Endophytic fungi from flowers, capsules and seeds of *Eucalyptus globulus*

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Summary

The main goal of this work was to detect whether *Cytospora chrysosperma* and *Fusicoccum eucalypti* are present as endophytes of symptomless hypocotyls, cotyledons, flowers, capsules, peduncles of flowers in order to interpret an earlier finding of their presence in seeds, seedling stems and twigs of *E. globulus.* Segments from these organs as well as from bark and the xylem from flower peduncles were surface-sterilized and plated on 2% malt-agar. All plates were incubated at 24°C for six weeks or more depending on the growth rate of fungi. *C. chrysosperma* was asymptomatically present in flowers, capsules, hypocotyls, cotyledons and peduncles. *F. eucalypti* was isolated from asymptomatic flowers and capsules.
It is probable that *C. chrysosperma* spreads during seed germination colonizing seedling stems through hypocotyl and cotyledon.

Key words Endophyte, Infection, Latent pathogen, Ecology

Hongos endofitos de flores, cápsulas y semillas de *Eucalyptus globulus*

Resumen

El objetivo principal de este trabajo fue detectar si *Cytospora chrysosperma* y *Fusicoccum eucalypti* se encuentran presentes como endófitos en hipocótiles, cotiledones, capsulas, flores y pedúnculos de flores, todos asintomáticos, con la finalidad de discutir su presencia en las semillas y en tallos de plántulas y ramitas de *E. globulos* estudiados anteriormente. Los segmentos de estos órganos, así como del xilema y corteza de los pedúnculos florales, fueron sometidos a esterilización superficial e inoculados en agar-malta al 2%. Las placas se incubaron a 24°C durante seis semanas o más dependiendo de la velocidad de crecimiento de los hongos. *C. chrysosperma* se encontró en forma asintomática en flores, cápsulas, hipocótiles, cotiledones y pedúnculos en cambio *F. eucalypti* fue aislado de flores y cápsulas solamente. Es probable que *C. chrysosperma* se establezca durante la germinación de la semilla, colonizando el tallo de las plántulas a través del hipocótile y el cotiledón.

Palabras clave Endofito, Infección, Patógeno latente, Ecología

Eucalyptus globulus is one of the main tree species planted in Uruguay [1]. During the last 30 years *ca.* 250,000 ha, comprising 1.5% of the total area of the country, have been planted with *Eucalyptus* spp. Approximately 98% of the wood produced is exported as raw material for use in the paper industry [2]. As Uruguay is mainly composed of grasslands and natural forests represent only 3% of the country [3], these new exotic plantations constitute an important change in the landscape.

Some investigations have already carried out on the endophytic fungi of *Eucalyptus* spp. in the Southern Hemisphere [4-8] and particularly in Uruguay [9-11]. Some species such as *Cytospora chrysosperma* and *Fusicoccum eucalypti* that were frequently isolated from healthy and symptomatic twigs of *Eucalyptus* spp. are commonly considered to be latent pathogens [12-16].

Endophytes of reproductive organs of woody plants have scarcely been studied [17] and only lists of species fruiting on all organs of *Eucalyptus* are available [18]. The aim of the present investigation was to detect whether *C. chrysosperma* and *F. eucalypti* are present as

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endophytes of symptomless hypocotyls, cotyledons, flowers, capsules and peduncles of flowers in order to discuss their presence in seeds, seedling stems and twigs of *E. globulus* previously studied in order to discuss their presence in seedling stems and twigs previously studied.

MATERIALS AND METHODS

Material collection and fungal isolation. Flowers, peduncles of flowers, capsules and seeds of *E. globulus* trees from old wind protection stands located in SE Uruguay (33º 51' S, and 53º 50' W) were collected during February 1997. Eighty branches from 40 trees (two per tree) containing flowers and capsules at different maturation stages were pruned. The branches collected were from *ca* 2.50 m above ground. All branches were transferred to the laboratory, stored at 4.5°C and processed within 48 h after collection. All samples were asymptomatic.

Forty flowers and their peduncles were cut off from 40 branches, 40 unopened capsules were also cut off from another 40 branches. Four hundred seeds, 20 hypocotyls and 20 cotyledons were also analyzed. The percentage of seed germination on culture medium was calculated. From flower hypanthium, 160 segments of internal tissues (the ovarian and surrounding parenchyma), and 160 segments of 2 mm external tissues were analyzed. From unopened capsules, 160 segments of internal tissues, mainly young seeds, and 160 of 2 mm external tissues were also all separately analyzed. From the peduncles of flowers, 100 segments of bark and 100 segments of xylem were obtained. From hypocotyl and cotyledon of the 20 germinated seeds, 160 segments were cut off.

Surface sterilization of all materials was performed by immersion sequence ethanol 80% v/v, for one minute, sodium hypochlorite (4% active chloride), for two minutes, ethanol 80% v/v, for 0.5 minute and rinsed with sterile water and then dried on sterile filter paper [19]. Seeds were surface sterilized likewise. This method was selected for seeds because it allowed eliminating species with high rate growth, but not those that could be attached to the teguments. To test the effectiveness an imprint of surface sterilized seeds on growth medium was performed. A total of 840 segments of flowers, capsules and peduncles, 80 segments of hypocotyl, 80 of cotyledon and 400 seeds were plated on 2% malt-agar. All plates were incubated in the dark at 24°C for six weeks or more depending on the growth rate of the fungal isolates. Each colony was transferred to fresh medium to facilitate identification and then incubated under a mixture of cool white and near-UV light to induce sporulation in some cultures. Those that failed to sporulate after four-six weeks were considered to be sterile.

Analysis of the data. The relative frequency of colonization was calculated as the total number of isolates of a taxon divided by the total number of segments obtained from each material plated out. Data was examined by correspondence analysis, using STAT-ITCF (Service des Études Statistiques, Institute Technique des Céréales et des Fourrages, France) to detect differences between the frequency distribution pattern of fungi colonizing different organs. This analysis was carried out using the species with a relative frequency equal to or higher than 2% [20].

RESULTS

From the tested samples, 332 fungi were isolated from 28 taxa: 11 were isolated from flowers, 18 from capsules, 10 from peduncles and 10 from seeds (Table 1). Only one species, *C. chrysosperma*, was isolated from

Table 1. Distribution frequencies (%) of endophytic fungi of *Eucalyptus globulus* of flowers, capsules, seeds and peduncle of flowers.

	PX	PB	IF	EF	IC	EC	S
Acremonium strictum W. Gams.							0.3
Alternaria citri Ellis & Pierce apud Pierce.							0.3
Alternaria alternata (Fr.) Keissler.				9.4		10.6	
Aureobasidium pullulans var. *melanigenum* (de Bary) Arn. Hermanides-Nijhof.		3.0		3.8	5.0	7.5	
Aureobasidium pullulans var. *pullulans* (de Bary) Arn.	0.5	1.0			2.5	3.1	0.3
Botrytis cinerea Pers.ex Nocca & Balb.							0.3
Chaetomium globosum Kunze ex Steud.					0.6		
Chaetomium funicola Ellis & Everh.							4.5
Cladosporium cladosporioides (Fresen.) de Vries.	0.5		1.3	1.9	0.6	1.9	
Cytospora chrysosperma Pers. ex Fr.		2.5		3.8	25.6	28.8	5.0
Didymosphaeria sp.						0.6	
Epicoccum purpurascens Ehrenb.: Schlecht.						0.6	0.3
*Fairmaniella leprosa (*Fairm.) Petrak & Syb.		1.5		4.4	0.6	1.3	
Fusicoccum eucalypti Sousa da Cámara.				5.0	2.5	15.0	
Hormonema sp. MVFI 126		2.5		1.9		3.8	
Microsphaeropsis olivacea (Bonord.) Höhn.		0.5					
Nigrospora oryzae (Berk. & Br.) Petch.							0.5
Nigrospora sacchari (Speg.) Mason.						0.6	
Nigrospora sphaerica (Sacc.) Mason.				0.6	0.6	1.9	1.5
Periconia igniaria Mason & M. B. Ellis.					0.6		
*Pestalotiopsis guepinii (*Desm.) Stey.			0.6				
Phoma sorghina (Sacc.) Boer. Doern. & van Kest.				0.6			
Phomopsis archeri Sutton.						0.6	
Sclerophoma pytophila (Cda) Höhn.	0.5					0.6	
Sporothrix cyanescens de Hoog & de Vries.					0.6		
*Xylaria s*p. MVFI 70							0.3
basidiomycete MVFI 144		0.5					
coelomycete MVFI 130	1.5	2.5		0.6	3.1	0.6	
Total taxa: 28							
Total isolates: 332	6	28	3	51	68	124	52
Total segments and seeds: 1240	100	100	160	160	160	160	400

Symbols indicate: PX, peduncle xylem; PB, peduncle bark; IF, internal tissue of flowers; EF, external tissue of flowers; IC, internal tissue of capsules; EC, external tissue of capsules; S, seeds.

asymptomatic hypocotile and cotyledons. Segments of external tissues from flower and capsule colonized by more than one fungal taxon were rare and the segments of internal tissues were scarcely colonized except those of the capsule containing immature seeds.

C. chrysosperma was present in the external tissues of flowers, capsules, seeds and bark of peduncles. It was also isolated from the internal tissues of capsules and from 30 % of the hypocotyl and cotyledon segments. *F. eucalypti* was present in external tissues of flowers and capsules (Table 1). *Alternaria alternata, Aureobasidium pullulans, Farmaniella leprosa* and *Hormonema* sp. MVFI 126 were also commonly isolated. The 66 % of seeds were able to germinate and it was not inhibited by *C. chrysosperma*. *Chaetomium funicola* overgrew and ascomata were formed on seeds that failed to germinate.

The correspondence analysis performed on 10 taxa at frequencies equal to or higher than 2 %, showed that the three first axes accounted for 80 % of the total inertia. The first axis (36.4 % of total inertia) separates seeds characterized by *C. funicola* (69.5 %) from peduncle, flower and capsule tissues. The second axis (26.6 % of total inertia) show differences between the internal flower tissues characterized by *Cladosporium cladosporioides* (15.8 %) and the peduncle mainly related the.coelomycete MVFI 130 (40.3%). *A. alternata, F. eucalypti, F. leprosa, C. chrysosperma, Hormonema* sp., *A. pullulans* constitute a cluster reflecting similarities between external tissues (Figure 1). The ordination using axes 1 and 3 (16.3 % of total inertia) yields similar results and is not shown here.

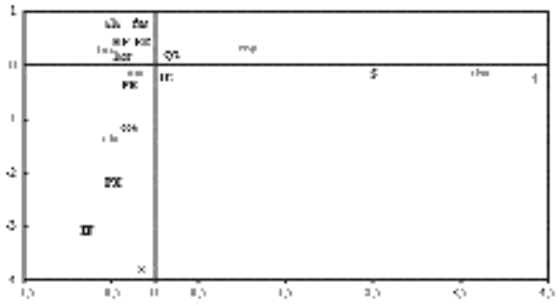

Figure 1. Correspondence analysis. Ordination of flowers, capsules, seeds and peduncles on the first and third axe. Symbols indicate: PX, peduncle xylem; PB, peduncle bark; IF internal tissue of flowers; EF, external tissue of flowers; IC internal tissue of capsules; EC, external tissue of.capsules; S, seeds; ala: *Alternaria alternata*; coe: coelomycete MVFI 130; aur: *Aureobasidium pullulans* ; chf: *Chaetomium funicola*; cla: *Cladosporium cladosporioides*; cyt: *Cytospora chrysosperma*; fai: *Fairmaniella leprosa*; fus: *Fusicoccum eucalypti*; hor: *Hormonema* sp.; nsp: *Nigrospora sphaerica.*

DISCUSSION

The colonization of internal tissues of flowers and peduncles of flowers was very limited in contrast to that of external tissues. Moreover, all taxa isolated from the external tissues are also present in the external. It can therefore be assumed that the infection of the former occurs after the fungus has extensively colonized the external tissues [21]. On the other hand, capsules shared the same species with flowers but at higher frequencies, probably due to the different physiological condition of both organs [22].

The most frequent isolated species, *C. chrysosperma, F. eucalypti, A. alternata, F. leprosa, A. pullulans* and *Cladosporium cladosporioides* isolated from flowers, capsules, peduncles and seeds were also isolated from seedlings, twigs and leaves of *Eucalyptus* spp. [6,11,23]. *Acremonium strictum, Chaetomium globosum, C. funicola, C. cladosporioides* and *Epicoccum purpurascens* isolated in this study are species that naturally occur on seeds of several *Eucalyptus* species as saprotrophs [18]. *C. funicola* overgrew seeds that failed to germinate.

The tissues of capsules favored the colonization of *C. chrysosperma* and *F. eucalypti* as it was shown by the correspondence analysis. The presence of *C. chrysosperma* in the internal tissue of capsules containing mainly young seeds suggests that the colonization of seed teguments could be initiated at this stage. In turn, as the hypocotyl was colonized by *C. chrysosperma*, its presence in *E. globulus* seedling stems [11,23] could probably be initiated early during seed germination colonizing seedling stems through hypocotyl and cotyledon, as was observed during early germination of *Quercus garryana* seeds infected by *Discula quercina* [24]. The appearance of *C. chrysosperma* on symptomatic twigs of *E. globulus* [11] most likely results from expansion of the endophytic mycelium rather than from external infection [25]. In brief, *C. chrysosperma* colonizes vegetative tissues at an early stage of development and probably colonizes reproductive organs from them.

As *F. eucalypti* was present in flowers and capsules, but was absent in seeds, hypocotyl, cotyledons and peduncles of flowers (Figure 1), it is probable that the infection was initiated from the external tissues. *F. eucalypti* (=*Dothiorella eucalypti* (Berk. and Broome) Sacc.) a conidial state of *Botryosphaeria ribis* [26] produces seed capsule necrosis, abortion and twig die back of *Eucalyptus camaldulensis* [26]. In the present study this species has colonized asymptomatically although the fungus might be a latent pathogen. Diminished host resistance or lesions produced by e.g. stress due to drought or frost, could allow the fungus to take over and produce capsule necrosis. This species was isolated in Uruguay from healthy and symptomatic stems of *E. globulus* [23]. It was also frequently isolated from xylem and bark of healthy and symptomatic twigs of *Eucalyptus grandis* grown under drought stress during the summer months combined with several days of frost. It was probable that these conditions may have incited lesions on the bark of twigs and allowed the saprotrophic expansion of this fungus. The fact that *Botryosphaeria* exists endophytically in healthy tissues has important implications for Uruguay forestry industry. The presence and relative frequency of this species in healthy tissues may provide a reflection of the inherent susceptibility for *Eucalyptus* species to this fungus. It was proposed the possibility to eliminate endophytic infection in trees of high value [17] such as those selected for cloned plants. But this procedure implies, in turn, to reduce other endophytic fungi with real or potential active biological properties.

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