Endophytic fungi of twigs and leaves from *Ilex paraguariensis* in Brazil

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The main purpose of this work was to examine the endophytic fungal composition of leaves and twigs of *Ilex paraguariensis* growing within its natural distribution area, in two sites in the South East of Brazil along an altitudinal gradient. Twigs, blades, main vein and petiole segments were plated onto 2% Potato Dextrose Agar. The diversity of fungal endophytes was low, as it was the number of tissue-specific fungal species. *Guignardia philoprina*, *Xylaria* spp. and several sterile mycelia were the main taxa present in all the tissues of the two sites. *Xylaria* was the genus with the highest number of species, *Xylaria cubensis* being the most frequent species. The sterile mycelia were more common in leaves blade than in twigs. Simple Correspondence Analysis evidenced differences mainly between twigs of two sites due to different frequencies of isolates than to species composition. This situation could arise because plants from these sites were planted under conditions which do not differ enough to be selective for fungal species distribution.

Keywords: *Guignardia philoprina, Xylaria* spp., sterile mycelia, biodiversity, yerba mate.

Ilex paraguariensis St. Hilaire (yerba mate) is a typical wild plant from tropical regions of South America (Aranda, 1986). It is distributed in Argentina, Brazil and Paraguay and occupies ca. 540,000 Km^2, mainly between 21 to 30° S and 48 to 56° W (Oliveira & Rota, 1985). Along this area the mean temperature of the warmest months is lower than 22 °C and the mean annual rainfall is around 1,500 mm. In the South East of Brazil *I. paraguariensis* occurs as a perennial shrub in the *Araucaria* forest, particularly on soils with low nutrient and high aluminum content. It grows between *ca* 500 to 1,000 m over the sea level and it is not very frequent outside of this altitude. This species has been used as a crop plant since about 1730 (Aranda, 1986). Dried leaf blades, petioles and small twigs are used to prepare a caffeine rich infusion which has also nutrient and therapeutic properties (Costa, 1989). Seed germination is low (12–

35 %) due to physiological dormancy, hence, vegetative propagation by means of stakes is a frequent practice (Kaspary, 1991).

Grigoletti & al. (1992) have recorded the main phytopathological problems of *I. paraguariensis* plantations. *Botrytis* sp., *Colletotrichum* sp. and *Cylindrocladium* sp. were considered the most important seedling pathogens and *Phyllosticta yerbae* Speg. and *Asterina mate* Speg. the causal agents of leaf spots. These pathogens, however, were considered unimportant and chemical control was not recommended (Carpanezzi & al., 1985).

Endophytes are commonly present in all living tissues, the majority being not associated with disease symptoms (Stone & Petrini, 1997). Some studies on endophytic fungi were performed in temperate regions of South America (Bertoni & Cabral, 1988; Bettucci & Alonso, 1997; Bettucci & al., 1997; Bettucci & Saravay, 1993; Bettucci & al., 1999; Lupo & al., 2001) and in tropical regions (Rodrigues & Samuels, 1990; Rodrigues, 1994; Rodrigues & Petrini, 1997). Fungal endophytes of *I. paraguariensis*, however, were not in the scope of any research. Consequently, the main goal of this work was to study the endophytic mycobiota from leaves and twigs of *I. paraguariensis* growing in two sites along an altitudinal gradient in Rio Grande do Sul in the South East of Brazil. Furthermore, we were also interested to evaluate the presence of endophytic species on twigs and leaves in view of their potential phytopathological incidence.

Materials and methods

Study area

Two sites planted with *I. paraguariensis* were selected on an altitudinal gradient in Rio Grande do Sul, at the South East of Brazil. Site Mato Leitąo was located in the Depressão Central region at an altitude of 200 m (29° 32' S and 53° 52' W) with an annual mean temperature of 19 to 20 °C and with an annual rainfall of 1500 to 1750 mm. Site Veranopolis was located in the Encosta Superior do Nordeste region, at an altitude of 705 m (29° 32' S and 53° 52' W), with an annual mean temperature of 14° to 16 °C and with an annual rainfall of 1750 to 2000 mm. Rainfall, in both sites, is distributed evenly during the year.

Plantations were situated on podzolic soils (Mato Leitão) and litosols (Veranopolis). Plants are yearly thinned to obtain twigs and leaves for commercial purposes.

Material collection and fungal isolation

In both sites a one year old twig with its leaves was collected from each of 10 trees randomly selected along a 100 m transect. All

materials were taken to the laboratory in paper bags, stored at 5 °C and processed within 24 h. Samples were examined under a dissecting microscope to detect the presence of fungal fructifications.

Ten segments of approximately 2–5 mm in diameter and 5 mm length of each twig were cut off and the bark was stripped off the xylem. Ten strips of 25 mm^2 were taken from the leaf blades and ten segments were cut off from the petioles and central veins. All materials were surface sterilized using sodium hypochlorite (0.4 g active Cl /100 ml) during 2', washed with sterile distilled water and then dried on sterile filter paper, according to Fisher & al. (1986).

A total of 400 segments were thus examined at each site. The segments were placed onto 9 mm Petri dishes containing potato dextrose agar (PDA, Difco) and incubated at 24 °C for six weeks or more depending on the growth rates of fungi. Ten segments were placed on each plate. Each colony that emerged from the segments was transferred to a fresh medium to allow its identification. Black light was used to induce sporulation in some cultures. Those cultures that failed to sporulate after 6 weeks were considered sterile.

Isolates with cultural characteristics similar to *Xylaria* were identified comparing them with those of *Xylaria* spp. isolates obtained from both ascospores and stromatal tissue collected in Rio Grande do Sul (Hamme, 1993) and with descriptions from the literature (Rogers, 1984; Petrini & Petrini, 1985; Rogers & Samuels, 1986; San Martin Gonzalez & Rogers, 1989; Callan & Rogers, 1990; Callan & Rogers, 1993; Rodrigues & al., 1993).

Analysis of the data

The relative frequency of colonization was calculated as the percentage of isolates of a given taxon from each segment divided by the total number of segments plated out.

Endophytic populations resulting from all materials were examined by simple correspondence analysis using STAT-ITCF (Service des Études Statistiques, Institute Technique des Céréales et des Fourrages, France). This analysis was carried out using those species with a relative frequency of 5 % or more isolated from any material (Howard & Robinson, 1995).

Results

Inspection under stereoscopic microscope revealed the absence of sexual or conidial state in any segment before incubation onto culture medium.

A total of 527 isolates belonging to 33 taxa were obtained from the 800 segments of twigs and leaves (Tab. 1). Segments were mostly colonized by 0 to 2 taxa. Multiple colonization by more than two fungi appeared to be rare.

Fungal assemblages from Mato Leitão and Veranopolis appeared to be composed by few taxa. *Guignardia philoprina* (Berk. & Curt.) van der Aa, several species of *Xylaria* and sterile mycelia were the most frequent taxa in all materials at both sites (Tab.1).

Simple correspondence analysis carried out on 10 taxa with a frequency equal to or higher than 5% showed that the first three axes explain 82.2% of the total inertia, indicating a good fit of the data to the model (Fig. 1). The first axis accounted for 49.7% of the total inertia and separated twig fungal assemblages of Veranopolis from the remainder tissues (Fig. 1). Sterile mycelia contributed with 54.9% and mainly characterized twigs from this site. An assemblage of several taxa was associated to all materials of Mato Leitão and the remainder of Veranopolis. *G. philoprina* was the most important one contributing with 9% of the total inertia of the axis. The second axis accounted for 18% of the total inertia and explained mainly differences between xylem and the bark of twigs from both sites. Sterile hyaline mycelium 1, sterile hyaline mycelium 3, dark sterile mycelium 4, *Phomopsis* sp. and *X. holmbergii* Speg. accounted for 73.5% of the inertia of this axis. Sterile hyaline mycelium 3 and *Phomopsis* sp. were associated with xylem, and dark sterile mycelium with the bark from twigs of Veranopolis. *X. holmbergii* characterized xylem pieces of Mato Leitão. Although the analysis evidenced that different leaf tissues have similar fungal composition, the third axis that accounted for 14.4% of the total inertia (display not shown) indicated that *X. cubensis* (Mont.) Fr. contributed to the characterization of the main vein and petiole from leaves of two sites (13.6% of the inertia).

Discussion

The diversity and frequency of endophytic fungi of *I. paraguariensis* found in both sites was very low. This is in agreement with results from tree species that are periodically thinned (Sieber-Canavesi & Sieber, 1998). Moreover, the fungal composition of twigs and leaves of *I. paraguariensis* planted in the two sites was similar. Differences evidenced by simple correspondence analysis between twigs of Mato Leitão and Veranopolis were mainly due to different frequencies of isolates more than to different mycobiota composition. This situation could arise because trees from these sites were planted within the natural distribution area under conditions that do not differ enough to be selective for fungal species distribution. In turn,

Tab. 1. – Endophytic fungi of *I. paraguariensis* twigs and leaves. Frequency of colonization [%].

		Mato Leitão				Veranopolis			
	code	**P**	**L**	**B**	**X**	**P**	**L**	**B**	**X**
Alternaria alternata (Fr.) Keissl.	aal	4			3		5		
Cladosporium cladosporioides de Vries		3	4	1	3		1		
Colletotrichum gloeosporioides Penz.			1	3			1	1	
Fusarium sp. 1						2			
Fusicoccum sp.			2					1	
Guignardia philoprina (Berk. & Curt.) van der Aa	gui	30	37	38	8	37	41	9	5
Hadronema sp.				2					
Neurospora tetrasperma Shear & Dodge		2		1					
Phomopsis sp.	pho	2		7		1	1	4	4
Xylaria adscendens Fr.: Fr.		3						1	
Xylaria comosa (Mont.) Fr.				1		3			
Xylaria cubensis (Mont.) Fr.	xcu	11	4	7		10		3	1
Xylaria deserticola Speg.		1	2			1			
Xylaria holmbergii Speg.	xho	1		1	5	2	2	1	
Xylaria montagnei Hamme & Guerrero						2	1	3	
Xylaria pseudoapiculata Hamme & Guerrero		2							
Xylaria spp.		2	2	4		4	2	2	1
Sterile dark mycelium 2	sd2	7	11	5	2	1	3	1	
Sterile dark mycelium 4	sd4					6	1	18	1
Sterile dark mycelia	sdm		1	2	2			10	2
Sterile hyaline mycelium 1	sh1	5	23	5	8	8	10	3	3
Sterile hyaline mycelium 3	sh3			11	2	2	1	6	6
Rare taxa		3	2	3		1	1	2	
Total isolates		76	89	91	33	80	70	65	23
Total segments		100	100	100	100	100	100	100	100

Rare taxa isolated once and in only one tissue: *Coniochaeta* sp.; *Epicoccum nigrum* Link; *Fusarium* sp. 2; *Gliocladium roseum* Bainier; *Leptosphaeria* sp.; *Libertella* sp.; *Periconia* sp., *Xylaria arbuscula* Sacc.; *Xylaria* aff. *curta* Fr.; Undetermined 1; Undetermined 2. Symbols indicate: P: main vein and petiole; L: blade; B: bark; X. xylem. The frequency of colonization was calculated as the percentage of isolates of a given taxon from each segment divided by the total number of segments plated out.

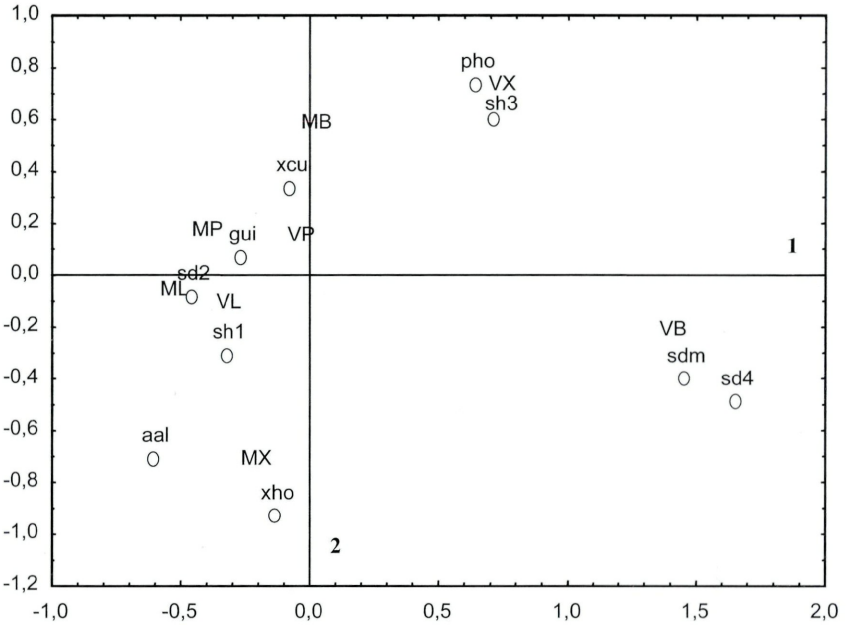

Fig. 1. – Simple correspondence analysis. Ordination of twigs and leaves from Mato Leitão and Veranopolis on the two first axes. Total inertia explained by the first three co-ordinates: 82 %. Variables are the relative frequencies of isolation of species with frequency equal or higher than 5%.–Symbols for the species are indicated in Table 1; M, Mato Leitão; V, Veranopolis; P, main vein and petiole; B, bark; X, xylem.

it is not surprising that the leaf tissues, blade and petiole, shared a similar composition. Fungi isolated here could be probably present in the wild plants or adapted to *I. paraguariensis*.

From all materials *G. philoprina*, sterile mycelia and *Xylaria* spp. were the most frequent taxa.

Species of *Guigniardia* have frequently been recorded in the South East of Brazil as endophytes of other plant species (Pereira & al., 1993; Bertoni & Guerrero, 1996). *G. philoprina* is known to be also present on other species of *Ilex* such as *I. opaca* Sol. ex Aiton and *I. aquifolium* L., producing in some cases brownish spots extending over large areas of the leaf, but this species has so far not been recorded as a pathogen of *I. paraguariensis* (van der Aa, 1973). *G. philoprina* has been found here to be a saprotrophic endophytic colonizer of *I. paraguariensis* but it has been described as pathogen of other *Ilex* species; therefore, monitoring of plantations will be necessary. Chemical control, on the other hand, should be avoided, as leaves and twigs are mainly used to prepared the mate infusion.

Xylaria was the genus with the highest number of species found to live endophytically in *I. paraguariensis*, as is the case in other tropical plants from rain forests (Dreyfuss & Petrini, 1984). Although in this study they were present at low frequency, all tissues contained some of them. *Xylaria cubensis* was the most frequent species in tropical palms in Brazil (Rodrigues & Samuels, 1990). Stromata of *Xylaria* were previously found on dead branches of *Araucaria* (Hamme, 1993; Hamme & Guerrero, 1994a; b) located in the proximity of the study area. Therefore, dead branches of *Araucaria* could probably constitute the source of inoculum that infected young tissues of *I. paraguariensis*.

The numerous sterile mycelia recorded from most of the plant samples may represent species that have sexual or conidial states which fail to form under laboratory conditions. Similar cases have been reported for young twigs of a variety of plants by Bills & Polishook (1992) and Smith & al. (1996). *Phomopsis* sp. colonized nearly all tissues, thus confirming that species of this genus are also common endophytes (Barengo & al., 2000).

Two rare but interesting phoenicoid species, *Neurospora* and *Coniochaeta*, were present as endophytes. These species are frequently found in rain tropical forests after fire (Wicklow, 1988) and could be very important for mineral immobilization in soil. In fact, fire frequently occur in the study area.

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