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Studies on diversity of foliar fungal endophytes of naturalised trees from Argentina, with a description of *Haplotrichum minutissimum* sp. nov

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Abstract

An ecological investigation of foliar endophytic fungal communities on *Broussonetia papyrifera*, *Celtis occidentalis* and *Ligustrum lucidum* was carried out in the natural reserve Dr. Carlos Spegazzini, Buenos Aires, Argentina. The study yielded 21 taxa. *Alternaria* and *Xylaria* were the most abundant ones and both were isolated from the three hosts. *Phomopsis ligustri-vulgaris*, *Pycnidiella resinae*, *Scytalidium acidophilum*, *Thermomyces lanuginosus* and the genus *Haplotrichum* are cited for the first time as endophytic microorganisms, while *P. resinae*, *Scytalidium* aff. *acidophilum* and *T. lanuginosus* are cited for the first time in Argentina. A new species, *Haplotrichum minutissimum*, is described. © 2008 Elsevier GmbH. All rights reserved.

Keywords: Biodiversity; *Broussonetia papyrifera*; *Celtis occidentalis*; Diatrypales; *Ligustrum lucidum*; *Pycnidiella resinae*

Introduction

Endophytic fungi inhabit, at least for some time along their life cycle, the inner tissue of plants without producing any external symptoms (Petrini, 1991). Studies of endophytes have been motivated by the observation that grazing mammals may exhibit severe symptoms after consuming endophyte-infected pasture grasses. However, because of differences in endophyte life cycles and growth forms, it is not possible to extrapolate results from studies on grasses to studies on host plants of other life forms, such as trees (Hammon and Faeth, 1992; Saikkonen et al., 1996). In Argentina, there are several studies performed on grass endophytes (Iannone and Cabral, 2006; Novas et al., 2003, 2005, 2007, among others). However, just a few of them focus on trees as hosts (Cabral, 1985; Durán et al., 2005; Weber et al., 2005). Trees are free of endophytes in

the bud; after bud break the leaves are gradually infected by airborne spores of many different endophyte species. In trees, foliar endophytes are not directly transmitted to descendants (Elamo et al., 1999).

Dr. Carlos Spegazzini reserve is, to our knowledge, the second natural Mycological reserve in the world (the other one is La Chenez in Switzerland). It is characterised by aged introduced trees, and constitutes the last natural reserve within an urban area. *Broussonetia papyrifera* (L.) L'Herit (Moraceae), *Celtis occidentalis* L. (Ulmaceae) and *Ligustrum lucidum* Ait. (Oleaceae), all exotic trees for Argentina, are species described in the area. *B. papyrifera* and *L. lucidum* are considered invasive plants (IMBIAR: Base de datos sobre invasiones biológicas en Argentina 2006) and both have a high impact in protected areas due to their fast colonisation rate. There are no records of endophytic fungi studied for these hosts.

On the mentioned trees in this protected area, during a study about the biology of Diatrypaceae (Ascomycetes)

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family, three species belonging to this family were identified in decomposed pieces of fallen wood. Analysing the possibility of an endophytic phase of these fungi (Carroll et al., 1977) the objective of the present work was to characterise the foliar endophytic communities of the three hosts and to identify symptomless infections of Diatrypaceae occurring in living tissue.

Material and methods

Study system and sampling

Dr. Carlos Spegazzini reserve is located in Lomas de Zamora district, Buenos Aires province. The soil pH is slightly acid, ranging from 5.4 to 5.8. The maximum and minimum average 5-year span temperatures are 22.2 and 10.7 °C, respectively. The land occupies 300 ha approximately and the plant community is characterised by a naturalised forest (De Magistris, 1996). Three different sampling sites were chosen at random within the study area.

Leaves and petioles of three introduced tree species, *B. papyrifera* and *L. lucidum*, native to China, and *C. occidentalis*, native to North America, were screened for the presence of endophytes. Three healthy leaves and three petioles were collected from each host species at each site. The material was transported to the laboratory in sterile polythene bags and processed within 24 h of collection.

Surface sterilisation

The leaves were washed thoroughly in running water and sterilised using the following immersion sequence: for *Ligustrum* and *Celtis*, 1 min in 70% ethanol, 2 min in a 4% bleach solution, and 30 s in 50% ethanol. Because of its hairy leaves the procedure for *Broussonetia* was slightly modified as follows: 3 min in 70% ethanol, 2 min in the bleach solution and 30 s in 50% ethanol. The leaves were then cut with a sterile scalpel into six 1 cm² segments and transferred to 90 mm Petri dishes containing malt extract agar (MEA). Plates were incubated at 24 °C for 6 weeks and examined periodically. Outgrowing mycelia were isolated, purified, transferred onto slants containing MEA and stored at 4 °C.

Identification of fungi

Cultures were made on the base of two replicates of each isolate grown on MEA and potato dextrose agar (PDA), for at least 10 days. Pure cultures were examined periodically for sporulation. Observations and measurements were taken from fresh material mounted in distilled water, 5% KOH and phloxine for optical

microscopy (M) and Melzer's reagent for the amyloid reaction (I). Those isolates that remained sterile were incubated at 24 °C with a cycle of 12 h UV light and dark to induce sporulation. The cultures that failed to sporulate within 2 months of incubation were designated as sterile mycelium and sorted to morphotypes according to their cultural characteristics.

Fungal identification methods were based on standard mycological manuals and, when necessary, on specialised bibliography (e.g. Ellis, 1971, 1976; Gruyter and Noordeloos, 1992; Kirk et al., 2001; Sutton, 1980; von Arx, 1970). Identified species were preserved in the Culture Collection of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (BAFCcult). Samples for epifluorescence light microscopy (EFM) were prepared in 0.05% calcofluor (Romero and Minter, 1988). Drawings were made using a camera lucida and photographs were taken with an Olympus c-5060 wide zoom Digital camera. Species diversity was calculated in terms of the Shannon–Wiener Index (Hs).

Results

We identified 53 out of the 64 isolates obtained, and 15.8% of the isolates were considered as sterile mycelia. *Xylaria* and *Alteranaria* were the most abundant taxa and isolated from all three hosts (Table 1). The Shannon index values for the overall fungal community of *B. papyrifera*, *C. occidentalis* and *L. lucidum* were 2.8, 3.7 and 3.9, respectively.

Twelve out of the 14 isolates obtained from *B. papyrifera* belonged to four genera, *Nigrospora* and *Alternaria* being the most common ones. *C. occidentalis* presented the highest endophyte biodiversity as 20 out of the 22 isolations were assigned to 10 genera. Most of these isolates belonged to *Xylaria* and *Fusarium*. Sixteen out of the 19 isolates obtained from *L. lucidum* were assigned to eight genera, most of them belonging to *Trichoderma*, *Xylaria* and *Alternaria*.

Thermomyces lanuginosus was the only taxon isolated from petioles and not from leaves, except for three not identified "Mycelia sterilia".

A new species, *Haplotrichum minutissimum*, is described.

Remarks of some species

***Haplotrichum minutissimum* Novas and Carmarán, sp. nov. (Figs. 1–5)**

Etymology: Refers to the small size of conidia.

Ad fungos conidiales hyphomycetes pertinens. Coloniis effusis, pallide, hyphis sterilibus hyalinis septatis, ramosis. Conidiophoris hyalinis ramosis. Cellulis conidiogenis

Table 1. Occurrence of fungi on samples of leaves and petioles from *Broussonetia papyrifera*, *Celtis occidentalis* and *Ligustrum lucidum* in Dr. Carlos Spegazzini reserve, Argentina

Taxa	Host					
	B. p.		C. o.		L. l.	
	L	P	L	P	L	P
<i>Alternaria alternata</i> (Fr.) Keissl.	2	–	–	–	2	1
<i>Alternaria cheiranthi</i> (Lib.) P.C. Bolle	4	–	–	–	2	–
<i>Alternaria chlamydospora</i> Mouch.	–	–	1	–	–	–
<i>Cladosporium tenuissimum</i> Cooke	–	–	1	1	–	–
<i>Cladosporium oxysporum</i> Berk. & M.A. Curtis	–	–	–	–	1	–
<i>Colletotrichum crassipes</i> (Speg.) Arx	–	–	–	–	1	–
<i>Colletotrichum gloeosporioides</i> (Penz.) Penz. & Sacc.	–	–	–	–	1	–
<i>Chaetomium globosum</i> Kunze	–	–	1	–	–	–
<i>Fusarium culmorum</i> (W.G. Sm.) Sacc.	–	–	3	–	–	–
<i>Fusarium semitectum</i> Berk. & Ravenel	–	–	1	–	–	–
<i>Geotrichum candidum</i> Link	–	–	–	–	1	–
<i>Haplotrichum minutissimum</i>	–	–	1	–	–	–
<i>Humicola nigrescens</i> Omvik	–	–	1	–	–	–
<i>Nigrospora sphaerica</i> (Sacc.) E.W. Mason	2	2	–	–	–	–
<i>Phoma chenopodiicola</i> Gruyter, Noordel. & Boerema	–	–	2	–	–	–
<i>Phomopsis ligustri-vulgaris</i> Petr.	–	–	–	–	1	–
<i>Pycnidium resiniae</i> (Ehrenb.) Höhn.	–	–	–	–	1	–
<i>Scytalidium aff. acidophilum</i>	–	–	1	–	–	–
Morphotype sp. 1	1	–	–	–	–	–
Morphotype sp. 2	1	–	–	–	–	–
Morphotype sp. 3	–	–	2	–	–	–
Morphotype sp. 4	–	–	1	–	–	–
Morphotype sp. 5	–	–	1	–	–	–
Morphotype sp. 6	–	–	–	–	1	–
Morphotype sp. 7	–	–	–	–	1	–
Morphotype sp. 8	–	–	–	–	–	1
Morphotype sp. 9	–	–	–	–	–	1
Morphotype sp. 10	–	–	–	–	–	1
<i>Thermomyces lanuginosus</i> Tsikl.	–	1	–	–	–	–
<i>Trichoderma harzianum</i> Rifai	–	–	–	–	6	–
<i>Xylaria</i> spp.	–	1	6	–	5	–

List of abbreviations: of host plants: B. p., *Broussonetia papyrifera*; C. o., *Celtis occidentalis*; L. l., *Ligustrum lucidum*; of tissue sample: L, leaves; P, petioles.

simplicibus, 2–5 µm crassis, denticulis sporogenis simplicibus, sinuatus, cylindratis brevis vel prominentis. Conidiis hyalinis, ovoideis, unicellularis, 10–4 × 3–4 µm papilla basali instructis. Teleomorphis ignota.

Colonies: Light brown to grey, velvety, growing slowly, later becoming dense, powdery. Conidiophores: semi-macronematous, densely caespitose, ascending, more or less erect, frequently branched, hyaline. Conidiogenous cells: polyblastic, integrated, sparsely septate, terminal or intercalary, cylindrical to fusiform, bearing short denticles at conidiation loci. Conidia: holoblastic, dry, unicellular, smooth, predominantly limoniform and bearing a single apiculate papilla, hyaline, (10–) 5.5 (–4) µm long × 3–4 µm wide.

Habitat: On living leaves of *C. occidentalis* L. (Ulmaceae).

Material examined: Argentina, Buenos Aires, Lomas de Zamora, Santa Catalina, culture isolated from *C. occidentalis* L. (Ulmaceae), December 2005, M.V. Novas and C.C. Carmarán (BAFC cult 3179; holotype).

Notes: The species of *Haplotrichum* Link are anamorphs of *Botryobasidium* Donk (Holubová-Jechová, 1969) and were considered for a long time under the names *Oidium* (Holubová-Jechová, 1976; Linder, 1942) and *Acladium* (Ellis, 1976; Partridge et al., 2001). Among the previously described species (Castañeda Ruíz, 1996; Partridge et al., 2001), only *Haplotrichum conspersum* (≡ *Acladium conspersum*) (conidial size 15–18 × 10–15 µm), *H. ramosissimum* (≡ *A. ramosissimum*) (conidial size 8–21 × 7–10 µm) and *Acladium* state of *Botryobasidium ellipsosporum* (conidial size 20–24 × 11–12.5 µm), somewhat resemble *H. minutissimum* because of the conidial shape and the

characteristics of the conidiophore. However, conidia (10–) 5.5 (–4) μm long \times 3–4 μm wide are only present in *H. minutissimum*. In Argentina, the *Haplotrichum* species that have been previously recorded are *H. gracile* (Linder) Hol. and *H. curtisii* (Berk.) Hol. on stumps of *Eucalyptus viminalis* Labill. by Romero et al. (1989). (Plate. 1)

***Pycnidiella resinae* (Ehrenb) Höhn**

The genus *Pycnidiella* L. is monotypic. *P. resinae* is a species registered for several trees, but always on Gymnosperm resins. The genus is characterised by pycnidial conidiomata, not beaked, superficial and microplectenchymatous – see Petch (1943) and Sutton (1980). On the other hand, the genus *Zhithiostroma* Höhn includes species with features similar to *Pycnidiella* but differing in having stromatic multilocular conidiomata with pale brown textura angularis walls (Petch, 1943; Sutton, 1980). The teleomorphs described for *Zhithiostroma* belong to Nectriaceae while that for *Pycnidiella* belongs to *Sarea* Fr. genus (Agyiaceae) (Kirk et al., 2001).

The Argentinean material (Figs. 6–10) showed features that placed it in the genus *Pycnidiella*; however, the features of its conidia (globose to ellipsoidal), conidiophores, and cultural characteristics (pink-orange mycelium developing short lateral conidiogenous cells; conidia produced in abundance giving the colony a slimy appearance; and in 2–3 weeks, superficial hyphae forming yellow floccose pustules and below a pycnidial-like conidiomata development) related it more closely to *Zhithiostroma* (Booth, 1959). Unfortunately, we did not obtain the teleomorph. Although members of the Nectriaceae family have been commonly found in Santa Catalina, no members of Agyiaceae (family of *Sarea*) have been reported for this area. The species concept of *P. resinae* seems to be strongly influenced by the substrate and probably some material assigned to *Zhithiostroma* would actually belong to *Pycnidiella* – the findings point to the necessity for an in-depth revision of *Pycnidiella* and *Zyithiostroma*. Based on the results of this study we decided to assign the examined material to the genus *Pycnidiella*.

Scyatlidium* aff. *acidophilum

This isolate fits the description of *S. acidophilum*, but this species was obtained from very particular substrates and showed very poor morphological characters (Figs. 11 and 12). For this reason we decided not to place it definitively until further studies are carried out.

Discussion

The endophytic mycobiota of *B. papyrifera*, *C. occidentalis* and *L. lucidum* was studied here for the

first time. *Phomopsis ligustri-vulgaris*, *P. resinae*, *Scyatlidium acidophilum*, *T. lanuginosus* and the genus *Haplotrichum* are registered for the first time as endophytic microorganisms, while *P. ligustri-vulgaris*, *P. resinae*, *S. acidophilum* and *T. lanuginosus* are recorded for the first time for Argentina.

We obtained 15.8% of isolates that did not sporulate. This amount agrees with previous studies such as those by Petrini et al. (1982), who reported that 15% of endophytes isolated from evergreen shrubs in Oregon did not sporulate, and also those by Fisher et al. (1994), who reported different isolate frequencies of sterile mycelia from different tissues of *Quercus ilex* from different sites.

The number of species recovered from leaves was much higher than that from petioles. Only 6% of the fungal species were recovered from both tissue types suggesting that most of these fungal species exhibit organ specificity, as it is also the case in other host plants (Bill and Polishook, 1992; Petrini, 1986, 1991).

For the mycota recovered from the three host plants a diversity index was calculated. The highest value was obtained for *Ligustrum*, that from *Celtis* was close to it, and a lower value was found for *Broussonetia*. The three introduced hosts obviously support a rich fungal diversity that comprises few dominant species and many rare species. This is reflected also in the number of new records for the country and the new taxon described.

By definition, an endophyte cannot be considered to cause diseases (Sinclair and Cerkauskas, 1996). However, genera and species than can cause diseases are regularly isolated as endophytes (Photita et al., 2004). Some of the endophytes isolated in the present study are generally assumed to be potential fungal pathogens. These species are *Alternaria alternata*, *Cladosporium oxysporum*, *Colletotrichum gloeosporioides*, *Fusarium culmorum*, among others. However, none of the endophytes isolated in this work has been previously recorded as a pathogen, with the exception of *A. alternata* for *L. lucidum*.

Species initially known to be pathogenic, which have been isolated as endophytes are not necessary pathogens. It has been suggested that virulent pathogens can undergo mutations rendering the fungus a nonpathogenic strain of the original pathogen (Freeman and Rodriguez, 1994). In the present study, we observed that the degree of correlation between the fungal pathogens recorded for these plant species (Farr, 1989) and the isolated endophytes is low, thus supporting the Freeman and Rodriguez (1994) hypothesis.

In the context of the present study, it is not possible to decide if the endophytes isolated from healthy leaves and petioles are potential pathogenic strains. They may be nonpathogenic strains and experiments following Koch's postulates are required to confirm any pathogenicity.

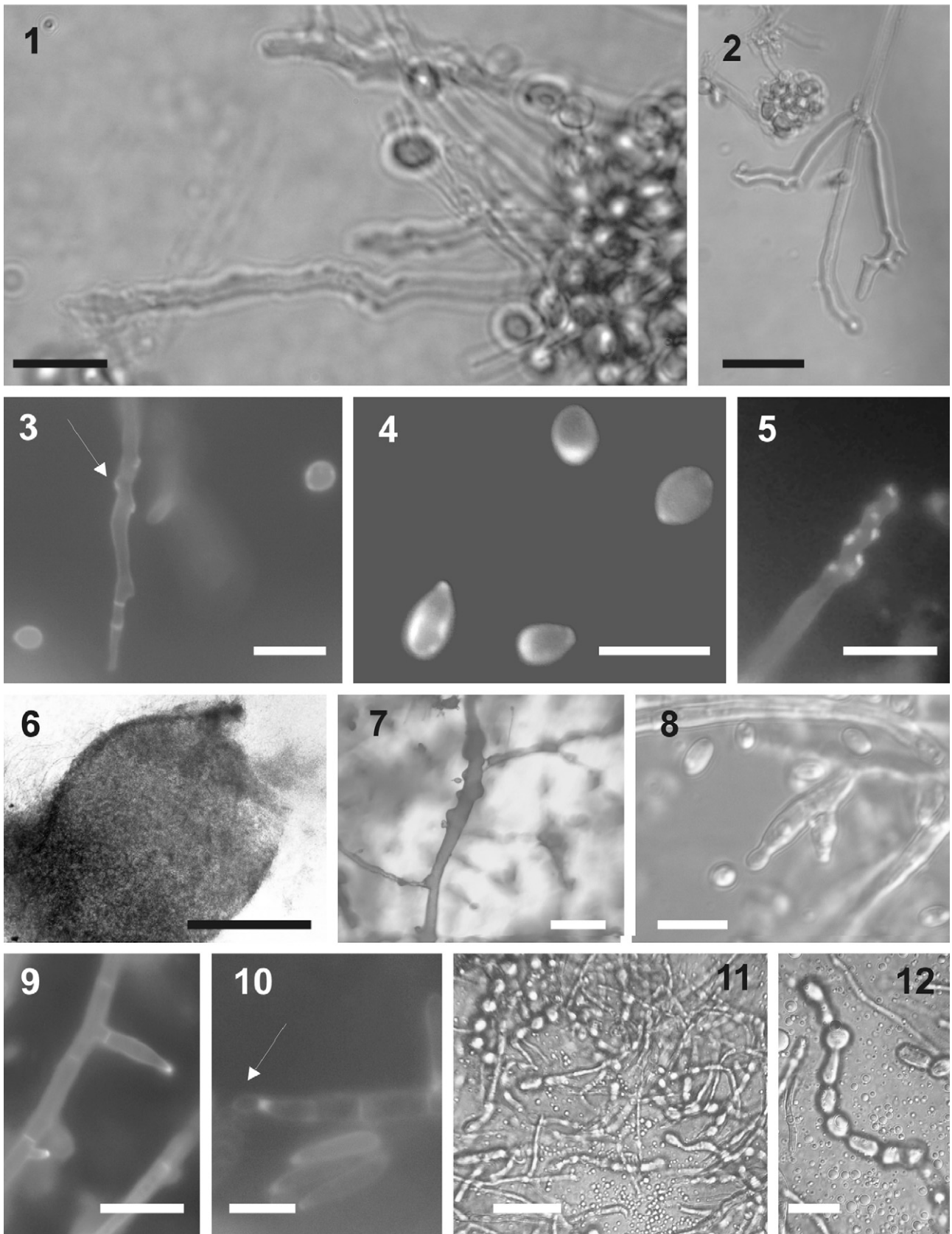


Plate 1. Figs. 1–5. *Haplotrichum minutissimum*. 1, 2. Conidiophores and conidia. 3–5. Conidiogenous cells and conidia (EFM). Figs. 6–10. *Pycniella resinae*. 6. Pycnidial conidiomata. 7. Slimy appearance of the colony. 8. Conidiogenous cells and conidia. 9. Conidiogenous cells. 10. Conidiogenous cell with young conidia. Figs. 11 and 12. Chains of thick-walled arthroconidia of *Scytalidium acidophilum*. Bars: 1 = 10; 2 = 20; 3 = 10; 4 = 10; 5 = 10; 6 = 150; 7 = 20; 8 = 5; 9 = 10; 10 = 5; 11 = 20; 12 = 10 μ m.

B. papyrifera and *L. lucidum* are considered invasive plants (INBIAR), and, relying on this fact, one could consider them a sources of potential inoculum for native, susceptible plants, especially in protected areas. Nevertheless, as discussed above, the probability of potential risk is low.

Fungi are megadiverse, vital for the ecosystem, yet their conservation lags a long way behind that of plants and animals. Hotspots are areas of high diversity or sites that contain threatened species. Tropical trees have already been considered to be special hotspots of fungal species diversity (Arnold and Lutzoni, 2007). The presence of a high diversity of fungal species living within the tissues of studied hosts, all with a wide distribution, suggests their potential as an important fungal reservoir to be taken into account in conservation issues.

Endophytic Xylariaceae have been documented in all lineages of land plants (Brunner and Petrini, 1992). However, no clear benefit to living host plants has been reported for this family (Davis et al., 2003). The Diatrypaceae family is nowadays placed in the Xylariales order (Kirk et al., 2001). Most of Diatrypaceae species have been described as saprophytic, predominantly on angiosperm bark. In Argentina, there has been little work on Diatrypaceae in general. All studies have focused on species descriptions and taxonomy (Carmarán, 2002; Carmarán and Romero, 1992; Romero and Carmarán, 2003). At present, to our knowledge, there are only a few records of a symptomless infection by Diatrypaceae: *Libertella* sp. Desm. (Carroll et al., 1977), *Cryptosphaeria populina* (Chapela, 1989) and *Cryptosphaeria lignyota* (Hutchison, 1999).

We have identified *Diatrype leprosa* in dead branches of *B. papyrifera* and *C. occidentalis*, and *Peroneutypa scoparia* (Carmaran et al., 2006) in dead branches of *L. lucidum* still attached to the tree. Nevertheless, we did not obtain Diatrypaceae fungi in living tissue of leaves and petioles. These present results appear to suggest that the endophytic strategy, at least in leaves and petioles, could be restricted to some species of the family and might not be a general characteristic of the group. We are planning to conduct a similar assay in living wood of these three hosts in order to obtain more evidence related to the ecological role of these fungi.

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