Keane 1984; Wingfield *et al.*, 1995; Crous 1998
M. yunnanensis Barber, Dell & T.I. Burgess	unknown	*E. urophylla*	South-west China	Burgess, *et al.*, 2007

(=) Synonymous

1.2.3.4 *Mycosphaerella* spp. reported in Portugal

In 1881 *M. molleriana* was recorded for the first time in Portugal (Crous & Wingfield, 1997a; Crous, 1998) like was said before, but during the nineties some other species were reported, such as, *M. walkeri* (Crous, 1998) and *M. africana* (Crous, 1998).

In 2004, a new species was also recorded in Madeira, *M. madeirae* (Crous *et al.*, 2004). In 2006, other species were reported: *M. communis*, *Mycosphaerella heimii* Bouriquet ex Crous, *M. lateralis* Crous & M.J. Wingf., *M. marksii*, *M. nubilosa*, *M. parva* (Crous *et al.*, 2006) (Table 3). However, serious damage on commercial *Eucalyptus* plantations was only reported on recent years when repeated and serious defoliation of young *E. globulus* were observed (1998) (Carlos Valente, *personal communication*).

Table 3 - Review of all species of *Mycosphaerella* in Portugal with respective year, site of collection and year and references.

Leaves collected	Recorded	Site in Portugal	Species	References
-	1881	-	***Mycosphaerella molleriana*** (Thüm.) Lindau	Crous *et al.*, 1995; Crous & Wingfield, 1997; Aptroot, 2006
Jul. 1995	1997	Abrantes		Crous & Wingfield 1997a
-	2006	-		Crous *et al.*, 2006
Jun. 1995	1998	-	***Mycosphaerella walkeri*** R. F. Park & Keane	Crous, 1998
Jun. 1995	1998	-	***Mycosphaerella africana*** Crous & M. J. Wingf.	Crous, 1998
Apr. 2000	2004	Madeira	***Mycosphaerella madeirae*** Crous & Denman	Crous *et al.*, 2004
-	2006	-	***Mycosphaerella communis*** Crous & J.P. Mansilla	Crous *et al.*, 2006
Mar. 2005	2007	Aveiro		Silva *et al.*, *submitted*
-	2006	-	***Mycosphaerella heimii*** Bouriquet ex Crous	Crous *et al.*, 2006
-	2006	-	***Mycosphaerella lateralis*** Crous & M.J. Wingf.	Crous *et al.*, 2006
Mar. 2005	2007	Aveiro		Silva *et al.*, *submitted*
-	2006	Madeira	***Mycosphaerella marksii*** Carnegie & Keane	Crous *et al.*, 2006
-	2006	-	***Mycosphaerella nubilosa*** (Cooke) Hansf.	Crous *et al.*, 2006
Nov. 2004	2007	Torres Vedras		Silva *et al.*, *submitted*
Mar. 2005	2007	Torres Vedras		Silva *et al.*, *submitted*
-	2006	-	***Mycosphaerella parva*** R.F. Park & Keane	Crous *et al.*, 2006
Mar. 2005	2007	Aveiro		Silva *et al.*, *submitted*
Mar. 2005	2007	Torres Vedras		Silva *et al.*, *submitted*
Mar. 2005	2007	Aveiro	***Mycosphaerella grandis*** Carnegie & Keane	Silva *et al.*, *submitted*
Mar. 2005	2007	Aveiro	***Mycosphaerella vespa*** Carnegie & Keane	Silva *et al.*, *submitted*
Mar. 2005	2007	Torres Vedras		

1.2.3.5 Multi-gene phylogeny for species of *Mycosphaerella*

Initially Parl & Keane (1982a) established the ascospores germination patterns as a tool to help species identification followed by Crous (1998). However, morphological identification stills difficult. So, it must be completed by molecular tools.

Different approaches using molecular biology have been done. Firstly, based mostly on the phylogeny of the Internal Transcribed Spacer (ITS) region (Crous *et al.*, 2001a, 2001b; Crous *et al.*, 2004 and 2006; Hunter *et al.*, 2004). Carnegie *et al.* (2001) used RAPD-PCR (Random Amplitication of Polymorphic DNA) to analyse differences in *Mycosphaerella* species and afterwards Kularatne *et al.* (2004) designed species-specific primers - RFLP (Restriction fragment length polymorphism) to differentiate *Mycosphaerella* species associated with MLD on *E. globulus*. Furthermore Maxwell *et al.* (2005) developed species-specific primers for the identification and detection on the ITS1, 5.8S, and ITS2 domain of the rDNA gene, specially for *M. cryptica*, *M. lateralis*, *M. marksii*, *M. nubilosa* and *M. parva*. Hunter *et al.* (2006b) showed that sequences for the ITS region give sufficient resolution to distinguish most taxa except for critical taxa and used DNA sequences for ITS, Elongation factor 1-alpha (EF-1α), Actin (ACT) and Large Subunit (LSU) of the rRNA operon and combined data of all regions. Recently, Hunter *et al.* (2006a) with the aim of helping the understanding of the genetics and the distribution of *M. nubilosa* population, used polymorphic microsatellite markers.

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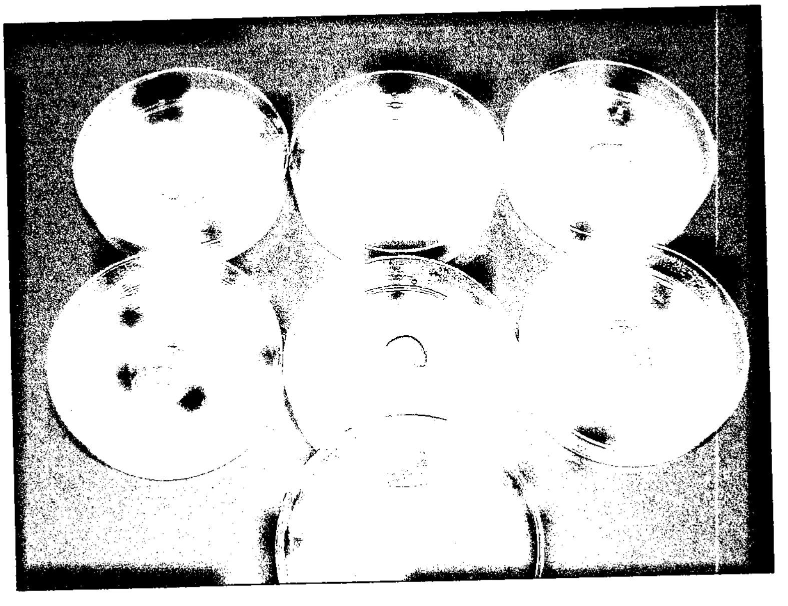

CHAPTER 2

Mycosphaerella species occurring on *Eucalyptus globulus* in Portugal

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Key words

Ascomycetes, ITS, *Mycosphaerella* Leaf Disease, Pathology, ribosomal RNA operon.

Abbreviations

AGE - agarose gel electrophoresis
CI - consistency index
DNA – desoxy ribonucleic acid
DTT - dithiothreitol
EDTA - ethylenediaminetetraacetic acid
HI - homoplasy index
ITS - internal transcribed spacer
MEA - malt extract agar
MLD - *Mycosphaerella* leaf disease
MP - maximum parsimony
NJ - neighbour-joining
PCR – Polymerase chain reaction
rDNA - ribosomal DNA
TBR - tree bisection and reconnection
TL - tree length
RI - retention index
UV - ultraviolet

Abstract

Mycosphaerella leaf disease is caused by species of *Mycosphaerella* and several anamorphic form *genera* that have been connected to *Mycosphaerella*. Until recently, *Mycosphaerella* leaf disease of eucalypts was largely ignored in Portugal. Serious damage to *Eucalyptus globulus* was only reported in recent years when frequent and severe defoliation of young *E. globulus* was observed. The severity of this disease prompted a preliminary study of the *Mycosphaerella* species associated with major symptoms of a leaf blotch disease in commercial plantations of *E. globulus* in Portugal, which is presented here. The species were identified by molecular methods based on the ITS1-5.8S-ITS2 cluster, together with morphological characterization. In addition to confirming the species previously recorded, two further species (*Mycosphaerella grandis* and *Mycosphaerella vespa*) are reported for the first time from Portugal.

Introduction

Commercial plantations of eucalypts in Portugal are almost exclusively of *Eucalyptus globulus* Labill., covering 646 700 ha, which represents 20.6% of the total forested area (DGRF, 2007). Plantations are concentrated in the wetter coastal regions where productivity is higher, and attack by pathogens is generally low. However, serious damage to *E. globulus* was reported in recent years when repeated and serious defoliation of young *E. globulus* trees was observed in Aveiro. The principle cause of this disease was suspected to be *Mycosphaerella* (Carlos Valente, *personal communication*).

Approximately 62 species of *Mycosphaerella*, and about 34 anamorphs have been reported associated with *Mycosphaerella* leaf disease of *Eucalyptus* spp. worldwide (Crous, 1998; Dick & Dobbie, 2001; Crous *et al.*, 2004, 2006; Maxwell *et al.*, 2003; Hunter *et al.*, 2004; Burgess *et al.*, 2007; Carnegie *et al.*, 2007). Morphological identification of *Mycosphaerella* species and their anamorphs is complicated by the problems with preparing single spore cultures and the slow growth of the fungus in culture. Furthermore, morphological characteristics and ascospores germination patterns are similar for several species (Crous *et al.*, 2004) and some anamorphs do not sporulate easily or do not sporulate at all. For these reasons, it is often impossible to identify *Mycosphaerella* species on eucalypts without DNA sequences analyses (Crous *et al.*, 2006). An additional complication is that several species can occupy the same lesion (Crous *et al.*, 2004).

Until 1892 only four *Mycosphaerella* species were known, namely, *Mycosphaerella molleriana* (Thüm.) Lindau, *Mycosphaerella cryptica* (Cooke) Hansf., *Mycosphaerella suttoniae* Crous & M.J. Wingf. (described as *Cercospora epicoccoides* Cooke & Massee) and *Mycosphaerella nubilosa* (Cooke) Hansf.. Between 1918 and 1998, 27 more species were described (Park & Keane, 1982a; Park & Keane, 1984; Crous *et al.*,1993a; Crous *et al.*, 1993b; Carnegie & Keane, 1994; Crous & Alfenas, 1995; Crous & Swart, 1995; Crous & Wingfield, 1996; Carnegie & Keane, 1997; Crous & Wingfield, 1997a; Crous & Wingfield, 1997b; Crous, 1998; Crous *et al.*, 1998; Carnegie & Keane, 1998). In the last seven years approximately 31 species have been described as new on eucalypts (Dick & Dobbie, 2001; Maxwell *et al.*, 2003; Crous *et al.*, 2004a; Hunter *et al.*, 2004; Crous *et al.*, 2006; Carnegie *et al.*, 2007; Burgess, *et al.*, 2007). This increase in the number of described species in recent years is related to the application of molecular techniques that can differentiate similar morphological species.

The first report of *Mycosphaerella* on eucalypts outside of Australia was in Portugal in 1881 when *M. molleriana* was described from a specimen collected from Lusitania (Crous & Wingfield, 1997a). During the 1990's two further species were reported from Portugal, namely, *Mycosphaerella africana* Crous & M. J. Wingf. and *Mycosphaerella walkeri* R. F. Park & Keane (Crous, 1998). In 2004 *Mycosphaerella madeirae* Crous & Denman was described as a new species from Madeira (Crous *et al.*, 2004). Two years later, *Mycosphaerella communis* Crous & J.P. Mansilla, *Mycosphaerella heimii* Bouriquet & Crous, *Mycosphaerella lateralis* Crous & M.J. Wingf., *Mycosphaerella marksii* Carnegie & Keane, *M. nubilosa* and *Mycosphaerella parva* R.F. Park & Keane were reported as new records from Portugal (Crous *et al.*, 2006).

The above reports of *Mycosphaerella* species on Eucalypts in Portugal were based on mainly random collections and there has been no systematic survey of these pathogens in this country. Therefore, the aim of this study was to make a preliminary study of the *Mycosphaerella* species associated with *Mycosphaerella* leaf disease in commercial plantations of Portuguese eucalypts.

Materials and methods

Isolates

Two young *E. globulus* plantations were selected in two separate regions, Torres Vedras and Aveiro, where MLD symptoms were observed during spring 2004. During autumn 2004 and spring 2005, as soon as the first lesions appeared, 50 symptomatic leaves were collected randomly from each plantation. Lesions were examined with a stereomicroscope and single spore cultures were obtained by the method described by Crous (1998). A collection of 43 isolates was maintained on 2% MEA slopes at 24 ºC.

DNA extraction and amplification

Molecular characterization of single spore cultures was based on sequence analysis of part of the nuclear rRNA operon spanning the 3' end of the 18S rRNA gene, the first internal transcribed spacer, the 5.8S rRNA gene, the second ITS region and the 5' end of 28S rRNA gene (ITS). Genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen GmbH, Germany) following the manufacturer's instructions. The ITS1-5.8S-ITS2 cluster was amplified with primers ITS1: 5' TCC GTA GGT GAA CCT GCG G and ITS4: 5' GCT GCG TTC TTC ATC GAT GC (White *et al.*, 1990). The cycling conditions were 96 °C, 5 minutes initial denaturation and cycles of 96 °C, 30 s, 55 °C, 30 s, 72 °C, 90 s were repeated 30 times. A final elongation step of 7 min at 72 °C was also included. PCR products were separated by electrophoresis on 1% agarose gels and visualised with UV light after staining with ethidium bromide. The amplification products were purified and sequenced in both directions. PCR reactions were prepared in a total volume of 50 µL including 1.5 µL of genomic 1/10 dilution DNA, 1.25 U of *Taq* DNA polymerase (ABgene), 1x Reaction buffer (100 mM KCl, 20 mM Trix-HCl, pH 8.0 (at 25 °C), 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Nonidet P40, 50% (v/v) glycerol) (ABgene) 20 pmol of each primer, 0.2 mM of each dNTPs, and 2.5 mM $MgCl_2$. PCR amplification products were purified using JETquick spin column (Genomed GmbH) and sequencing reactions were prepared with primers ITS1 and ITS4 with Kit BDT v1.1 (Applied Biosystems). Separation of PCR products by capillary electrophoresis at ABI PRISM 3700 (Applied Biosystems Inc., Foster City, California) and the results analysed with Software Sequencing Analysis 3.7.

Phylogenetic analysis

All sequences were checked with BioEdit version 7.0.5.3 (Hall, 1999) and ambiguities corrected. The ITS sequences generated in this study were added to sequences of other *Mycosphaerella* species obtained from GenBank (www.ncbi.nlm.nih.gov) and aligned with Clustal X (Thompson *et al.*, 1997). All sequences generated in this study were deposited in GenBank (Table 1) and alignments were deposited in TreeBASE.

Phylogenetic analyses of sequence data were done using PAUP version 4.0b10 (Swofford, 2002). All characters were unordered and of equal weight and alignment gaps were treated as a fifth character state. MP analyses were performed using the heuristic search option with 1000 random taxa addition and TBR as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Robustness of the branches was evaluated by 1000 bootstrap replications (Hillis & Bull, 1993). Other measures included TL, CI, RI and HI.

The Kimura-2-parameter nucleotide substitution model (Kimura, 1980) was used for distance analysis. All characters were unordered and of equal weight. Bootstrap values were obtained from 1000 NJ bootstrap replicates.

Results

The dataset consisted of 64 isolates including the one outgroup isolate. Incomplete parts at the start and end of the sequences (positions 1–24 and 561–608) were excluded from the analyses. After alignment the dataset consisted of 536 characters including alignment gaps. In the MP analysis 292 characters were constant, 58 were variable and parsimony-uninformative. MP analysis of the remaining 186 parsimony-informative characters resulted in a single tree (TL = 409, CI = 0.846, RI = 0.978, HI = 0.154). MP and NJ analyses yielded trees with similar topology and similar bootstrap support for the branches. The MP tree (Fig. 1) could be resolved into three clades.

Clade 1 was well-supported with bootstrap values of 100% in both MP and NJ analyses and included isolates identified as *M. molleriana* and *Mycosphaerella vespa* Carnegie & Keane in one sub-clade and *M. nubilosa* and *Mycosphaerella juvenis* Crous & M. J. Wingfield in a second sub-clade. Two isolates (EFN NX7A and EFN Y16F) clustered with *M. molleriana* and *M. vespa* while four clustered with *M. nubilosa* and *M. juvenis*.

Clade 2 was also well resolved with a bootstrap value of 100 % in both analyses and contained isolates previously identified as *Mycosphaerella grandis* Carnegie & Keane and *M. parva*. Most of the isolates from this study (29 isolates) clustered in this clade. Isolate EFN X40D formed a branch separate from the main clade. Only 2 bp differences separated this branch from other isolates in clade 2. Sub-clades within clade 2 were separated by short branches with low bootstrap support.

The third major clade (Clade 3), supported by high bootstrap value of 100 % in both analyses, contains representatives of *M. lateralis* and *M. communis*. These two species reside in two separate, well-supported (> 90% MP and 100% NJ) clades. Two of the EFN isolates clustered with *M. lateralis* and six with *M. communis*.

Discussion

In this study we characterized the species of *Mycosphaerella* that were found during a preliminary survey of MLD in commercial *Eucalyptus* plantations in Portugal. The identifications were complemented with information on the collection sites, dates and host, which contributes to a better understanding of the distribution and relative importance of the species. Previous reports of *Mycosphaerella* species in Portugal have not included such data (e.g. Crous *et al.*, 2006).

Since 1995 only *M. africana*, *M. molleriana* and *M. walkeri* were recorded from Portugal. In 2004 *M. madeirae* was reported from Madeira and then in 2006 *M. communis, M. heimii*, *M. lateralis*, *M. marksii*, *M. nubilosa* and *M. parva* were recorded by Crous *et al.* (2006). In addition to this last report, with the exception of *M. heimii* and *M. marksii*, we report *M. grandis* and *M. vespa* as new records for Portugal. Thus, until now, a total of 12 species of *Mycosphaerella* have been recorded on Portuguese eucalypts.

In the phylogenetic study, five species were clearly distinguished with high bootstrap support in both MP and NJ analyses. However, isolates previously identified under different names clustered within each species clade. The status of some of these species is not entirely clear. For example, Carnegie & Keane (1994) described *M. grandis* in Australia as a *sp. nov.* because of its ascospores morphology and lesions characteristics. On the other hand other works characterized it as a synonym of *M. parva* (Crous, 1998; Crous *et al.*, 2004; Maxwell *et al.*, 2005; Hunter *et al.*, 2006). In this study these two species clustered in the same clade with a low bootstrap (only 60 % bootstrap support in MP) separated by origins *M. parva* with specimens from Spain and Portugal and *M. grandis* with specimens from Chile. Observation of germination patterns suggests that we could have two distinct species, in which case a more detailed study with other gene sequences would complete this idea.

Two species clustered together in each of the two sub-clades in clade 1. The distinction between *M. molleriana* and *M. vespa* is also unclear. Hunter *et al.* (2006) described these two species as synonyms because of phylogenetic analysis, however we have more changes in phylogram and germination patterns observed for specimens EFN NX7A and EFNY16F are closely to description of *M. vespa* in Carnegie & Keane, 1998.

Mycosphaerella lateralis and *M. communis* were clearly distinguished in the phylogentic study and morphologically. Thus, *M. lateralis* has *Dissoconium dekkeri* as anamorph while *Dissoconium commune* is the anamorph of *M. communis*

Of particular concern is the confirmation of the presence of *M. nubilosa* on eucalypts in Portugal. This species is well known as an aggressive pathogen of eucalypts (Carnegie *et al.*, 1997). Thus it is important that rapid screening tools are developed to aid in the detection of this pathogen. It is also important to select tolerant planting material in order to reduce its impact.

Mycosphaerella nubilosa was first described as *Sphaerella nubilosa* by Cooke (1892). Subsequently Park & Keane (1984) examined various collections of *M. molleriana* and concluded that it resembled *M. nubilosa* in general morphology and Crous *et al.* (1991) showed that is impossible to distinguish the two species. In that case the older name of *M. molleriana* (1881) would take preference over *M. nubilosa* (based on *Sphaerella nubilosa* 1892). Crous & Wingfield (1996) described *M. juvenis* as *sp. nov*, commonly associated with leaf spots on juvenile leaves of *E. nitens*. Crous *et al.* (2001) supported *M. juvenis* as a species distinct from *M. nubilosa* and *M. molleriana* based on ITS phylogeny, possibly because the ex-type strain of *M. juvenis* was not included in that phylogeny analysis (Crous *et al.*, 2004). Besides Crous *et al.* (2004) used *M. juvenis* ex-types cultures and showed that were identical to *M. nubilosa* and some strains of *M. juvenis* produced an *Uwebraunia* anamorph, despite the fact that *M. nubilosa* did not. Finally, the confusion surrounding *M. juvenis* and *M. nubilosa* was resolved by Crous *et al.* (2004) because the spores germination patterns in MEA at 24ºC were type C (germinating from both ends parallel to germ tubes) like for *M. nubilosa*. When germination patterns were re-evaluated after 24h spores became distorted like in type F, similar to those observed for *M. juvenis* in Crous (1998).

More than one species was frequently found on a lesion. *M. communis* and *M. nubilosa* always occurred alone on the lesion. *M. grandis* and *M. vespa* were observed either alone or with *M. parva* on the same lesion. *M. parva* also occurred alone, while *M. lateralis* always occurred in association with *M. parva*. It is important in future pathogenicity tests to analyse these relationships and relate them to individual virulence and aggressiveness of the coexisting species.

Park & Keane (1982b) showed that *M. parva* were isolated only from older lesions and *M. nubilosa* were isolated from young blighting lesions. We also observed this and only found *M. nubilosa* alone, maybe before others species arrived or developed on spots caused by *M. nubilosa*.

M. lateralis always occurred in this study with *M. parva*. Jackson *et al.* (2004) showed that there was no evidence that *M. lateralis* could parasitize *M. cryptica* or *M. nubilosa*. However, *M. lateralis* is frequently found associated with other *Mycosphaerella* species leaf spots. Nevertheless its ecological position needs to be determined (Crous *et al.*, 2006).

M. grandis was regularly observed linked with older lesions caused by *M. tasmaniensis* Crous & M.J. Wingf., *M. nubilosa* and *M. cryptica* (Cooke) Hansf.

(Milgate *et al.*, 2001) and occurred alone or with one or a combination of *M. cryptica*, *M. gregaria*, *M. marksii*, *M. nubilosa* or *M. mexicana* on the same lesion (Maxwell, 2004) but in our work *M. grandis* was observed in combination only with *M. parva*. Carnegie and Keane (1994) suggested that *M. grandis* is a pathogen and *M. parva* a saprophyte on old *M. nubilosa* lesions.

Further work should be done to clarify these species complex like study of mating-type genes of critical species based on the work of Groenewald *et al.* (2006) that will design degenerate primers from homologous sequences in worldwide group and amplify part of the mating-type genes. The work will focus on determining species barriers between different species of *Mycosphaerella* and sequence analyses will be used to study population structure of the pathogen from different regions in Portugal. Furthermore is need to identify more *sp. nov.* in the world because Crous *et al.* (2006) had estimated that only 14% of *Mycosphaerella* spp. from eucalypts have been described. It seems that many new species will be reported in future.

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Table 1 - Isolates included in the sequence analysis of *Mycosphaerella* species.

Species	Isolate No.	Origin	Host plant	Date	Collector	GenBank Accession No.	Source
M. communis	EFN X8	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN NX8A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN NX30A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN NX30B	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN NX30C	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X38A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	CPC 11700	Spain	*E. globulus*	-	P. Mansilla	DQ302948	Crous *et al.*, 2006
M. communis	CBS 114238	Spain	*E. globulus*	-	J.P.M. Vazquez	AY725541	Crous *et al.*, 2004
M. grandis	EFN X4A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X21C	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X21D	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X29A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X53A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	CMW 8557	Chile	*E. globulus*	-	A. Rotella	DQ267583	Hunter *et al.*, 2006
	CMW 8554	Chile	*E. globulus*	-	M.J. Wingfield	DQ267584	Hunter *et al.*, 2006
M. juvenis	CBS 115669	South Africa	*E. nitens*	-	M.J. Wingfield	AY725548	Crous *et al.*, 2004
M. lateralis	EFN NX6B	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X13A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	CPC 11789	Portugal	*Eucalyptus sp.*	-	J.P. Sampaio	DQ302975	Crous *et al.*, 2006
	CPC 11706	Spain	*E. globulus*	-	P. Mansilla	DQ302972	Crous *et al.*, 2006
M. lateralis	CMW 4935	Zambia	*Eucalyptus sp*	-	-	AF309625	Hunter *et al.*, 2004

Table 1 - Isolates included in the sequence analysis of *Mycosphaerella* species (cont.).

Species	Isolate No.	Origin	Host plant	Date	Collector	GenBank Accession No.	Source
M. madeirae	CBS 112895	Portugal (Madeira)	*E. globulus*	-	S. Denman	AY725553	Crous *et al.*, 2004
	CPC 3746	Portugal (Madeira)	*E. grandis*	-	S. Denman	DQ302976	Crous *et al.*, 2006
M. molleriana	CBS111164	Portugal	*E. globulus*	-	S. McCrae	AF309620	Hunter *et al.*, 2006
M. nubilosa	EFN Y8B	Portugal	*E. globulus*	Spring 2005	L. Neves		This study
	EFN M7A	Portugal	*E. globulus*	Autumn 2004	L. Neves		This study
	EFN M17 B	Portugal	*E. globulus*	Autumn 2004	L. Neves		This study
	EFN M32	Portugal	*E. globulus*	Autumn 2004	L. Neves		This study
M. nubilosa	CBS116005	Australia	*E. globulus*	-	A.J. Carnegie	AF309618	Crous, 1998
M. nubilosa	CPC 11246	Spain	*E. globulus*	-	M.J. Wingfield	DQ302992	Crous *et al.*, 2006
	CPC 11723	Portugal	*E. globulus*	-	A.C. Alfenas	DQ302996	Crous *et al.*, 2006
	CPC 11767	Portugal	*E. globulus*	-	A.J.L. Phillips	DQ302998	Crous *et al.*, 2006
	CPC 11882	Portugal	*E. globulus*	-	A.J.L. Phillips	DQ302999	Crous *et al.*, 2006
	CPC 11885	Portugal	*Eucalyptus sp.*	-	A.J.L. Phillips	DQ303000	Crous *et al.*, 2006
M. parva	EFN X6	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X9C	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN NX13A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X15A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X21A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X22A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X27A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X37A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study

Table 1 - Isolates included in the sequence analysis of *Mycosphaerella* species (cont.).

Species	Isolate No.	Origin	Host plant	Date	Collector	GenBank Accession No.	Source
M. parva	EFN NX46A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X49C	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X50B	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN Y2B	Portugal	*E. globulus*	Spring 2005	L. Neves		This study
	EFN Y27D	Portugal	*E. globulus*	Spring 2005	L. Neves		This study
	EFN Y32B	Portugal	*E. globulus*	Spring 2005	L. Neves		This study
	EFNG21126BC	Portugal	*E. globulus*	Autumn 2004	L. Neves		This study
	CPC 11273	Spain	*E. globulus*	-	M.J. Wingfield	DQ303001	Crous *et al.*, 2006
	CPC 11888	Portugal	*Eucalyptus sp.*	-	A.J.L. Phillips	DQ303005	Crous *et al.*, 2006
	EFN NX7B	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN X29B	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN Y10A	Portugal	*E. globulus*	Spring 2005	L. Neves		This study
	EFN Y10B	Portugal	*E. globulus*	Spring 2005	L. Neves		This study
	EFN Y12B	Portugal	*E. globulus*	Spring 2005	L. Neves		This study
	EFN Y23A	Portugal	*E. globulus*	Spring 2005	L. Neves		This study
	EFN Y27A	Portugal	*E. globulus*	Spring 2005	L. Neves		This study
	EFN Y27C	Portugal	*E. globulus*	Spring 2005	L. Neves		This study
	EFN X40D	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
M. vespa	EFN NX7A	Portugal	*E. globulus*	Spring 2005	C. Valente		This study
	EFN Y16F	Portugal	*E. globulus*	Spring 2005	L. Neves		This study
	CBS117924	Tasmania	*E. globulus*	-	Unknown	DQ267590	Hunter *et al.*, 2006
M. vespa	CMW 11558	Australia	*Eucalyptus sp.*	-	Unknown	DQ303059	Crous *et al.*, 2006
Botryosphaeria parva	STE-U 4438	-	-	-	-	AY343467	Crous *et al.*, 2006

CBS - Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands
CPC - Culture collection of Pedro Crous, housed at CBS
CMW - Culture collection of Mike Wingfield, housed at Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa
EFN – Culture collection of Estação Florestal Nacional (National Forest Research Station) Oeiras, Portugal
STE-U: Culture collection of Stellenbosch University, South Africa. Isolate numbers from Crous (1998)
Ex-type strains are indicated in **bold** print.

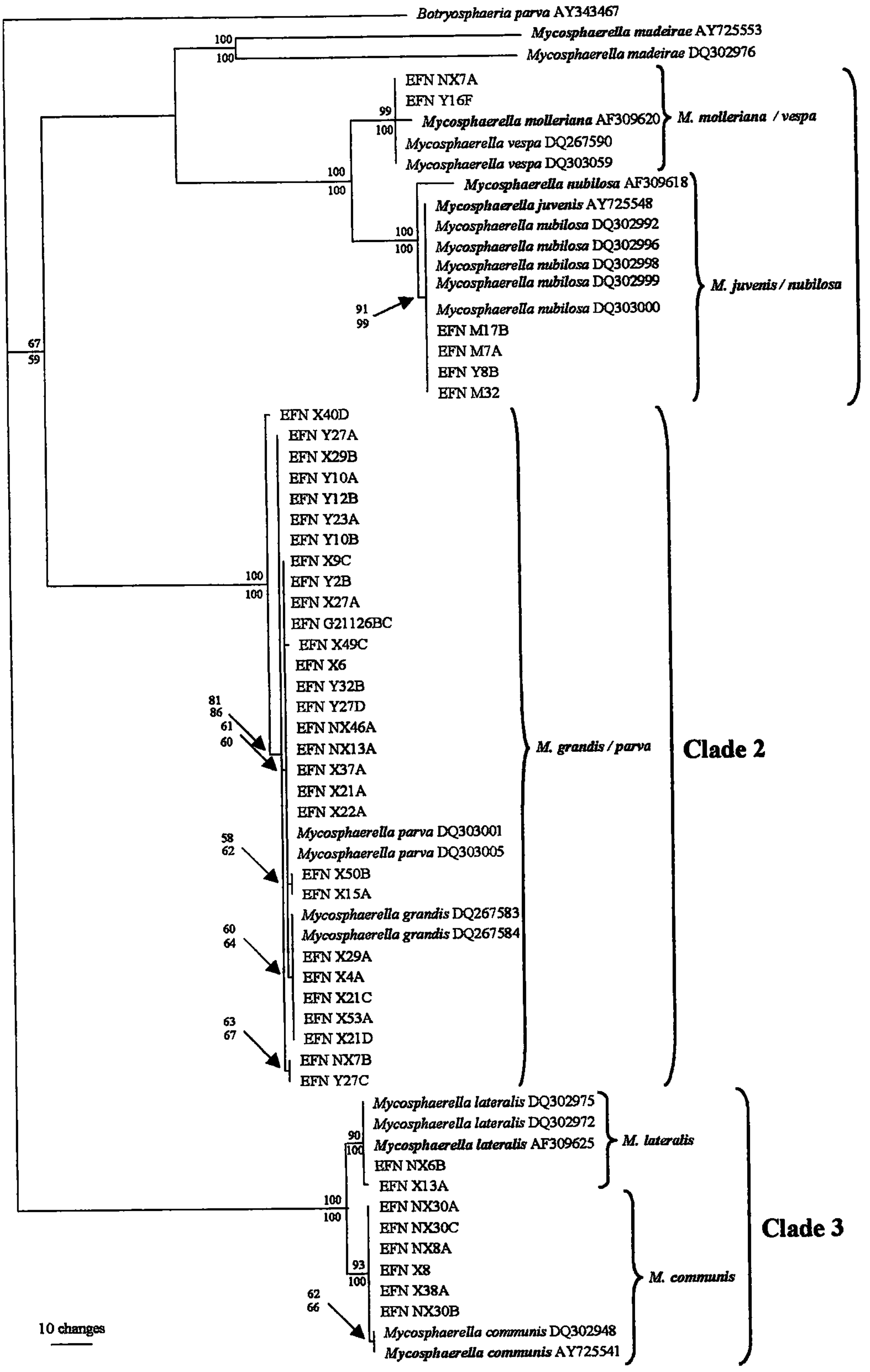
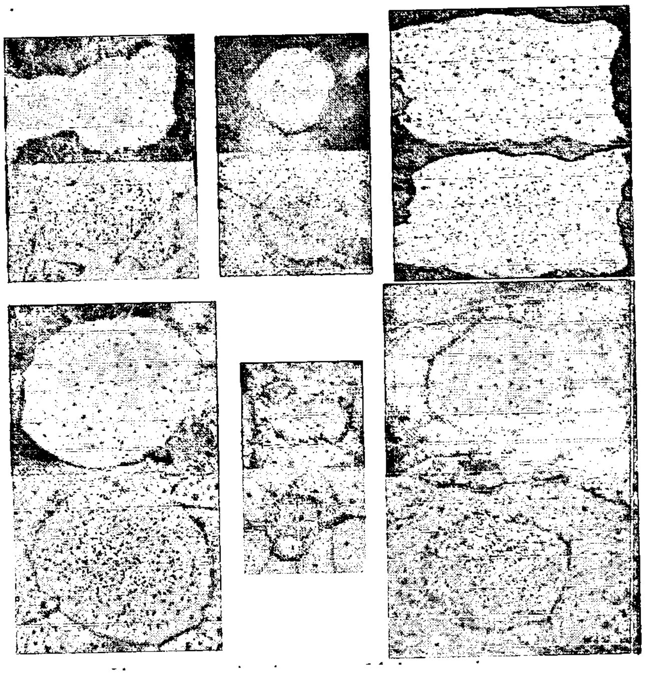


Fig. 1 – MP tree using bootstrap method with heuristic search (no. replicates = 1000): random taxon sampling; branch-swapping algorithm (tree-bisection-reconnection). TL = 409, CI = 0.846, RI = 0.978, HI = 0.154 from DNA sequence alignment on Internal Transcribed Spacer (ITS) rDNA data of *Mycosphaerella* spp. occurred on *Eucalyptus* spp. leaves. The tree was rooted to a *Botryosphaeria parva*. MP bootstrap values are given above the branch with NJ bootstrap values below the branches. The scale bar shows 10 changes. Ex-type strains are indicated in bold print.



CHAPTER 3

Evaluation of *Mycosphaerella* impact in eucalypts plantations in Portugal

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Abstract

Mycosphaerella leaf disease (MLD) is one of the most important leaf pathogens of eucalypts plantations worldwide, however only recently it has become relevant in Portugal. Caused by a complex of *Mycosphaerella* species, this disease reduces the photosynthetic area and can cause tree defoliation. In extreme cases it causes reduction of volume of wood produced. In order to relate the observed symptoms of MLD with the presence of the pathogen and at the same time obtaining a first evaluation of eucalypts clones and families, two experimental plantations were established in sites where the disease has been detected. Data on the percentage of affected crown (necrosis or defoliation) were collected and some of the *Mycosphaerella* species present were identified (*M. africana*, *M. communis*, *M. grandis*, *M. lateralis*, *M. marksii*, *M. nubilosa*, *M. parva, M. vespa* and *M. walkeri*).

Resumo

A "doença das manchas das folhas do eucalipto" é uma das mais importantes nas plantações de eucalipto, tendo-se só recentemente tornado relevante em Portugal. Esta doença, causada por um complexo de espécies de *Mycosphaerella*, reduz a área fotossintética da árvore, podendo causar desfolha, com consequente redução da taxa de crescimento e de volume de madeira produzido. Com o objectivo de relacionar os sintomas observados com

a presença do agente patogénico e avaliar a susceptibilidade de clones e famílias de eucalipto, foram estabelecidas duas plantações experimentais em locais onde foi detectada a doença. Foram recolhidos dados relativos à percentagem de área da copa afectada (por necroses ou desfolha) e identificadas as espécies de *Mycosphaerella* associadas (*M. africana*, *M. communis*, *M. grandis*, *M. lateralis*, *M. marksii*, *M. nubilosa*, *M. parva*, *M. vespa* and *M. walkeri*).

Introduction

Eucalyptus globulus is a valuable forestry species due to its rapid growth rate and high quality wood fibre suited to a wide range of paper products. However, sustainable production from plantations will only be possible if major pests and diseases can be avoided or their impact on tree growth and wood quality are kept below economically significant levels. In Portugal, forestry and forest products represent a major industry sector of substantial economic, social and strategic importance. *E. globulus* plantations occupy an area of 646 700 ha, representing 20.6% of the total forest area (DGRF, 2007).

Mycosphaerella leaf disease (MLD - also referred to as *Mycosphaerella* leaf blotch and *Mycosphaerella* leaf spot disease) is one of the most important diseases of plantation-grown eucalypts in the world. It is a very complex and relatively poorly understood disease, mainly because it might better be referred to as a complex of diseases, which are caused by a multiplicity of *Mycosphaerella* species. In fact, nearly 62 species belonging to the *Mycosphaerella* genera have been related to MLD symptoms in *Eucalyptus* spp. worldwide.

The first record of *Mycosphaerella* species on *Eucalyptus* outside its native range comes from Portugal. This species was identified as *Mycosphaerella molleriana* (Thüm.) Lindau in 1881 (Crous & Wingfield, 1997) and afterwards *Mycosphaerella africana* Crous & M. J. Wingfield and

Mycosphaerella walkeri R. F. Park & Keane (Crous, 1998) were also reported. However, serious damage in industrial plantations of *E. globulus* was only reported in recent years when leaf blotch and serious defoliation of young eucalypt plantations was observed. Some more species were then reported: *Mycosphaerella madeirae* Crous & Denman (Crous *et al.*, 2004), *Mycosphaerella communis* Crous & J.P. Mansilla, *Mycosphaerella heimii* Bouriquet ex Crous, *Mycosphaerella lateralis* Crous & M.J. Wingf., *Mycosphaerella marksii* Carnegie & Keane, *Mycosphaerella nubilosa* (Cooke) Hansf. and *Mycosphaerella parva* R.F. Park & Keane (Crous *et al.*, 2006). Recently based on molecular methods together with morphological characterization *Mycosphaerella grandis* Carnegie & Keane and *Mycosphaerella vespa* Carnegie & Keane were reported for the first time from Portugal (Silva *et al.*, *submitted*).

The aim of this work was to relate observed symptoms with the presence of the pathogen and at the same time obtain a first evaluation of the susceptibility of a range of eucalypt clones and families showing resistance to this disease. Two experimental stands were established and parameters for disease evaluation in the field were collected. Symptomatic leaves were collected from both experimental stands and species were identified by their general morphology, mode of ascospore germination and cultural characters.

Material and Methods

Experimental stands

During spring 2004 two experimental stands were established in Torres Vedras and Aveiro.

In the Torres Vedras stand 35 *E. globulus* clones, 2 hybrid clones with *E. globulus* and 13 full-sib families were tested using repetitions of 6 two-tree plots per each genotype. Two treatments were applied: a fungicide treated block (bitertanol or tolyfluanid was applied every two weeks until the autumn 2005) and an untreated block. Disease evaluation was recorded every two weeks on a single branch for each tree. Percentage of leave area affected was expressed as 4 levels (level 1 – 1 to 24%; level 2 – 25 to 49%; level 3 – 50 to 75% and level 4 – more than 75%).

In the Aveiro stand 10 *E. globulus* clones and 1 family were tested using repetitions of 10 trees per each genotype. Two treatments similar to the Torres Vedras stand were applied. Disease evaluation was based on percentage of crown defoliation expressed as 4 levels (level 1 – 1 to 24%; level 2 – 25 to 49%; level 3 – 50 to 75% and level 4 – more than 75%).

Morphological characterization of fungi

During spring 2005, as soon as the first lesions in new leaves were reported, 50 leaves were collected randomly from each plantation. For morphologic characterization of *Mycosphaerella* species lesions were observed under the stereomicroscope and one lesion per leaf selected. Stereomicroscope photographs of each lesion were obtained and used for description of leaf lesion dimensions, shape and color. Wherever possible, 30 repeated measurements (600x) were made of ascospores dimensions. Germination patterns of ascospores were determined after 24 h on malt extract agar medium (MEA) and single spore cultures obtained. Pure cultures were maintained in MEA 2 %, at 24 °C. Linear growth of single spore cultures was assessed after 1 month at 24°C in the dark. Colony colours of the surface and reverse were also obtained (Crous, 1998; Carnegie & Keane, 1998; Crous *et al.*, 2004).

Results

Results of disease evaluation in Torres Vedras stand are showed in figure 1. The 50 genotypes tested presented higher levels of disease severity expressed as percentage of leave area affected on fungicide untreated plot. Analysis of results from untreated plot permitted to distinguish some *E. globulus* clones (numbers 17, 18, 19, 21 and 25) showing resistance to MLD disease. Other *E. globulus* clones (numbers 2, 9, 15, 21, 22, 27 and 29) and hybrid clones with *E. globulus* (numbers 37 and 38) presented moderated resistance and full-sibs families with some *E. globulus* clones showed the highest sensitivity to disease.

Results of disease evaluation in Aveiro stand are showed in figure 2. With exception of clone 4 and 10, the genotypes tested showed significant differences in the severity of the disease, expressed as defoliation percentage, between treated with fungicide and untreated plot. Analysis of results from the plot without fungicides application, permitted to distinguish different behavior of *E. globulus* clones varying from sensitive (clone 1) to resistant (clone 10). Trees of seminal origin showed a median level of defoliation.

In both plantations (figure 1 and 2) fungicide application could not completely control the disease development but reduced severity level attained.

Results of identification of *Mycosphaerella* species based on general morphology are showed in table 1. Differences in species percentage between plantations are not important. With the exception of *M. lateralis* that occurred only in Aveiro, all the other species were detected in both plantations.

M. communis and *M. nubilosa* occurred always alone on lesions. Although *M. grandis, M. parva* and *M. vespa* occurred alone in some lesions, *M. parva* were observed in association with *M. grandis* and *M. vespa*, at a maximum of two species per lesion. *M. lateralis* occurred always in association with *M. parva*.

Morphologic characteristics of lesions observed in the present study agreed with type descriptions for *M. nubilosa* (Park & Keane, 1982), *M. parva* (Park & Keane, 1982), *M. grandis* (Carnegie & Keane, 1994), *M. communis* (Crous *et al.* 2004), *M. lateralis* (Crous & Wingfield, 1996), *M. vespa* (Carnegie

& Keane 1998), *M. africana* (Crous & Wingfield, 1996), *M. marksii* (Carnegie & Keane, 1994), and *M. walkeri* (Park & Keane, 1984), except for small differences.

The identification of *M. communis, M. grandis, M. lateralis, M. nubilosa, M. parva* and *M. vespa* was confirmed by molecular biology tools (Silva *et al.*, *submitted*).

Dimensions of *M. nubilosa* lesions here not given in the type description (Park & Keane 1982). In this study lesions were round or irregular with 3-15 mm diameter, sometimes coalescent, forming larger irregular blotches pale brown, frequently surrounded by a raised thin brown margin. Ascospore germination on MEA after 24 h was type C, with germination from both ends, germ tubes parallel to long axis of spore, not darkening or distorting. Cultures presented 14–18 mm after 1 month on MEA at 25°C in the dark, with irregular margins, olivaceous grey at both surfaces.

Leaf spots of *M. parva* were subcircular with 4-15 mm diameter, light brown, surrounded by a raised border and thin, dark brown margin. Ascospore germination on MEA after 24 h was type N. Ascospores and germ tubes became uniformly brown, distorted and verruculose. Cultural features here not given in the type description (Park & Keane 1982). In this study cultures were 15-25 mm per month on MEA at 25° in the dark, olive in surface and green on reverse.

Leaf spots of *M. grandis* were described in Carnegie & Keane (1994) to be confined to the margin of the leaf extending from the tip almost back to the petiole. In this study we did not confirmed this particular distribution pattern on leaf surface. Ascospore germination on MEA after 24 h was type N. Ascospores and germ tubes became dark with gross distortion and producing several germ tubes.

Lesions of *M. communis* were sub-circular to circular, 4–12 mm diameter, medium brown, surrounded by a thin, raised, concolorous border. Ascospores germination on MEA after 24 h were type F with ascospores not darkening and germinating from both ends, with germ tubes parallel to the long axis of the spore and distorting prominently upon germination. Cultures were 29-36 mm per month on MEA at 25° in the dark (not 20–35 mm as in Crous *et al.*, 2004), irregular, erumpent, uneven, folded, aerial mycelium moderate to sparse, hazel in surface and olivaceous-black on reverse.

Leaf spots of *M. lateralis* were subcircular, 3-12 mm diameter, gray-brown, surrounded by raised borders, medium brown on the adaxial surfaces concolorous on the lower surfaces. Ascospore germination on MEA after 24 h was type I with germination from both ends, germ tubes parallel to long axis of spore, not darkening, constricted at septum, developing lateral branches. Cultures were 32-37 mm per month on MEA at 25° in the dark, not 53 mm as in Crous & Wingfield (1996) or 15–25 mm as in Maxwell (2004), with an even margin, cream aerial mycelium, gray olivaceous on reverse.

Lesions of *M. vespa* were circular to irregular, mostly less than 5 mm diameter, rarely confluent, light-brown to red-brown becoming grey with age, with a red-brown margin that is often raised. Although wasps are often found in cavities within lesions they were not found in this study. Ascospore germinating at 25°C after 24 h on MEA was type C with germination from the apices of both cells, parallel to the long axis of the spore. Occasionally, a third germ tube grows at an acute angle from the median septum end of one of these cells. Cultures were grey-green to brown, often becoming dendritic towards the outer margins. Aerial hyphae were light grey to white and fluffy, becoming sparser towards the edge of cultures.

Lesions of *M. africana* were smaller (1-2 mm) and easy to distinguish because of its dark brown borders frequently surrounded by diffuse red-purple margins. Ascospore germination on MEA after 24 hours was type G with irregular germination from both ends, or from different positions in cells, with two or more germ tubes, darkening and distorting.

Lesions of *M. marksii* were bigger than *M. africana* lesions (3-20 mm) with red-purple margins. Ascospore germination on MEA after 24 hours was type B with germination from both polar ends with germ tubes parallel to long axis of spore, not darkening or distorting.

Lesions of *M. walkeri* were very similar to those of *M. marksii*. Ascospore germination on MEA after 24 hours was type C, not darkening at germination, germ tubes parallel to long axis of spore, with the spore becoming swollen and constricted. Conidia of *Sonderhenia eucalypticola* (A.R. Davis) H. Swart & J. Walker were observed in some lesions of *M. walkeri*.

Discussion

Differences in disease severity among species of eucalyptus have been described pointing *E. globulus* as one of the most susceptible (Carnegie *et al.*, 1994; Dungey *et al.*, 1997; Tejedor, 2004). A comparison of results between the two plantations is difficult because measured parameters were distinct (percentage of leave area affected / defoliation percentage). Also quantity and quality of genotypes tested were different. Nevertheless these first field trials allow detecting important differences between genotypes (*E. globulus* clones and families) in the susceptibility to MLD. These results show the potential benefits of selecting resistant *E. globulus* material to plantations in areas where *Mycosphaerella* leaf disease is a problem.

Fungicide application could not completely control the disease development but reduced severity level attained.

The composition of *Mycosphaerella* complex of species recorded in both plantations showed no important differences, with the exception of *M. lateralis* that occurred only in Aveiro. These results should be considered with some reserves due to difficulties of differentiation of *Mycosphaerella* species based only on morphologic characteristics. Further work is needed in order to obtain an accurate identification of *Mycosphaerella* complex of species occurring in *E. globulus* Portuguese plantations.

From the twelve *Mycosphaerella* species previously recorded from Portugal nine (*M. nubilosa*, *M. parva*, *M. grandis*, *M. communis*, *M. lateralis*, *M. vespa*, *M. africana*, *M. marksii* and *M. walkeri*) were detected in this survey, although only one collection of leaves was made. This study suggests that the number of species present in *E. globulus* plantations is higher, making difficult the attribution of pathogenic/saprophytic role to individual species.

This difficulty is increased in part because more than one species may occur in the same lesion. In this study *M. parva* were observed in association with *M. grandis, M. vespa* and *M. lateralis*. Park and Keane (1982) reported *M. parva* to colonize old lesions of *M. cryptica* (Cooke) Hansf. and *M. nubilosa* whereas Maxwell (2004) reported it occurring alone or with one or a combination of *M. cryptica*, *M. gregaria* Carnegie & Keane, *M. marksii*, *M. nubilosa* or *M. mexicana* Crous on the same lesion. Carnegie and Keane (1994) reported *M. grandis* to be associated with lesions of *M. gregaria* and *Aulographina eucalypti* (Cooke & Massee) Arx & E. Müll..

Further research is needed to complete identify species involved in MLD, with comprehension of their biological life cycle and attribution of individual role in MLD development.

Full identification of the complex of *Mycosphaerella* species causing economical losses in Portuguese eucalypts plantations will be essential for devise future control strategies. During the 2007 spring, new field trials were established with the most resistant and susceptible genotypes screened in the 2005 reported trials. These field trials will provide further information in the resistance/susceptibility of different genotypes to be included in plant production in future stands establishment.

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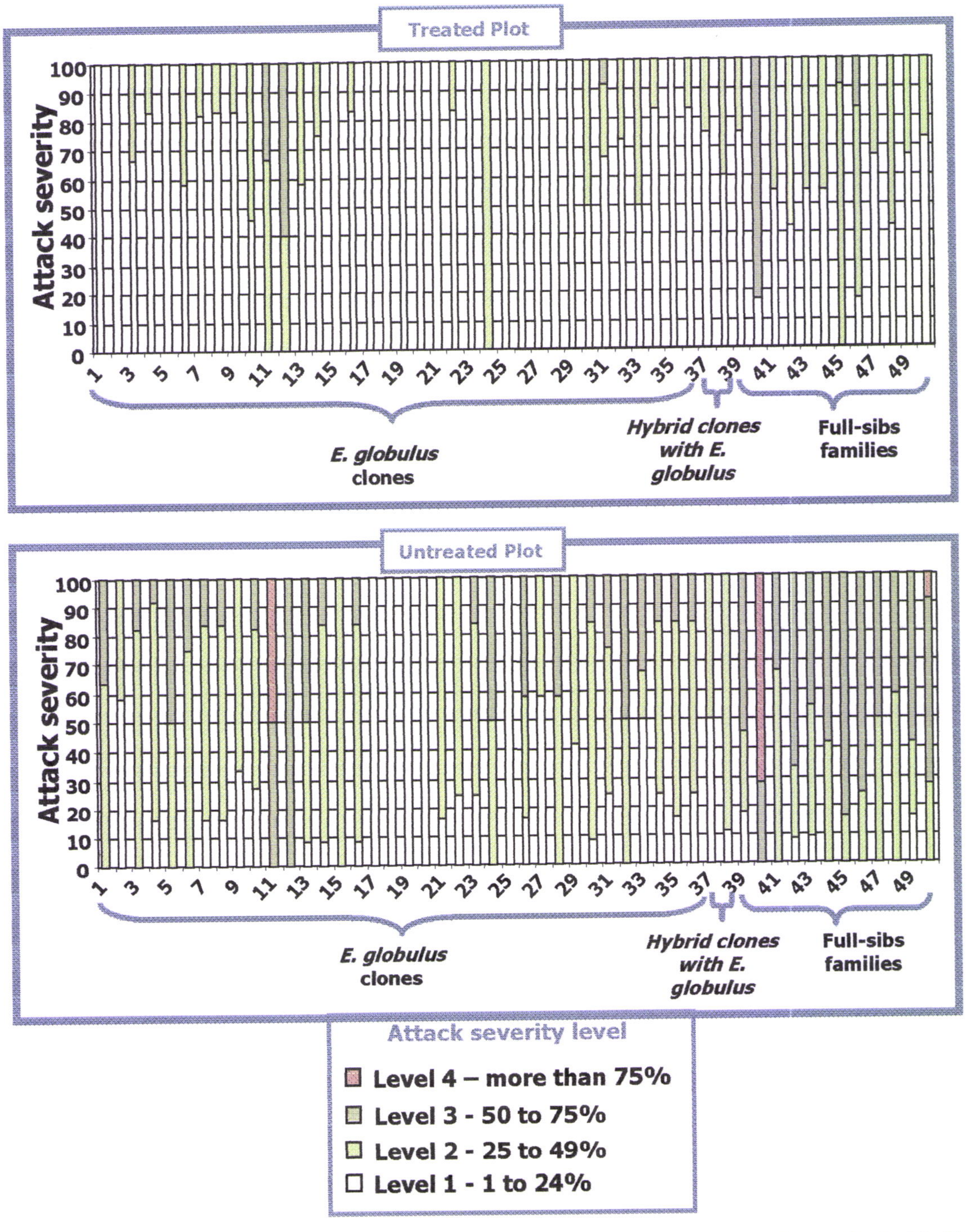


Figure 1 – Distribution of disease severity levels per 50 *E. globulus* genotypes tested in Torres Vedras stand. Levels are expressed as percentage of leave affected area. Results are separated per plot, treated and untreated with fungicides.

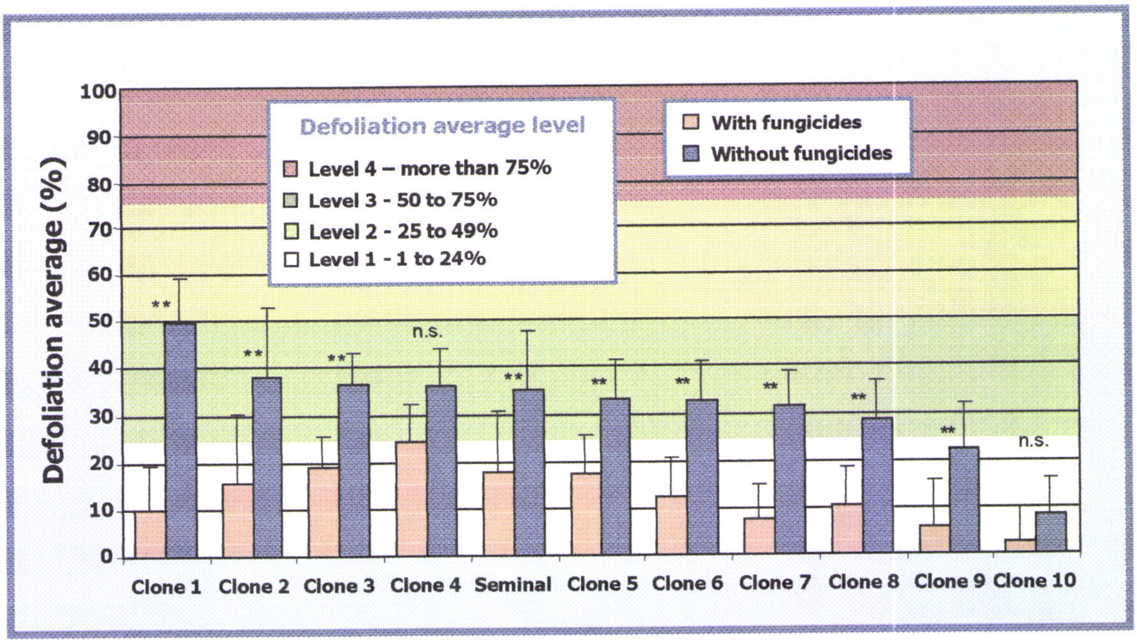
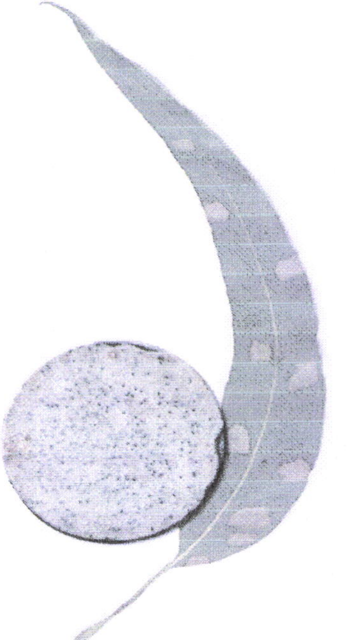


Figure 2 – Average of crown defoliation in 11 *E. globulus* genotypes, treated and untreated with fungicides, in Aveiro stand.

Table 1 – Percentage of *Mycosphaerella* species detected in Torres Vedras and Aveiro plantations. Data from 1 lesion per leaf, from 50 leafs collected from each plantation.

***Mycosphaerella* species**	**Torres Vedras (%)**	***Aveiro (%)***
M. nubilosa	22.81	17.46
M. parva	12.28	20.63
M. grandis	28.07	19.05
M. communis	10.53	7.94
M. lateralis	-	6.35
M. vespa	15.79	3.17
Other species (*)	10.53	25.40

(*)Other *Mycosphaerella* species included *M. africana*, *M. marksii* and *M. walkeri*.



CHAPTER 4

GENERAL DISCUSSION

Mycosphaerella species is one of the main *Eucalyptus* pathogens. In Chapter 1 is presented a literature review of both fungi and host. Nevertheless, *Mycosphaerella* species previous records did not include complete information about collections sites, dates and hosts.

In order to better understand the distribution and *Mycosphaerella*'s relative importance, a phylogenetic study using ITS gene region to cluster isolates from two young *E. globulus* plantation was accomplished. This work contributed to the identification of two new species in Portugal.

In addition to the molecular analysis presented in Chapter 2, the samples were characterized based on morphological methods. In some species, in particular *M. vespa* and *M. molleriana*, different ascospore germination patterns were observed which showed that these species may not be synonyms like was refereed on previous works. These results emphasize the importance of methodologies conjugation in order to obtain a classification.

Also in Chapter 3 was established the first evaluation on Eucalypts clones and families susceptibility to MLD, this data are extremely important for wood pulp enterprises.

This work was the first step to understand the real MLD on eucalypts situation in Portugal. Although the species identification was not completed, the methodology was tested and set up. In the future, is necessary a complete survey extended to all important areas of eucalypt plantations; such as the determination of barriers between different species of *Mycosphaerella*, sequence different regions of genome, and completed with pathogenicity tests.