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FUNGI ASSOCIATED TO BARK LESIONS OF *Eucalyptus globulus* STEMS IN PLANTATIONS FROM URUGUAY¹

Raquel Alonso², Susana Tiscornia², Acelino Couto Alfenas³ e Lina Bettucci²

ABSTRACT – Trees with stem bark lesions are frequently observed in *Eucalyptus globulus* Labill. plantations, particularly in the central west region of Uruguay. These lesions constitute a problem for trunk decortifications at harvest and they also facilitate the access of fungi that could cause wood decay. Seven, three and one-year-old plantations, located at three sites in close proximity were selected. Four types of trunk lesions were present in trees regardless the age of plantation and more than one type was found in each plantation. The aim of this study was to investigate the fungal composition associated with these lesions and compare them to healthy tissues and try to find out the origin of these symptoms. Another purpose was to elucidate the real role of the fungi considered pathogens by means of experimental inoculations. Segments from lesions and healthy tissues yielded 897 fungal isolates belonging to 32 taxa, 681 isolates from bark lesions and 216 from healthy tissues. Both healthy and symptomatic tissues showed similar fungal species composition, but with differences in frequencies of colonization. *Cytospora eucalypticola* Van der Westhuizen, *Botryosphaeria* spp., *Pestalotiopsis guepinii* (Desm.) Stey. and *Penicillium* spp. were the dominant species isolated. As symptoms were not reproduced after experimental inoculation with *Botryosphaeria ribis* Grossenb. & Duggar and *B. eucalyptorum* Crous, & M.J. Wingf, it could be suggested that these lesions were originated by unfavorable environmental conditions. The frost that occurred for several days out of season and flooding may have been involved in the development of bark lesion.

Keywords: Fungi, *Botryosphaeria* and *Eucalyptus*.

FUNGOS ASOCIADOS AS LESÕES DA CASCA DO CAULE DE *Eucalyptus globulus* EM PLANTAÇÕES NO URUGUAI

RESUMO – As lesões na casca de *Eucalyptus globulus* Labill. são frequentemente observadas nas plantações da Região Centro-Oeste do Uruguai. Constituem problema para o descortçamento na colheita e, além disso, facilita a penetração de fungos apodrecedores da madeira. Selecionaram-se plantações com 1, 3 e 7 anos de idade, em três localidades próximas, para serem estudadas. Quatro tipos de lesões em caule foram encontrados nas árvores, independentemente da idade destas. Numa mesma plantação, havia mais de um tipo de lesão. Os objetivos deste estudo foram identificar a composição da micobiota associada a cada tipo de lesão da casca, compará-la com a dos tecidos sadios e investigar a origem desses sintomas. Outro objetivo foi verificar o papel de alguns colonizadores considerados patógenos, através de inoculações experimentais. Obtiveram-se 897 isolamentos correspondentes, a 32 taxa, 681 de casca lesionada e 216 dos tecidos sadios. Tanto os tecidos lesionados quanto os sadios mostraram composição micobiota específica similar, mas diferentes na frequência de colonização. *Cytospora eucalyptica* Van der Westhuizen, *Botryosphaeria* spp., *Pestalotiopsis guepinii* (Desm.) Stey. e *Penicillium* spp. foram as espécies dominantes isoladas. Devido à não reprodução dos sintomas depois da inoculação experimental com *Botryosphaeria ribis* Grossenb. & Duggar e *B. eucalyptorum* Crous & Wingf, foi sugerido que as lesões aqui estudadas fossem originadas de condições ambientais desfavoráveis. A ocorrência de geadas fora de estação associada à inundações pode ter influenciado o desenvolvimento dessas lesões na casca.

Palavras-chave: Fungos, *Botryosphaeria* and *Eucalyptus*.

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1. INTRODUCTION

In recent years, forestry has become a dynamic sector of the Uruguayan economy. Plantations of *Pinus* spp. and *Eucalyptus* spp., mainly *Eucalyptus globulus* Labill, increased to more than 200.000 ha (RESQUÍN e BALMELLI, 2005). Nearly 98% of the produced wood is exported as raw material for use in the paper industry. Diseases and pests as well as unfavorable environmental conditions present a great thwart to the forest industry and its economic viability (KLIEJUNAS et al., 2001). Bark lesions are a major problem in *E. globulus* trees at different ages. These lesions cause problems with decortication at harvest and they also facilitate the access of wood rotting fungi (MARTÍNEZ, 2005).

Fungi associated with healthy trunks of *Eucalyptus globulus* and *E. maidenii* (SIMETO et al., 2005) and associated to canker and asymptomatic twigs in *E. globulus* and *E. grandis* Hill ex Maiden have been studied in Uruguay. *Cytospora eucalypticola* Van der Westhuizen and *Botryosphaeria* spp. were present in both healthy and symptomatic tissues (BETTUCCI and ALONSO, 1997; BETTUCCI et al., 1999). *C. eucalypticola* Van der Westhuizen and *Botryosphaeria* spp. are commonly associated with stem cankers and are considered opportunistic pathogens (SMITH et al., 1994). *Colletogloeopsis zuluense* Wingf., Crous & Cout. (CORTINAS et al., 2006), *Chrysosporthe cubensis* (Bruner) (GRYZENHOUT et al., 2004), *Holocryphia eucalypti* Venter & Wingf. (GRYZENHOUT et al., 2004) and *Ceratocystis fimbriata* (Ell. Et Halst.) Davidson were recognized as the main pathogens in *E. grandis* in Uruguay but not recorded in *E. globulus* (VENTER et al., 2002; COUTO et al., 2004; FERREIRA et al., 2006).

Sieber et al. (1995) pointed out that the development of lesions in stems and twigs of young plants could be sometimes associated to a remarkable negative effect of abiotic factors.

The aim of this work was to investigate the fungal species associated with different bark lesions in trees at different ages, to compare them with that of healthy tissues and to find out the origin of the symptoms. An additional purpose was to evaluate the ability of *Botryosphaeria* isolates to produce lesions.

2. MATERIALS AND METHODS

This study was carried out in three *E. globulus* plantations, located at neighboring sites but with different

soil types, in the central west region of Uruguay (32° 45' S and 58° 10' W) (Table 1). The soil of site 1 is well drained, whereas those of sites 2 and 3 are moderately to low drained (PYÑEIRÚA GARCÍA, 1995). All plants were obtained from Australian seeds. Seedlings were grown in polyethylene bags containing composted pine bark as substrate with the addition of a mineral nutritional solution. When they were nearly six months old, they were transferred to the field showing a good growth rate. Plantations in site 1 were the oldest and those of site 3, the youngest.

Two types of bark lesions were observed at site one (types I and II), two at site three (types I and IV) and three at site two (types I, II and III) (Table 2). The symptoms studied were characterized by bark lesions. In the symptom of type I, bark was superficially cracked in the axial direction, with green healthy tissues underneath and xylem unexposed. In the symptom of type II, the bark was axially cracked, the xylem was exposed and the margin of the lesion presented protruding lips. The symptom of type III was characterized by annular cracked bark with an enlarged internode at nearly 2m from the ground. The youngest trees were affected by the symptom of type IV characterized by cracked bark with a purple discoloration at 1.50 m height. Table 2 shows the frequency of each lesion at different sites.

The climate is temperate humid. The mean annual precipitation was nearly 1100 mm between 1992 and 1998, ranging from 980 mm (1994) to 1577 mm (1993). There was frost during several days in autumn, winter and spring and there was also alternation of drought and high precipitation periods occurred (DIRECCIÓN NACIONAL DE METEOROLOGÍA) (Table 1).

At each site, 100 trees were inspected as for the presence of any type of symptom. Bark samples were collected from 10 trees with each type of bark lesion, and from 10 trees with healthy bark, in each site, in March 1998. The symptomatic bark was removed with a sterile scalpel from the center and margin of the lesions. In the healthy trees, the bark samples were also obtained with a sterile scalpel at the same height of the trunk from which the symptomatic samples were obtained.

All materials were taken to the laboratory in paper bags, examined under a dissecting microscope as for the presence of fructification, stored at 5° C and processed within 24 h.

Table 1 – Plant and site characteristics**Tabela 1** – Características das plantas e localidades

	Site 1	Site 2	Site 3
Plantation sites	Molino Petiso	Las Acacias	Santo Tomás
Seedlings planted in the field	1991	1995	1997
Age (years) of plants	7	3	1
Height (m)	16	9	2.5
Diameter at the breast height (cm)	18	12	12.4
Spacing (m) between plants	3.5x2.2	3x2.4	3x2.4
Number of days with frost between April and June	13*	20**	0***
Number of days with frost between July and November	28*	33**	16***

* 1992; ** 1996; ***1998. Values were recorded during the year, after seedlings were planted in the field.

* 1992 ** 1996 e ***1998 = . valores registrados durante o ano, após o plantio no campo.

Table 2 – Type and percentage of the bark lesions examined**Tabela 2** – Característica e porcentagem de lesões da casca analisadas

Symptoms	Site 1	Site 2	Site 3
Type I. Bark superficially cracked in axial direction with green healthy tissues underneath; xylem unexposed	45	76	7
Type II. Bark axially cracked, xylem exposed, margin of lesion with protruding lips	23	5	-
Type III Annular enlarged internode (20-25 cm) at 1.80 – 2.20 m stem height with cracked bark but xylem unexposed	-	24	-
Type IV. Purple cracked bark, xylem exposed	-	-	34

For fungal isolation, segments of approximately 1 x 3 x 5 mm (radial x tangential x axial) from healthy bark and from injured bark of each lesion were dissected. The surface sterilization was performed by immersion in 80 % ethanol for 1 min, and then in sodium hypochlorite (4 g active Chloride per 100 ml) for 2 min. The segments were then washed with sterile distilled water and dried on sterile filter paper. A total of 900 segments, 300 from healthy and 600 from symptomatic tissues (Table 3), were plated onto 90 mm Petri dishes containing 2 % malt extract agar, pH 4, with 10 segments per plate. The plates were incubated at 24 °C for six weeks or more, depending on the growth rates of the fungi. As colonies emerged from the segments, they were transferred to a fresh medium (2% malt extract agar). The identification was carried out by means of micro and macromorphological characteristics, according to the current mycological methods. Isolates of Basidiomycetes were characterized by culture characteristics and the presence of extracellular oxidative enzymes (STALPERS, 1978).

Botryosphaeria anamorphs obtained from healthy and symptomatic tissues were identified by means of molecular methods. The amplification of DNA sequences by polymerase chain reaction (PCR) was performed

for the entire ITS regions 1 and 2 and 5.8S rDNA gene using universal primer pair ITS-5/ ITS-4 (WHITE et al., 1990) on a Gene-tech SPCR1 MKII – Termoblock, using the following parameters: 35 cycles of 70 s at 94 °C, 45 s at 52 °C and 90 s at 72 °C, preceded by 5 min at 94 °C and ending with a 5 min elongation step at 72 °C. The PCR products obtained were sequenced using primers ITS-5/ ITS-4 on an automatic sequencer ABI PRISM 377 using a Sequencing Kit (Applied Biosystems Foster City, CA). The sequences obtained were compared with those recorded in Gen Bank using Blast NCBI program (WHEELER et al., 2000)

Two groups of *Botryosphaeria* isolates could be delimited according to the morphological and molecular characteristics. One isolate of each group was used to evaluate its pathogenicity to *Eucalyptus globulus*. One-year-old plants of *E. globulus* actively growing in a field located in the study area, without apparent environmental stress were inoculated. The stems of five *Eucalyptus* plants were disinfected with 70% ethanol and 5 mm-diameter disks of epidermis tissue were lifted. 4 mm diameter agar disks containing active growing mycelium obtained from the margin of a ten-day colony were placed on experimentally injured and wrapped in a single layer of Millipore® tape to prevent desiccation

and cross contamination. Five *Eucalyptus* plants were inoculated without superficial injury to evaluate the fungal ability as for epidermis penetration. In all trees, inoculation with isolates of each species was performed at breath height. The controls were inoculated with sterile MEA disks. The entire trial was repeated once in the same plantation. The evaluation was performed 3 months later.

3. RESULTS AND DISCUSION

Out of the 900 segments plated, a total of 897 isolates belonging to 32 taxa were obtained. 216 were from 300 segments of healthy tissues and 681, from 600 segments of bark lesions. Nearly 62 % of the species occurred with a relative frequency of more than 2 % (Table 3). From the segments of healthy tissues and bark lesions incubated, few species and isolates were obtained, as previously observed in *E. globulus* and *E. maidenii* stems (SIMETO et al., 2005). The fungal composition of healthy tissues was, in general, similar to those with lesions but with lower frequency (Table 3). The dominant species colonizing bark lesions were *C. eucalypticola*, *Botryosphaeria* spp. and *Pestalotiopsis guepinii*

(Desm.) Stey., *Eupenicillium* sp., *Penicillium* spp., and sterile mycelia were also dominant in some lesions. *P. guepinii* is a common endophyte found in *Eucalyptus* spp. and in other temperate and tropical tree species (BILLS e POLISHOOK 1992; BETTUCCI et al., 1997; BAYMAN et al., 1998; BARENGO et al., 2000). *Peniophora*, several sterile mycelia, *Botryosphaeria* spp. and *C. eucalypticola* are species also commonly isolated from *Eucalyptus* spp. and native Myrtaceae in Uruguay (BETTUCCI and ALONSO, 1997; BETTUCCI et al. 2004; SIMETO et al., 2005). *C. eucalypticola* was nearly absent from the lesions of the youngest plants (purple cracked bark with exposed xylem) in site 3, but they were very frequently isolated from bark cracked with xylem unexposed (symptom I, of site 2) and with xylem exposed (48 %) (symptom II, site 1). The potential pathogenicity of *Cytospora eucalypticola* isolates was evaluated by experimental inoculation on *E. globulus* and *E. grandis* stem and did not evidence the ability to incite symptoms, although they were recovered from tissues at the inoculation court (ALONSO et al. 2005).

Table 3 – Relative frequency of fungal colonization of lesions and healthy tissues
Tabela 3 – Frequência relativa de colonização por fungos nas lesões e tecidos sadios

Taxa	Code	Site 1			Site 2			Site 3		
		I**	II	T	I	III	T	I	IV	T
<i>Aureobasidium pullulans</i> (de Bary) Arnaud	aur	2	-	4	-	-	-	-	1	1
<i>Bartalinia robillarioides</i> Tassi	bar	-	-	-	-	-	-	-	5	-
<i>Coniella petrakii</i> Sutton	con	-	-	-	-	-	-	-	-	3
<i>Cytospora chrysosperma</i> Pers.: Fr.	cyt	15	48	10	63	37	14	1	3	1
<i>Eupenicillium</i> sp.	-	-	73	-	-	-	-	-	-	-
<i>Botryosphaeria</i> spp.	fus	26	5	16	3	52	37	13	76	9
<i>Nigrospora sphaerica</i> (Sacc.) Mason.	nig	-	-	-	2	-	-	-	-	-
<i>Penicillium decumbens</i> Thom	-	17	-	1	37	-	2	1	-	-
<i>Penicillium spinulosum</i> Thom	-	-	-	6	-	7	-	-	-	-
<i>Pestalotiopsis guepinii</i> (Desm.) Stey.	pes	37	2	2	29	9	38	72	24	10
<i>Sporothrix</i> sp.	spo	-	1	2	-	-	-	-	-	-
Ascomycete MVFI 399	asc	-	-	2	-	-	-	-	-	-
<i>Peniophora</i> sp. MVFI 288	ba1	-	-	-	-	-	-	4	-	-
Basidiomycete MVFI 289	ba2	-	-	-	-	-	-	2	-	-
Coelomycete MVFI 303	coe	-	-	3	-	-	-	-	-	-
Sterile hyaline mycelium MVFI 382	sh1	-	-	4	-	-	-	-	-	-
Sterile hyaline mycelium MVFI 400	sh2	-	-	-	-	-	-	-	-	4
Sterile dark mycelium MVFI 397	sd1	-	-	12	-	-	3	6	-	4
Sterile dark mycelium MVFI 394	sd2	-	-	3	-	-	-	-	-	10
Sterile dark mycelium MVFI 392	sd3	-	-	-	-	-	-	-	-	12
Rare taxa*	-	1	1	1	2	1	0	1	2	2
Total isolates	-	98	130	66	136	106	94	100	111	56
Total segments	-	100	100	100	100	100	100	100	100	100

*: *Acremonium strictum* W. Gams.; *Alternaria alternata* (Fr.) Keissler; *Aspergillus* sp.; *Colletotrichum gloeosporioides* (Penz.) Sacc.; *Hainesia lythri* (Desm.) Hohn.; *Mucor* sp.; *Phoma sorghina* (Sacc.) Boer. Doren. & van Kest.; *Sordaria fimicola* (Roberge: Desmaz.) Ces. & De Not.; *Xylaria* sp.; Basidiomycete MVFI 228; Basidiomycete MVFI 246; Sterile hyaline mycelium MVFI 395.

One group of isolates of *Botryosphaeria* identified by means of molecular methods showed a high homology with *B. ribis* (98%) and the other group, with *B. eucalyptorum* (100%) (SMITH et al., 2001). These species were indistinctly obtained from all bark lesions and healthy tissues. From the experimental inoculation, *Botryosphaeria* isolates were recovered from the inoculated point, but never from the control, nor superficially inoculated plants. It was observed the absence of symptoms, except for some purple discoloration around the fungal infection point or aseptic control lesion. It suggests that *B. ribis* and *B. eucalyptorum* obtained from the lesions analyzed in this study were not the causal agents of the symptoms observed in plantations. Conversely, the experimental infection with *Botryosphaeria* species on *Eucalyptus* in South Africa resulted in bark lesions of different entities (SMITH et al., 1994). It is probable that there is a susceptibility variation among *Eucalyptus* species (SMITH et al., 1996) as well as variation in virulence of *Botryosphaeria* strains (SMITH et al., 2001). In Uruguay, *Botryosphaeria* spp. was found to be associated with healthy tissues and twig bark cankers of *E. grandis* exposed to drought during summer months, combined with several frosts during the early autumn (BETTUCCI and ALONSO 1997). Apparently, any of the fungal species associated to bark stems could be related with the origin of the lesions described here. Then, it is possible hypothesized that abiotic conditions, such as several days with frost and the alternation of drought and water logging, where soils are not well drained, were involved in the origin of these lesions. *E. globulus* plants selected for high growth rate in Uruguay have a high water translocation efficiency and low stomatal conductivity probably resulting in a handicap when they are exposed to a flooded soil in winter (UNIVERSIDAD..., 1999; DE MENEZES et al., 2006). Under this unfavorable condition, several physiological changes are produced in plants manifested as senescence or wounds in photosynthetic tissues (SMIRNOFF, 1993). It was also observed that during periods of freezing temperatures, both stems and branches of some trees may also develop cracks (KOZLOWSKI et al., 1991; PLIETH, 1999; HARA et al., 2003).

In recent years, 5-15 % of tree losses were detected in *E. globulus* plantations located in sites 2, 3 and elsewhere by *Inocutis Jamaicensis*, a white heart-rot fungus that infect heartwood through bark lesions (MARTINEZ, 2005; KUNIEDADEALONSO et al., 2007).

Consequently, a great effort is being carried out to obtain selected genotypes adapted to the Uruguayan environmental condition, so that the incidence of these symptoms can be reduced (RESQUÍN and BALMELLI, 2005; RESQUÍN, 2007).

4. CONCLUSIONS

The fungal community on bark lesions was similar to those of healthy tissues. Most of the species were also found as endophytes in previous studies.

Isolates of *B. ribis* and *B. eucalyptorum* obtained from the lesions analyzed were not the causal agents of the symptoms observed in plantations.

Abiotic conditions such as frost, alternation of drought and water logging, where soils are not well drained, could be involved in the origin of these lesions in *E. globulus* plants.

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