Endophytic fungi of twigs and leaves of three native species of Myrtaceae in Uruguay

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The main purpose of this research was to examine the endophytic fungal composition of leaves and twigs of *Blepharocalyx salicifolius*, *Myrceugenia glaucescens* and *Acca sellowiana* (Myrtaceae-Myrtoideae) growing within their natural distribution area in southern central Uruguay. Twig and leaf segments were plated on 2% MEA. The percent of colonization ranged from 27% (*M. glaucescens* xylem) to 78% (*Blepharocalyx* bark). *Xylaria enteroleuca*, *Pestalotiopsis guepinii*, and sterile mycelia colonized several tissues of all host species. Simple correspondence analysis showed differences in endophyte composition between *M. glaucescens* and the other two hosts, suggesting host preferences. Conversely, *A. sellowiana* and *B. salicifolius* were characterized by similar species composition and the slight difference observed was due to some species isolated in only one of them or in both but with different relative frequency of isolation. Ordination resulting from cluster analysis showed that the endophytic composition of twig xylem and bark was similar in *B. salicifolius* and *A. sellowiana*. In *M. glaucescens* tissue preference was not evident. The endophytes *Sphaeropsis sapinea*, *Botryosphaeria dothidea* and *Colletotrichum gloeosporioides* found here were also present in *Eucalyptus* spp. cultivated as non-native in Uruguay but the endophytic diversity of the three hosts was nearly similar to that of *Eucalyptus* spp.

Keywords: *Xylaria enteroleuca*., sterile mycelia, fungal diversity, neotropical Myrtaceae.

Several genera of Myrtaceae are typically distributed in tropical and subtropical forests southeastern of South America (Brazil, Uruguay, northeast Argentina, south-central Paraguay), mainly between 20–35° S and 48–56° W, with a few genera restricted to the Andean highlands of the northwest (Landrum, 1981; 1986). In Uruguay three species of Myrtaceae, *Myrceugenia glaucescens* (Cambessèdes) Legr. & Kaus., *Blepharocalyx salicifolius* (Hook. & Arn.) Legr. and *Acca sellowiana* (O. Berg) Burret, are some of the evergreen shrub or small trees of low hills, riparian and ravine vegetation. *A. sellowiana* is also cultivated in subtropical areas as an ornamental plant and edible fruit (Legrand & Klein, 1977).

Endophytes are commonly present in all living plant tissues without causing disease symptoms (Carroll, 1986). Although, these fungi can produce symptoms in some cases when appropriate ecological and physiological conditions occur after living for a certain period as neutral symbionts (Stone & Petrini, 1997; Bissegger & Sieber, 1994).

In recent years fungal endophytes and some potential pathogens occurring in non-native *Eucalyptus* planted in South America and particularly in Uruguay have been studied (Bertoni & Cabral, 1988; Bettucci & Alonso, 1997; Bettucci & al., 1997; Bettucci & Saravay, 1993; Bettucci & al., 1999; Lupo & al., 2001). However, no research has been carried out on fungal endophytes of wild Myrtaceae. Therefore, the main goal of this work was to study the composition of endophytic assemblages of these native Myrtaceae and, at the same time, to detect whether or not differences in tissue preference exist. An additional goal was to compare the endophytic composition of these native plants with that of non-native *Eucalyptus* spp. cultivated in Uruguay, and to evaluate whether or not some fungal species, which are present as endophytes in native species, could also colonize *Eucalyptus* spp.

Materials and methods

Sampling site

The selected site was the "Quebrada de los Cuervos", located in the northeast of Uruguay, from 32°53'S, 54°30'W to 33°06'S, 54°30'W. This site is a ravine, deeper than 100 m; the Arroyo Yerbal Chico is running through the lower part. The climatic type according to Koeppen-Geiger classification (Strahler & Strahler, 1992) is *Cfa*, temperate with rains during all year. The mean annual temperature ranges from 11° to 23° C and the annual precipitation amount ranges from 900 to 1300 mm. The vegetation growing on the ravine is mainly composed by species of *Acca*, *Blepharocalyx*, *Eugenia*, *Hexachlamys*, *Myrcianthes*, *Myrceugenia*, *Myrrhinium*, *Psidium* (Myrtaceae), and other native species such as *Syagrus romanzoffiana* (Cham.) Glassm and *Ocotea acutifolia* (Nees.) Mez. (Basso & Pouso, 1992). *A. sellowiana*, *B. salicifolius* and *M. glaucescens* are small evergreen trees or shrubs that are very common in this habitat. A mixed stand of 4,500 m^2, along the slope of the ravine, was delimited for plant species sampling.

Material collection and fungal isolation

From 10 randomly selected trees of *A. sellowiana*, *B. salicifolius* and *M. glaucescens*, growing in proximity to one another, 20 asymptomatic twigs with leaves were collected from each plant species. All materials were taken to the laboratory in paper bags, stored at 5° C and processed within 24 h.

Ten segments of approximately 2–5 mm in diameter and 5 mm in length were cut from 10 twigs of each species, and the bark was stripped off the xylem. From 20 leaf blades, 10 discs of 4 mm in diameter were dissected. Ten segments from 20 petioles were also examined, except from *B. salicifolius* in which the petiole is very small. Segments from each tissue discriminated by host tree were pooled and surface-sterilized by sequential immersion in 80% ethanol for 1 minute, sodium hypochlorite (0.4 g active Cl/100 ml) for 2 minutes, washed with sterile distilled water, and then dried on sterile filter paper. To test the effectiveness of surface sterilization, segments imprint on growth medium was performed.

Segments from each tissue were randomly selected for plating. In sets of 10 segments per plate, 500 segments from *M. glaucescens* and *A. sellowiana*, and 400 segments from *B. salicifolius*, were placed onto 9 mm Petri dishes containing MEA 2% (Difco), pH 4.5, and incubated at 24° C for six weeks or more depending on the growth rates of fungi. Each colony that emerged from segments was transferred to fresh medium (MEA 2% Difco, PDA Difco and oatmeal agar) for identification and incubated at 24° C. Black light was used to induce sporulation in some cultures. Identification was performed by means of conventional mycological methods following description of the cultural and micromorphological characteristics of each isolate (Sutton, 1980; Ellis, 1971; 1976; Dennis, 1981; Gams, 1983; Tulloch, 1972). Isolates with cultural characteristics similar to *Xylaria* were identified comparing them with several descriptions of cultural characters (Rogers, 1984; Petrini & Petrini, 1985; Rogers & Samuels, 1986; San Martin Gonzalez & Rogers, 1989; Callan & Rogers, 1990; Callan & Rogers, 1993; Rodrigues & al., 1993). The identification was confirmed comparing ITS1 and ITS2 sequences with the GenBank data. Similarly, identification of isolates with micromorphological characteristics corresponding to *Cytospora* was also confirmed by means of ITS1 and ITS2 sequences (Simeto & al., 2003).

Those cultures that failed to sporulate after 6 weeks were considered sterile mycelia.

Data analysis

The relative frequency of isolation was calculated as the number of segments colonized by a given fungus, divided by the total number of segments expressed as percentage. To evaluate to which extent the complete fungal community was revealed by the sampling, the abundance distribution and species accumulation curves for each tissue of tree hosts were performed. Moreover the relative abundance curves were compared to lognormal theoretical model using the Kolmogorov-Smirnov test (Krebs, 1989). Diversity was measured for each tissue, organ and tree host by means of Shannon diversity index with the computer package MVSP for Windows (Kovach Computing, Anglesey, UK).

To evaluate differences in endophytic composition among hosts, a simple correspondence analysis using STAT-ITCF (Service des Etudes Statistiques, Institute Technique des Céréales et des Fourrages, France) was carried out using the sum of species frequencies, at least, in any tissue with a relative frequency of 5% or more. Species with lower frequency, but isolated from two host species, were also included (Howard & Robinson, 1995). A single-linkage UPGMA cluster analysis was performed on all species using the Sorensen's index of similarity with the computer package MVSP for Windows (Kovach Computing, Anglesey, UK) to examine the degree of similarity among the endophytic populations found in each tissue from the three hosts.

Results

From 1,400 segments, 771 isolates belonging to 35 taxa were obtained from twigs and leaves with the number of taxa ranging from 5 (*A. sellowiana* petiole) to 19 (*A. sellowiana* blade). Of all taxa isolated, 6 were present in the three hosts, 9 in two hosts and 20 were found exclusively in any host species. Of these 20, 14 were isolated from only one tissue and 11 from leaves, in general at low relative frequency. Conversely, the species isolated with the highest relative frequency (> 25% at least in one tissue) were in general, those common to two or three hosts. The endophyte community was dominated by *Pestalotiopsis guepinii* (Desm.) Stey., *Xylaria enteroleuca* (Speg.) Martin, *Colletotrichum gloeosporioides* (Penz.) Sacc., *Cladosporium cladosporioides* (Fresen.) de Vries and sterile mycelia common to the three hosts. *X. enteroleuca* was the species isolated with the highest relative frequency (Tab. 1).

Endophytic communities in all tissues of the three hosts fit with lognormal distribution ($P>0.05$) (Fig. 1). The percent of segments colonized ranged from 27% (*M. glaucescens* xylem) to 78% (*B. salicifolius* bark) (Fig. 2), with overall average colonization rates for each host tree of 59.5% for *A. sellowiana*, 60% for *B. salicifolius* and 48% for *M. glaucescens*. In leaves, the percentage of colonization of petiole was greater than that of lamina, and in twigs, bark was more colonized than xylem, in the three host species.

The cumulative species abundance (Fig. 3) shows the number of species found with each 10 additional segments plated out. The point

Tab. 1, – Endophytic fungi of leaves and twigs. Frequency of colonization (%).L: blade, P: petiole, B: bark, X: xylem.

		A. sellowiana				*B. salicifolius*			*M. glaucescens*			
	code	L	P	B	X	L	B	X	L	P	B	X
Alternaria alternata (Fr.) Keissler	alt	2.0							0.5			
Botryosphaeria dothidea (Moug. : Fr.) Ces. & De Not.	bot								5.5	9.0	11.0	5.0
Cladosporium cladosporioides (Fresen.) de Vries	cla	6.5					1.0	3.0	0.5			
Colletotrichum gloeosporioides (Penz.) Sacc.	col	2.5				3	3.0		6.5	4.0	2.0	
Colletotrichum musae (Berk. & Curt.) Arx										1.0		
Colletotrichum sp.									0.5			
Coniella delicata Sutton	con	3.0						1.0				
Coniothyrium sp.								1.0				
Coryneum calophylli (Syd.) Morgan-Jones	cor							5.0				
Cylindrocarpon magnusianum (Sacc.) Wolllenw.	cyl										7.0	1.0
Cytospora sp. FI 1277	cyt	13.5	10.0	5.0	4.0	8.0	33.0	18.0				
Diplodia sp.		0.5										
Diplodina sp.	dip								0.5			12.0
Discosporium eugeniae (Allesch.) Sutton	dis	2.0				14.5						
Epicoccum nigrum (Link) Schlecht.	epi			2.0					1.0			4.0
Fusicladium scribnerianum (Cav.) Ellis									0.5			
Gelasinospora retispora Cain										1.0		
Kuskia oryzae Hudson	kus	0.5				1.0						
Microsphaeropsis arundinis Sutton		1.0										
Microsphaeropsis pseudaspera Sutton		1.0		2.0	1.0							
Myrothecium sp.	myr	7.5										
Nigrospora sphaerica (Sacc.) Mason		1.0										

Nodulisporium sp.		nod	1.5								1.0		
Pestalotia pezizoides De Not.			0.5		1.0								
Pestalotia sp.		pest			2.0	2.0		2.0					
Pestalotiopsis guepinii (Desm.) Stey.		pes	17.0	3.0	28.0	22.0	1.0	9.0	8.0	1.5	5.0	2.0	1.0
Phialemonium dimorphosporum Gams & Cooke			0.5										
Phoma sp.		phm						6.0					
Phomopsis archeri Sutton		pho								1.0	17.0	36.0	1.0
Phyllachora melaleucae Syd. & P. Syd.		pyl					10.0						
Pleurocytospora vestita Petrak			1.5										
Sphaeropsis sapinea (Fr.) Dyko & Sutton		sph		9.0	7.0	8.0	0.5	17.0	7.0				
Xylaria enteroleuca (Speg.) Martin		xyl	5.5	51.0	8.0		0.5	10.0	6	1.5	8.0		1
Dark sterile mycelia		dsm	1.5		2		9.0		2.0	19	1		
Hyaline sterile mycelia		hsm		2.0		1.0	2.5		2.0	5.5	5	4.0	2.0
Total segments	1400		200	100	100	100	200	100	100	200	100	100	100
Total isolates	771		138	75	57	38	100	81	53	88	52	62	27
Total taxa	35		19	5	9	6	10	8	10	13	10	6	8

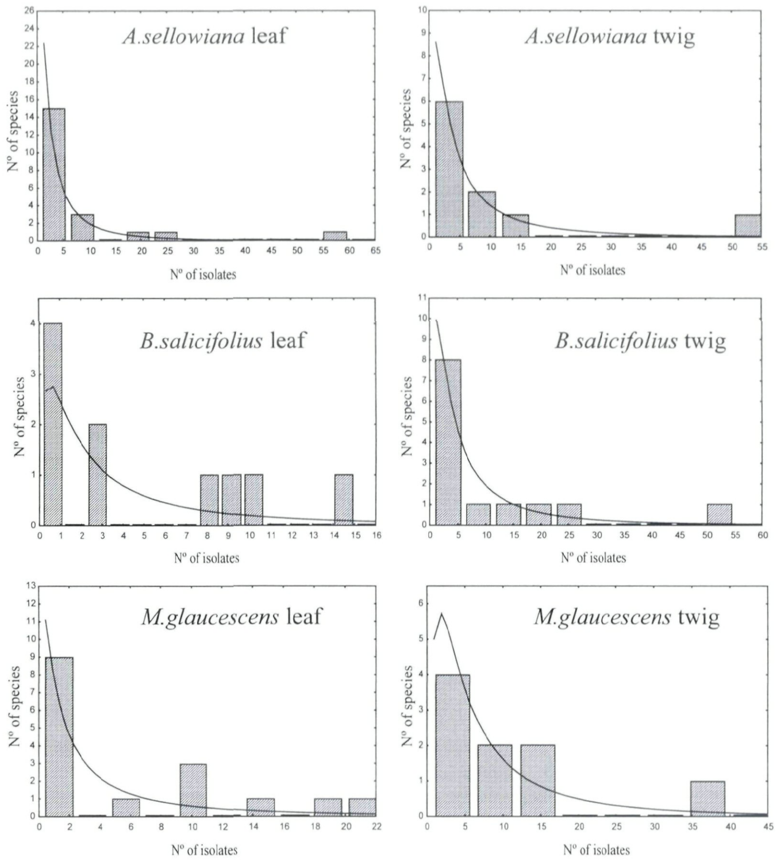


Fig. 1. – Lognormal of species abundance distribution from each tissue. Few species were isolated with high frequency and several were rare. The lognormal distribution expected (line) did not differ significantly ($P > 0.05$) from the observed data (Kolmogorov-Smirnov).

of achieving the asymptote varied for each tissue and host segments from 150–170 in the blade, 50–70 in the petiole, 50–90 in the bark, and 30–70 in the xylem.

Diversity was higher in leaves than in twigs for *A. sellowiana* and *M. glaucescens*, while for *B. salicifolius* the diversity in leaves and twigs was similar. Contrary to *A. sellowiana*, in *M. glaucescens* the diversity in the petiole was higher than in the blade and was also higher in the xylem than in the bark.

Endophytic communities of each plant host evidenced a higher diversity than that of separate organs from each host (Tab. 2). Simple correspondence analysis carried out on 23 species showed that the two first coordinate axes explained 100% of the total inertia, indi-

Tab. 2. – Measures of diversity for endophytic communities of tissues, organs and plant hosts.

	Diversity oh host plant	Blade	Petiole	Bark	Xylem	Leave diversity Blade and petiole	Bark and xylem
Acca selowiana							
H’	2,078	2,368	1,011	1,637	1,228	2,058	1,562
J	0,663	0,804	0,628	0,745	0,685	0,676	0,678
S	23	19	5	9	6	21	10
Blepharocalyx salicifolius							
H’	2,233	1,850		1,656	1,949		1,904
J	0,805	0,803		0,797	0,846		0,742
S	16	10		8	10		13
Myrceugenia glaucescens							
H’	2,175	1,822	1,909	1,267	1,637	2,149	1,731
J	0,768	0,710	0,829	0,707	0,787	0,775	0,788
S	17	13	10	6	8	16	9

H’: Shannon’ diversity index; J: evenness; S: total number of species in the community.

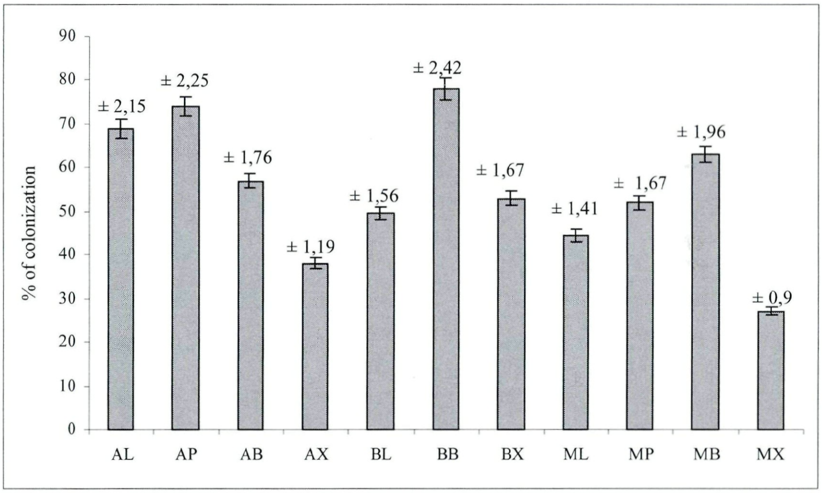
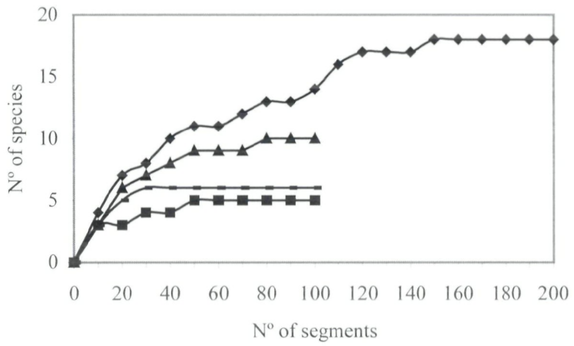
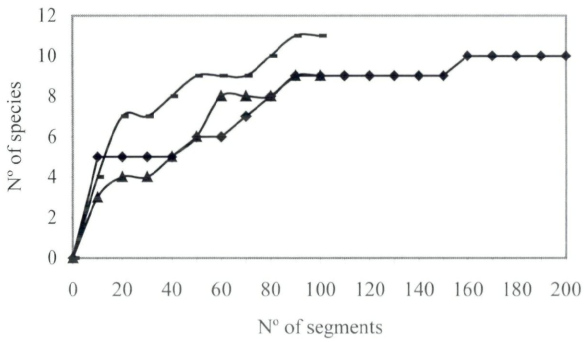


Fig. 2. – Number of tissues segments colonized in *A. sellowiana, B. salicifolius* and *M. glaucescens*. B: bark; X: xylem; L: blade; P: petiole. Bars indicate standard deviations.

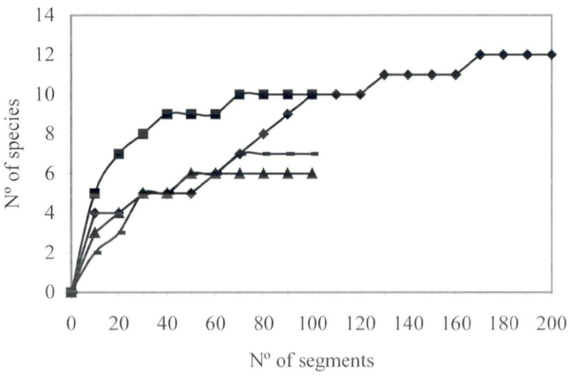
cating an excellent fit of the data to the model (Fig. 4). The axis 1 accounted for 73.3% of the total inertia and separated fungal assemblages of *M. glaucescens* from those of *A. sellowiana* and *B. salicifolius*. *Botryosphaeria dothidea* (Moug. : Fr.) Ces. & De Not.



Acca sellowiana



Blepharocalyx salicifolius



Myrceugenia glaucescens

Fig.3. – Species accumulation curves showing the number of species found with each 10 additional segments plated out. Symbols indicate: ◆: leaf; ■: petiole; ▲: bark; ▬: xylem.

and *Phomopsis archeri* Sutton contributed 47.4% to the inertia of this axis and colonized all tissues of *M. glaucescens*. An assemblage of several taxa was associated with the other two plant species, *Cytospora* sp. FI 1277 and *Sphaeropsis sapinea* (Fr.) Dyko & Sutton being the two most important fungal species. The axis 2 accounted for 26.7% of the total inertia and explained some differences between *A. sellowiana* and *B. salicifolius*. A set of species including *P. guepinii* and *X. enteroleuca*, mainly associated with *A. sellowiana*, contributes to 34.9% of the inertia of this axis. However, *P. guepiini* was isolated from all tissues but at a higher relative frequency in *A. sellowiana* than in other plants. *Cytospora* sp. FI 1277, *Discosporium eugeniae* (Allesch.) Sutton, *Phyllachora melaleucae* Syd. & P. Syd., that accounted for 37.9% of the inertia of this axis, were mainly associated with *B. salicifolius*. *P. melaleucae* only colonized leaves of *B. salicifolius*.

The ordination resulting from the cluster analysis performed on all isolated taxa (Fig. 5) showed that the endophytic composition of *M. glaucescens* constitutes a node that differed by 66% from those of the other two hosts; the similarity among tissues of this host ranged from 61–71%. Xylem and petiole of *A. sellowiana* showed the highest similarity (73%), mainly due to four of the five species isolated from both petiole and xylem. Conversely, *A. sellowiana* blade constitutes a separate node mainly explained by 5 rare taxa and *Myrothecium*, exclusively isolated from this host. When the analysis was carried on without rare species isolated from only one tissue, *B. salicifolius* blades showed a greater similarity (65%). The remainder tissues of *A. sellowiana* and *B. salicifolius* reflect moderate similarity, ranging from 57–61 %.

Discussion

The composition of the endophytic communities of *A. sellowiana* and *B. salicifolius* was similar, differing in their relative frequencies of isolation, suggesting that the host preference is low (Cannon & Simmons, 2002). Conversely, endophyte composition in *M. glaucescens* evidenced host preference. This preference, however, needs to be confirmed by additional research on endophytic fungi of several other Myrtaceae.

Some species considered almost exclusively endophytic (Bills & Polishook, 1992) were the dominant component of the endophyte communities of these three hosts. *P. guepinii* was commonly found in *Eucalyptus* spp. in Uruguay (Bettucci & Saravay, 1993; Bettucci & Alonso, 1997; Bettucci & al., 1997; Bettucci & al., 1999; Fisher & al., 1993). *Pestalotiopsis* spp. is a common endophyte in temperate

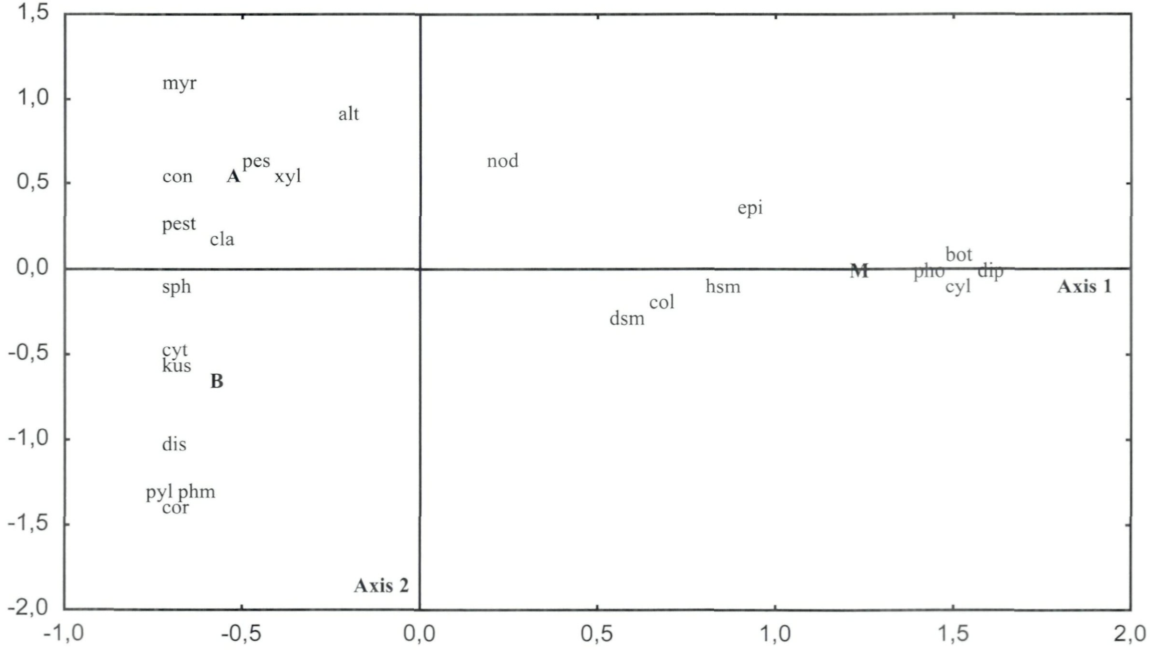


Fig. 4. – Simple correspondence analysis. Ordination of hosts according to the endophytic composition on the two first axes. Total inertia explained by the first two co-ordinates axes is 100%. Variables are the relative frequencies of isolation of species with frequency equal or higher than 5% and those with lower frequency but isolated from at least two host species. Symbols for the species are indicated in Tab. 1; A: *A. sellowiana*, B: *B. salicifolius* and M: *M. glaucescens*.

(Barengo & al., 2000; Bills & Polishook, 1992) and tropical (Bayman & al., 1998) tree species.

X. enteroleuca has been reported as endophyte of *Manilkara bidentata* (A.DC.) Chev. and orchids in Puerto Rico (Lodge & al.,1996). Moreover, several species of *Xylaria* have been isolated as endophytes of almost all tropical plants and, to a lesser extent, of temperate trees (Bayman & al., 1998; Dreyfuss & Petrini, 1984; Lodge & al., 1996; Rodrigues, 1994; Rodrigues & al., 1993; Rodrigues & Petrini, 1997; Rodrigues & Samuels, 1990; Takeda & al., 2003; Bills & Polishook, 1992; Fisher & al., 1986; Fisher & Petrini, 1990; Boddy & Griffith, 1989). In contrast to the distribution observed in *M. bidentata* (Lodge & al., 1996), *Xylaria* was isolated at higher frequencies in petioles than in blades of *A. sellowiana* and *M. glaucescens*. In addition *Xylaria* was absent from *M. glaucescens* twigs: consequently vertical transmission cannot be inferred.

B. dothidea and *Cytospora* spp. are common endophytes in *Eucalyptus* spp. (Bettucci & Alonso, 1997; Bettucci & al., 1997; Bettucci & al., 1999; Alonso & al., 2003) Cultural characteristics and ITSI and ITS II sequences of *Cytospora* sp. FI 1277 differed from those of the species recorded in *Eucalyptus* spp. plantations located in a similar sampling site of the native Myrtaceae, providing evidences that isolates of *Cytospora* sp. FI 1277 from native Myrtaceae and those from *Eucalyptus* are different species (data not shown). Conversely, *B. dothidea* isolates from *M. glaucescens* (Simeto & al., 2003) were similar to those isolated from *Eucalyptus* spp., *S. sapinea*, and *C. gloeosporioides* are also common endophytes of *Eucalyptus* spp. in Uruguay and in different other countries (Smith & al., 1996; Fisher & al., 1993). Therefore, there is no specific evidence that they have adopted *Eucalyptus* spp. as a host as they also colonize several other hosts and have a world-wide distribution. The diversity in twig endophytic communities of the three host was nearly similar to that found in *Eucalyptus* spp. (Bettucci & Alonso, 1997; Bettucci & al., 1997; Bettucci & al., 1999).

The absence of wood rotting Basidiomycetes in native Myrtaceae represents a remarkable difference with the composition of endophytic communities of *Eucalyptus* spp. twigs (Bettucci & Saravay, 1993; Bettucci & Alonso, 1997; Bettucci & al., 1997; Bettucci & al., 1999).

In general, the proportion of twig segments infected in tropical trees (Gamboa & Bayman, 2001; Cannon & Simons, 2002; Arnold & al., 2001; Arnold & Herre, 2003) exceed the one recorded in the neotropical Myrtaceae studied here, but is similar to that of temperate trees (Carroll, 1995). Instead, the diversity in twigs was, at least, similar to that of low and mid branches of *Guarea guidonia* (L.) Sleumer in Las Piedras, Puerto Rico (Gamboa & Bayman, 2001). In

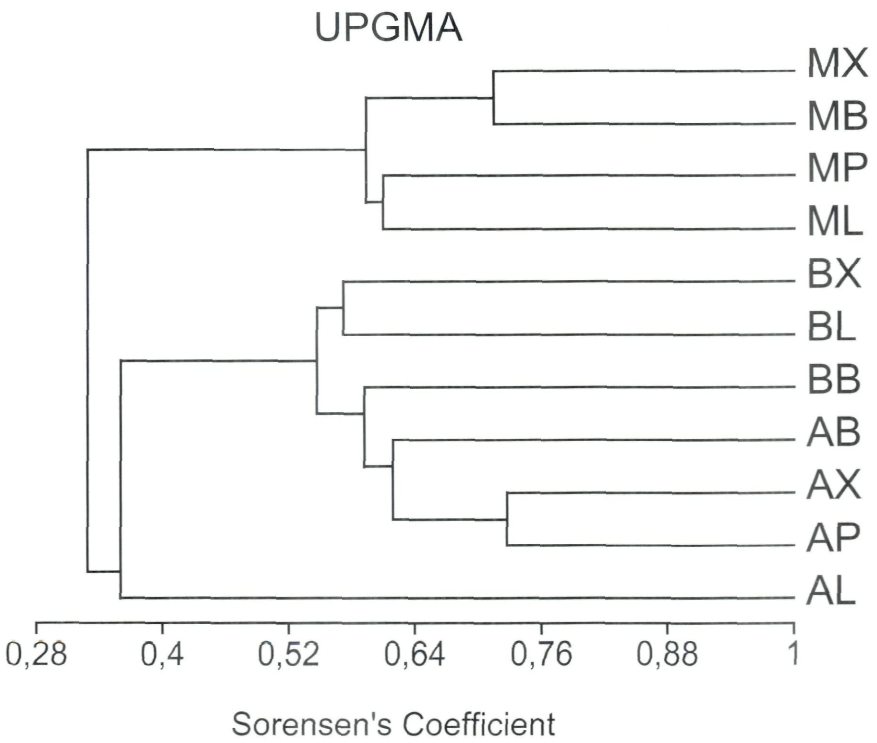


Fig. 5. – Single linkage UPGMA cluster analysis of endophytic communities resulting from tissues of each host using Sorensen's index of similarity. Symbols for tissues are indicated in Fig. 1. A: *A. sellowiana*, B: *B. salicifolius* and M: *M. glaucescens*; B: bark; X: xylem; L: blade; P: petiole.

Uruguay, Myrtaceae colonize the southern limit of their geographical distribution and present morphological characteristics that reflect adaptations to temperate climate (Landrum, 1986; Legrand. & Klein, 1977). Therefore, it is not surprising that these host species have a lower rate of infection with a diversity similar to that of endophytic communities of tropical trees. Moreover, the distribution of the isolation frequency of some species could reflect host and tissue preference as already described in trees of the temperate zones (Carroll, 1995).

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