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Mycosphaerella and *Teratosphaeria* species associated with *Mycosphaerella* Leaf Disease on *Eucalyptus globulus* in Portugal

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Abstract

Plantations of *Eucalyptus globulus* represent the main source of wood for the pulp and paper industry in Portugal and are affected by the complex of *Mycosphaerella* and *Teratosphaeria* species *(Mycosphaerella* leaf disease), which is an important foliage disease worldwide. This disease affect mainly young trees with juvenile-phase foliage, causing premature defoliation, decreased growth and wood production.

Species of *Mycosphaerella* sensu lato reported on eucalypts in Portugal are *M. communis*, *M. heimii*, *M. lateralis*, *M. madeirae*, *M. marksii M. walkeri*, *T. africana*, *T. molleriana*, *T. nubilosa* and *T. parva.* In order to complete the survey, symptomatic leaves were collected from *Eucalyptus globulus* plantations. Morphological and molecular characterization was used to give an indication of the species occurrence and most frequent species (*T. nubilosa*) and the composition of the MLD complex that did not change after the latest review.

Key words: *Mycosphaerella*; *Teratosphaeria*; Leaf Disease; MLD.

Resumen

Especies de *Mycosphaerella* y *Teratosphaeria* asociadas con la enfermedad *Mycosphaerella* de las hojas en *Eucalyptus globulus* en Portugal

Las plantaciones de *Eucalyptus globulus* representan la principal fuente de madera para la industria de pasta y papel en Portugal y se ven afectadas por el complejo de especies de *Mycosphaerella* y *Teratosphaeria* (enfermedad *Mycosphaerella* de las hojas), que es una enfermedad importante en todo el mundo. Esta enfermedad afecta principalmente a los árboles con fase juvenil de follaje, causando defoliación prematura, disminución del crecimiento y de producción de madera.

Las especies de *Mycosphaerella sensu lato* descritas sobre el eucalipto en Portugal son *M. communis, M. heimii, M. lateral, M. madeirae, M. marksii, M walkeri, T. africana, T. molleriana, T. nubilosa* y *T. parva*. Con el fin de completar el inventario, se obtuvieron hojas sintomáticas de plantaciones de *Eucalyptus globulus*. Se ha utilizado una caracterización morfológica y molecular para identificar la presencia de especies, la especie más frecuente (*T. nubilosa*) y la composición del complejo de MLD que no cambió después de esta última revisión.

Palabras clave: *Mycosphaerella*; *Teratosphaeria;* Enfermedad *foliar;* MLD.

Introduction

Mycosphaerella sensu lato is represented by more than 10,000 taxa (Crous, 2009) and some of them cause significant economic losses worldwide on plant hosts. M*ycosphaerella* leaf disease (MLD) on eucalypts is a significant disease worldwide that can reduce the growth of *Eucalyptus* when defoliation of the juvenile crown reaches more than 25% (Lundquist and Purnell, 1987; Dungey *et al.,* 1997) thus affecting the pulp and paper industry. Commercial plantations of eucalypts in Portugal are almost exclusively of *Eucalyptus globulus*,

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covering 646,700 ha representing 20.6% of the total forested area (AFN, 2007). MLD is considered to be the most damaging leaf disease of *Eucalyptus* in Australia (Park and Keane, 1984; Park, 1988; Park *et al.*, 2000; Carnegie, 2007) and is expected to become gradually more important worldwide in the future (Crous *et al.*, 2004). In recent years adult foliage were infected more often, like e.g. *T. nubilosa* in Australia (Barber *et al.*, 2008), in Uruguay (Perez *et al.,* 2009a), in Brazil (Perez *et al.,* 2009b) and *M. marksii* recorded in Uruguay always on adult leaves (Perez *et al.,* 2009).

In the 19th century, eucalypts were introduced in Portugal and Spain and *Teratosphaeria molleriana* (= *M. molleriana*) reported by von Thumen (1881) in Portugal was the first species of *Mycosphaerella* recorded outside Australia (Crous and Wingfield, 1997). Since 1999, serious damage on *E. globulus* has been reported characterized by a frequent and severe defoliation of young trees in the coastal regions assigned to MLD complex. In subsequent years more species were reported, including *T. africana* and *M. walkeri* (Crous, 1998), *M. madeirae* in Madeira (Crous *et al.,* 2004), *M. communis*, *M. heimii*, *M. lateralis*, *M. marksii*, *T. nubilosa*, *T. parva* (Crous *et al.,* 2006). In 2009, based on morphological and molecular analysis was suggested the occurrence of two more species, *M. grandis* and *M. vespa*, considered synonyms of *T. parva* and *T. molleriana* respectively by Hunter et al. (2006) (Silva *et al.,* 2009).

In Spain the situation is comparable and to the best of our knowledge the first report was in 1980 by Ruperez and Munoz. Only in 2004 more species were added like *M. communis, M. marksii*, *M. readeriellophora, M. toledana, T. molleriana, T. nubilosa*; in 2006 *M. lateralis, M. suberosa, M. walkeri, T. parva, T. pluritubularis* and in 2007 *M. aurantia* and *M. madeirae* (Crous *et al.,* 2004, 2006; Otero *et al.,* 2007). In 2009, *M. ellipsoidea, M. endophytica, M. flexuosa, M. gracilis, M. parkii*, *T. cryptica* were reported by De Blas *et al.* (2009). In 2011, *M. fori, M. punctiformis, T. africana* and *T. dimorpha* were reported by Sánchez Márquez *et al.* (2011).

The aim of this study was to observe species occurrence and the main species associated with MLD in ten locations distributed all over the coastal border in Portugal.

Materials and Methods

During autumn 2009 and spring 2010, ten young *Eucalyptus globulus* stands (1-2 years old) were surveyed throughout the country in regions that have suffered significant outbreaks of MLD (Table 1). As soon as the first lesions appeared, early spring or autumn, 5 trees were selected randomly and at least 10 symptomatic leaves were collected from each tree. In this study only the juvenile leaves were analyzed.

Lesions were examined with a stereomicroscope and characterized on the basis of colour, dimensions and shape. Wherever possible, 30 ascospores were measured at a magnification of × 600. Germination patterns of ascospores were determined after 24 h on 2% (w/v) malt extract agar (MEA) at 24 °C in the dark and single ascospore cultures obtained and colony colours of the top and reverse surface were recorded (Crous, 1998). The isolates were maintained on 2% (w/v) MEA slopes at 24 °C in the dark and deposited in the culture collection of LISFA (Herbarium Code of Unidade de Silvicultura e Produtos Florestais, Instituto Nacional de Investigação Agrária, I.P., L-INIA, Oeiras, Portugal).

All the unclear isolates morphological characterized were confirmed by sequence analysis based on the ITS1-5.8S-ITS2 cluster and for a preliminary taxonomical placement, each sequence was submitted to Basic Local Alignment Search Tool (BLAST) against the National Center for Biotechnology Information (NCBI) nucleotide databases. Genomic DNA was extracted (described by Silva *et al.*, 2009) and the ITS1-5.8S-ITS2 cluster was amplified with primers ITS1 and ITS4 (White *et al.*, 1990). Sequencing reactions and purification of amplification products were according to the procedure described by Silva *et al.* (2009).

Results

All isolates were processed for morphological methods and in few cases characterized with molecular methods. The diversity of MLD species by location is presented at Table 1 (Valongo, Trofa, Penafiel, Aveiro – further North and Ourém, Santarém, Cadaval, Torres Vedras, Bombarral 1, Bombarral 2 – further South). It showed less species in the MLD complex composition in north Portugal than in further south locations. *T. nubilosa* is the most frequent, present in 8 out of the 10 analyzed plantations; followed by *T. parva* and *M. lateralis* (6 of 10), *M. grandis* and *M. vespa* (4 of 10). The other species present were *M. communis* and *M. marksii* (3 of 10) and *T. molleriana* (2 of 10).

T. nubilosa was always isolated from young lesions on juvenile leaves. In contrast, the others species ob-

Table 1. Locations of the ten plantations studied along the coastal border, ordered from north to south, total number of fungal species identified at each location and its presence **(1)** and absence **(0)**: **(C)** *M. communis*; **(G)** *M. grandis*; **(L)** *M. lateralis*; **(MK)** *M. marksii*; **(V)** *M. vespa*; **(MLL)** *T. molleriana*; **(N)** *T. nubilosa*; **(P)** *T. parva* and number of other species unidentified

Location	Number of species identified	Species presence or absence								
		C	G	L	MK	V	MLL	N	P	Number of other species
Valongo	4	0	1	1	0	0	0	1	1	0
Trofa	1	0	0	1	0	0	0	0	0	0
Penafiel	1	0	0	0	0	0	0	1	0	0
Aveiro	4	1	0	1	0	1	0	1	0	0
Ourém	1	0	0	0	0	0	0	0	1	0
Santarém	1	0	0	0	0	0	0	1	0	0
Cadaval	5	0	1	1	0	1	0	1	1	0
Torres Vedras	11	1	1	1	1	1	1	1	1	3
Bombarral 1	5	0	0	0	1	0	0	1	1	2
Bombarral 2	10	1	1	1	1	1	1	1	1	2
% Total of occurrences		30	40	60	30	40	20	80	60	

served were found only on older lesions on juvenile leaves. *T. nubilosa* was the species best represented with 80% of occurrences contrasting with few isolates of *T. molleriana* and *M. vespa* with 20% and 40% isolates correspondingly.

A summarized description for the main characters used on morphological identification of *M. communis*, *M. grandis*, *M. lateralis*, *M. marksii*, *M. vespa*, *T. molleriana*, *T. nubilosa and T. parva* is presented below.

Germination of *M. communis* ascospores was type F (Crous, 1998) 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. Lesions were sub-circular to circular, 4-12 mm diameter, medium brown, surrounded by a thin, raised, concolorous border. Diameter of cultures were 27-38 mm after 1 month at 24 °C in the dark, irregular, erumpent, uneven, folded, aerial mycelium moderate to sparse, hazel in surface view, olivaceous-black on reverse.

Ascospores and germ tubes of *M. grandis* were type N (Crous, 1998), became dark with gross distortion and produced several germ tubes. Leaf lesions were round and angular, 5-7 mm diameter. Cultures were 16-25 mm diameter after 1 month at 24 °C in the dark, olive in surface view, green on reverse.

Ascospore germination of *M. lateralis* was type I (Crous, 1998) with germination from both ends, germ tubes parallel to the long axis of the spore, not darkening, constricted at septum, developing lateral branches. Leaf spots were subcircular, 3-12 mm diameter, grey-brown, surrounded by raised borders, medium brown on the adaxial surfaces concolorous on the lower surfaces. Cultures were 33-37 mm after 1 month at 24 °C in the dark, with an even margin, cream aerial mycelium, grey olivaceous on reverse.

Ascospore germination of *M. marksii* was type B (Crous, 1998), with germination from both ends germ tubes parallel to the long axis of the spore, not darkening or distorting. Leaf spots were subcircular to irregular, 4-20 mm diameter, light brown, delimited by raised borders, medium brown with red-purple margins. Cultures grew 22-26 mm after 1 month at 24 °C in the dark, with an even or uneven margin, aerial mycelium sparse, olivaceous grey on surfaces.

Ascospores of *M. vespa* were type C (Crous, 1998), with germination from both ends, parallel to the long axis of the spore, ends obtusely rounded, slightly constricted. Leaf lesions were circular to irregular, sometimes confluent, light-brown to grey and less than 5 mm. Cultures were 23-38 mm diameter after 1 month at 24 °C in the dark, olivaceous grey on both surfaces.

Ascospore germination of *T. molleriana* was type C (Crous, 1998) germination from one or both ends, germ tubes parallel to long axis of the spore, not darkening, constricted at septum. Leaf spots were subcircular to irregular, 2-11 mm diameter, light brown, medium brown border. Diameter of cultures was 10-39 mm after 1 month at 24 °C in the dark, aerial mycelium absent or sparse, iron gray on bottom and olivaceous-gray on top.

Ascospore germination of *T. nubilosa* was type C (Crous, 1998) with germination from both ends, germ tubes parallel to long axis of spore, not darkening or distorting. Lesions were 3-15 mm diameter, irregular or round, occasionally coalescent, forming larger irregular pale brown blotches, often surrounded by a raised thin brown margin. Cultures grew 15-19 mm after 1 month at 24 °C in the dark, with irregular margins, olivaceous grey on both surfaces.

Ascospores of *T. parva* and its germ tubes became uniformly brown, distorted and verruculose. Leaf spots were subcircular 4-15 mm diameter, light brown, surrounded by a raised border and thin, dark brown margin. Ascospore germination was type N (Crous, 1998). In this study cultures were 14-18 mm after 1 month at 24 °C in the dark, olive in surface view, and green on reverse.

The comparison obtained between sequences reads of the ITS1-5.8S-ITS2 cluster and the published sequences of the GenBank nucleotide sequences database confirmed the relation of the isolates with the highest similarity (Table 2).

Discussion

Morphological characteristics of species identified concurred with descriptions for *M. communis* (Crous *et al.,* 2004), *M. grandis* (Carnegie and Keane, 1994), *M. lateralis* (Crous and Wingfield, 1996), *M. marksii* (Carnegie and Keane, 1994), *M. vespa* (Carnegie and Keane, 1998), *T. molleriana* (Crous and Wingfield, 1997), *T. nubilosa* and *T. parva* (Park and Keane, 1982). *M. grandis* and *M. vespa* were considered synonyms of *T. parva* and *T. molleriana* respectively (Hunter *et al.,* 2006). However, in this study some differences in morphology were described as it was shown by Silva et al (2009).

The composition of species of MLD complex did not change after the latest revision in Portuguese plantations (Silva *et al.,* 2009). *T. nubilosa* is the dominant species and occurred mostly on young lesions on juvenile leaves confirming the aggressiveness of this species on eucalyptus. *T. molleriana* was reported in 1881 (130 years ago), after that only in 1995 in Abrantes (collected by S. McCrae), in 2005 in Torres Vedras (collected by H. Machado), in 2006 near Lisbon (collected by A. J. L. Phillips) and in this work, in Torres Vedras and Bombarral. All these reports refer to close locations in the centre of the country. It seems that *T. molleriana* did not spread out during all these years and not become problematic and aggressive as *T. nubilosa* did in a few years since the first detection. *M. marksii* is also confined to centre locations. The others reported species are spread out all over the country as *T. nubilosa*.

All the species reported in Portugal were also observed in Spain, except *M. heimii*. In Spain the diversity of species of MLD complex is higher than in Portugal with more 14 species detected: *M. readeriellophora, M. toledana* (2004)*; M. suberosa, T. pluritubularis* (2006); *M. aurantia* (2007); *M. ellipsoidea, M. endophytica, M. flexuosa, M. gracilis, M. parkii*, *T. cryptica* (2009) *M. fori, M. punctiformis* and *T. dimorpha* (2011) (Crous *et al*., 2004, 2006; Otero *et al*., 2007; de Blas *et al*., 2009; Sánchez Márquez *et al*., 2011).

Conclusions

In this study were identified *M. communis, M. grandis*, *M. lateralis, M. marksii, M. vespa*, *T. molleriana*, *T. nubilosa* and *T. parva,* five more unidentified species on Portuguese plantations, suggesting that the main species composition on MLD complex did not change after the latest revision (Silva *et al.,* 2009).

Until now, there are twice as many species reported from Spain than from Portugal and one particular high-

Table 2. Species identified on juvenile leaves of *Eucalyptus globulus* based in the percentage of similarity of each isolate to its closest match at the GenBank nucleotide sequences database

Sequence accession no.	Origin	Sequence-based identification	Maximum identity (%)
FJ515739	Portugal	*Mycosphaerella communis*	100
FJ515719	Portugal	*Mycosphaerella grandis*	100
EU851919	Uruguay	*Mycosphaerella lateralis*	99
EU851931	Uruguay	*Mycosphaerella marksii*	100
FJ515727	Portugal	*Mycosphaerella vespa*	100
EU851932	Uruguay	*Teratosphaeria molleriana*	99
FJ515731	Portugal	*Teratosphaeria nubilosa*	100
AY509779	Australia	*Teratosphaeria parva*	99

light is the absence of *T. cryptica* in Portugal, since this is one of the most aggressive species of MLD as *T. nubilosa*.

This study suggests that *T. nubilosa* is the main species causing MLD in Portugal. Nevertheless, a continuing effort on survey associated with a complete climatic characterization during different seasons to accurately attribute species aggressiveness will be necessary. Furthermore, the distribution was less diverse in north than in south locations thus more research is needed to understand and identify the factors implicated in differences in composition of MLD complex.

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