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Mycosphaerella leaf disease (MLD) outbreak on *Eucalyptus globulus* in Brazil caused by *Teratosphaeria* (*Mycosphaerella*) *nubilosa*

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Summary. Severe defoliation of young plantations of *Eucalyptus globulus* trees showing symptoms resembling Mycosphaerella leaf disease was observed in the province of Rio Grande do Sul, Brazil. The serious primary pathogen, *Teratosphaeria nubilosa* (= *Mycosphaerella nubilosa*) was repeatedly isolated from leaf spots and blotches on both juvenile and adult foliage and its identification was confirmed with DNA sequence comparisons. This pathogen was recently introduced into Uruguay and this report suggests a rapid range expansion in South America. Further spread of *T. nubilosa* northwards into Brazil's main *Eucalyptus*-growing areas as well as to other South American countries is probable and research towards developing resistant planting stock should be a priority in South America.

Key words: biological invasion, forest pathology, introduced pathogen, *Mycosphaerella nubilosa*.

Introduction

Commercial forestry in Brazil using non-native species covers an area of almost 6 million ha, of which 3.5 million ha are planted to *Eucalyptus* spp (BSS, 2007). Pure species and hybrids of *E. grandis*, *E. saligna* and *E. urophylla* are typically planted in tropical and subtropical climate regions and *E. dunnii*, *E. globulus* and *E. viminalis* in temperate zones (BSS, 2007, Gonçalves *et al.*, 2008). Pests and diseases seriously threaten these and other non-native plantation resources in Brazil and elsewhere in the world (Coutinho *et al.*, 1998; Alfenas *et al.*, 2004; Wingfield *et al.*, 2008).

Mycosphaerella leaf disease (MLD) caused by several species of *Mycosphaerella* and *Teratosphaeria* (Crous, 1998; Crous *et al.*, 2007) represents one of the diseases threatening *Eucalyptus* plantation forestry (Wingfield *et al.*, 2008). MLD reduces the photosynthetic capacity of leaves causing premature defoliation, shoot die-back and decreased growth resulting in significant losses (Carnegie, 2007, Hunter *et al.*, 2008, Lundquist and Purnell, 1987). Despite the fact that many species of *Teratosphaeria* and *Mycosphaerella* have been reported to infect *Eucalyptus*, *Teratosphaeria nubilosa* (= *Mycosphaerella nubilosa*) and *T. cryptica* (= *M. cryptica*) are considered the

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most important species causing MLD worldwide (Carnegie and Ades, 2002; Carnegie, 2007, Hunter *et al*., 2008). These two species are primary pathogens that infect young succulent living leaf tissue acquiring nutrients from them in a hemibiotrophic relationship with the host trees (Park and Keane, 1982; Hunter *et al*., 2008).

MLD is well-known in Brazil, but *M. marksii, M. parkii*, *T. suberosa* and *T. suttonii* associated with the disease in that country are not considered aggressive pathogens (Alfenas *et al*., 2004). These species are apparently mildly pathogenic or secondary colonists of necrotic tissue as many other *Mycosphaerella* and *Teratosphaeria* species on *Eucalyptus* (Crous, 1998; Crous *et al*., 2006; Crous *et al*., 2007). There are consequently no reports of MLD resulting in severe damage to *Eucalyptus* spp. in Brazil (Alfenas *et al*., 2004).

During the autumn of 2007, severe defoliation in young plantations of *E. globulus* was observed in the province of Rio Grande do Sul, the most southern province of Brazil (Fig. 1). Plantations older than six months were particularly severely affected, showing symptoms resembling MLD but substantially more severe than has been known in the past. There are no reports of the incidence and severity of this new outbreak of MLD in Brazil; however, in a preliminary study conducted in the Pinheiro-Machado and Piratini areas, Rio Grande do Sul, (Finkenauer and Arantes, 2007, unpublished) claim that between 50 to 90% of the *E. globulus* trees were showing clear symptoms of MLD. In addition, the death of 2.5 to 18.4% of the trees was associated with this disease on a 16 months old *E. globulus* plantation (Finkenauer and Arantes, 2007, unpublished). The change in the status of MLD in Brazil and the recent appearance of the serious primary pathogen *T. nubilosa* in the neighbouring Uruguay (Pérez *et al*., 2009), led to the suspicion that defoliation observed in Rio Grande do Sul might also be caused by this pathogen. The aim of this study was, therefore, to isolate and identify the causal agent of the new outbreak of MLD on *E. globulus* in Brazil.

Sampling was conducted in 'Fazenda São Luiz', 'Fazenda São Jose III', 'Fazenda Pedreiras' and 'Fazenda Verde Vale' plantations near Pedro Osório, Rio Grande do Sul, Brazil. Samples from all plantations showed identical symptoms; therefore, fungal isolations were performed only from 'Fazenda São Luiz' samples. One diseased leaf showing symptoms of MLD was collected from each of 50 *E. globulus* plants, chosen randomly. Fungal strains were isolated from these lesions using the method described by Crous (1998) and deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Germinating ascospores were observed and classified based on germination patterns (Crous, 1998). Fungal strains were grown on MEA (2% malt extract agar) and DNA was extracted from the mycelium as described by Pérez *et al*. (2009). The ITS region of the rDNA operon was amplified and sequenced using the primer pair ITS-1 and ITS-4 (White *et al*., 1990) with the same reaction mixture and PCR conditions as those utilized by Pérez *et al*. (2009). Analyzed sequences were compared with a worldwide collection of sequences for *T. nubilosa* deposited in GenBank as well as with representative *Mycosphaerella* spp. and *Teratosphaeria* spp., including those previously reported in Brazil. DNA sequence alignments were conducted in MAFFT v 6. employing the high-accuracy option of local alignment mode L-INS-I (Katoh *et al*., 2005) and manually aligned by inserting gaps when necessary in MEGA v 3.1 (Kumar *et al*., 2004). For parsimony analysis, heuristic searches were conducted in PAUP v 4.0b10 (Swofford, 2002) using the same parameters as those utilized by Pérez *et al*. (2009).

Leaf lesions were abundant on the current season's leaves (Fig. 1, a), which is characteristic of *Teratosphaeria* species that are primary pathogens (Park and Keane, 1982; Hunter *et al*., 2008). On juvenile foliage, leaf lesions were amphigenous, yellow to brown in color, round to angular in shape, frequently coalescing to form larger blotches across the leaf surface (Fig. 1, a and b). Heavily infected leaves were prematurely shed from the tree (Fig. 2, b) causing almost completely defoliation on young trees (Fig. 2). On adult foliage, lesions were also amphigenous more angular than those observed on juvenile foliage with prominent raised borders (Fig. 1, c and d). Lesions were yellow to brown in color as those on juvenile foliage, however, a light-brown pigmentation towards the borders was observed on mature adult leaves (Fig. 1, d). The ascospore germination pattern after 24 hours and the characteristics of the colonies on

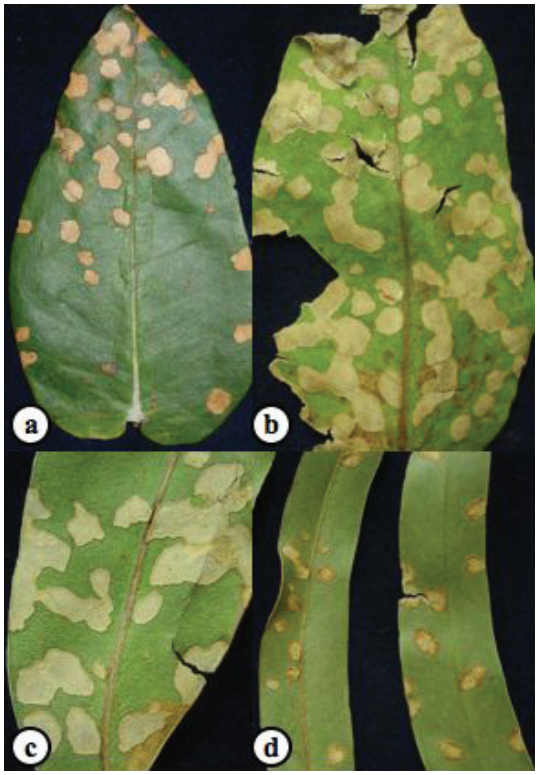

Fig. 1. *Teratosphaeria nubilosa* lesions on juvenile and adult *Eucalyptus globulus* foliage. a. Single and coalescing lesions on the current season's growth and b. prematurely shed juvenile leaves. c. Lesions on young adult leaves and d. mature adult leaves. Note the light-brown pigmentation towards the borders of the lesions.

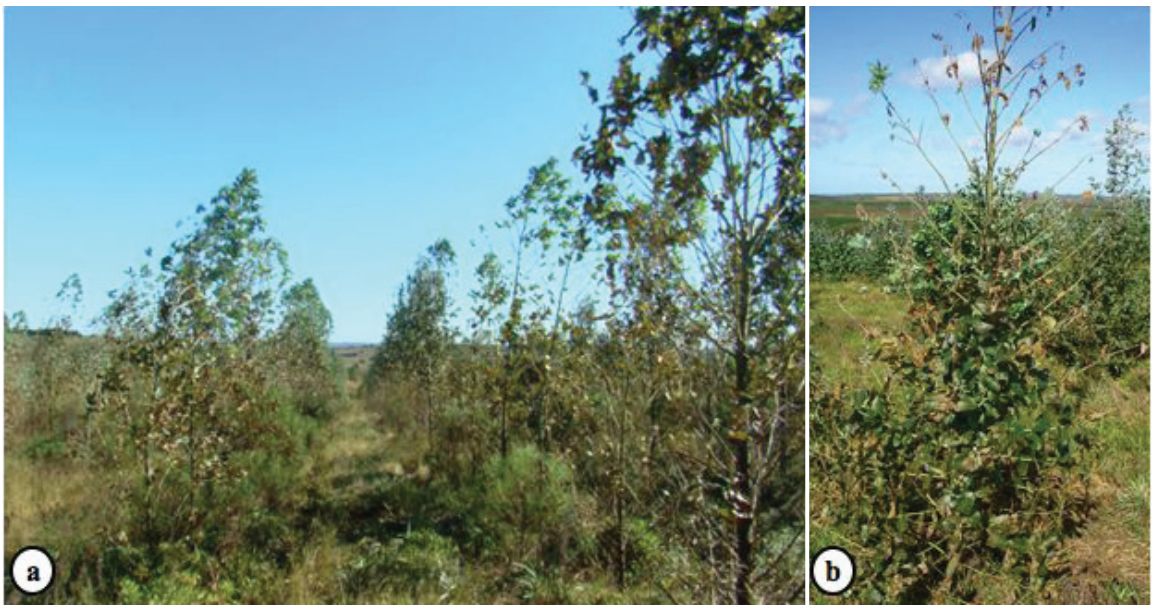

Fig. 2. Premature defoliation of young *Eucalyptus globulus* trees caused by *Teratosphaeria nubilosa*.

MEA were identical to those described for *T. nubilosa* elsewhere (Park and Keane, 1982; Crous, 1998; Hunter *et al.*, 2008).

PCR amplifications of the ITS region yielded amplicons of approximately 600 bp. The ITS sequences for all 20 isolates were identical, which justified to consider only one representative isolate for phylogenetic analyses (GenBank accession number GQ411058). Parsimony analyses resulted in a phylogenetic tree where the Brazilian isolates and all *T. nubilosa* isolates grouped together in a well supported clade showing 100% of bootstrap support (Fig 3). This clade included the ex-epitype culture of this species. The nature and severity of the new outbreak of MLD in Brazil, the presence of leaf spots and blotches on leaves from the current season's growth, the germination pattern of ascospores, the characteristics of the colonies on MEA and phylogenetic analyses based on the ITS region sequence data led to the conclusion that the outbreak of MLD in Brazil is caused by the primary pathogen *T. nubilosa*.

Teratosphaeria nubilosa was associated with severe defoliation on *E. globulus*, which is planted in temperate zones representing the minority of the Brazilian *Eucalyptus* plantations (BSS, 2007; Gonçalves *et al.*, 2008). However, *T. nubilosa* is also a primary pathogen of several *Eucalyptus* spp. widely planted in Brazil such as *E. dunnii, E. grandis, E. viminalis* and the hybrid *E. urophylla* × *E. globulus* (BSS, 2007, Hunter *et al.*, 2008). There are no data regarding the susceptibility to *T. nubilosa* of

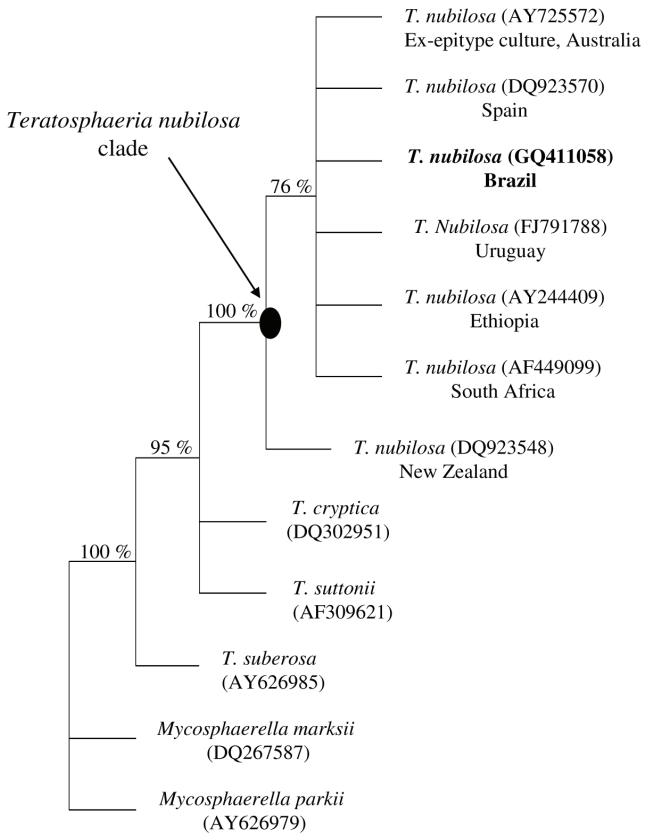

Fig. 3. Consensus phylogram obtained from the ITS sequence data using parsimony and heuristic search (Tree length =159, CI=0.7547, HI=0.2453, RI=0.7547). Bootstrap support values after 1000 randomizations are shown on the branching points. The arrow indicates the node defining the *T. nubilosa* clade. GenBank accession numbers are provided in brackets and the origin of the sequences behind each isolate.

the hybrid *E. grandis* × *E. urophylla* that is widely planted in Brazil. The appearance of the pathogen in the south of the country raises substantial concern regarding further spread north, to the main Brazilian *Eucalyptus* growing areas. Therefore, evaluation of the susceptibility to *T. nubilosa* of the Brazilian planting stock and monitoring of young plantations is strongly recommended.

The presence of *T. nubilosa* in Rio Grande do Sul, the province of Brazil neighboring Uruguay, suggests that this pathogen was introduced from Uruguay. The spread of the pathogen between the countries could have occurred naturally given that *T. nubilosa* produces windborne ascospores (Park and Keane, 1982; Hunter *et al.*, 2008), or by the movement of seeds or cuttings between these countries (Wingfield *et al.*, 2008). Using microsatellite markers, Pérez *et al.* (2009) suggested that the introduction of *T. nubilosa* into Uruguay most probably originated from the Iberian Peninsula. Populations of the pathogen from Rio Grande do Sul are now being compared with those from Uruguay and other parts of the world.

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