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Endophytic fungi from leaves of *Centella asiatica*: occurrence and potential interactions within leaves

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Abstract Fungal endophytes were isolated from leaves of *Centella asiatica* (Apiaceae) collected at Mangoro (middle eastern region of Madagascar, 200 km from Antananarivo). Forty- five different taxa were recovered. The overall foliar colonization rate was 78%. The most common endophytes were the non-sporulating species 1 (isolation frequency IF 19.2%) followed by *Colletotrichum* sp.1 (IF 13.2%), *Guignardia* sp. (IF 8.5%), *Glomerella* sp. (IF 7.7%), an unidentified ascomycete (IF 7.2%), the non-sporulating species 2 (IF 3.7%) and *Phialophora* sp. (IF 3.5%). Using sequences of the ribosomal DNA internal transcribed spacer (ITS) regions, major endophytes (IF > 7%) were identified as xylariaceous taxa or as *Colletotrichum higginsianum*, *Guignardia mangiferae* and *Glomerella cingulata*. Results from *in vitro* fungal disk experiments showed a strong inhibitory activity of the xylariaceous non-sporulating species 1 against *G. mangiferae* and *C. higginsianum* and of *C. higginsianum* against *G. mangiferae*. This can be explained by antagonism between dominant taxa.

Keywords *Centella asiatica* · *Colletotrichum higginsianum* · Endophytic fungi · Xylariaceae

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Abbreviations:

ASCO1	Unidentified ascomycete
CR	Colonization rate
IF	Isolation frequency
IMRA	Institut Malgache de Recherches Appliquées
MEAC	Malt extract agar with chloramphenicol
NSS	Non-sporulating species

Introduction

Endophytic fungi have been described as fungi that asymptomatically colonize healthy plant tissues, even though they may, after incubation or a latency period, cause diseases (Petrini 1991; Stone et al. 2000). Diverse associations with host plants have been reported, ranging from mutualistic relationships (Schulz et al. 2002) and cryptic commensalism (Deckert et al. 2001) to latent and quiescent pathogens (Sinclair and Cerkauskas 1996). The stability or the variability of the asymptomatic interaction depends on numerous factors such as environmental stress, senescence of the hosts, virulence of the endophytes and the host defense response (Schulz and Boyle 2005). Endophytes may be beneficial to the hosts in conferring resistance to insects and herbivores (Clay 1988), drought tolerance (West 1994), protection against pathogens (White and Cole 1985) and enhanced vegetative growth (Porter et al. 1979).

Endophytic fungi have been encountered from any plant ever investigated. In contrast, endophytic fungi in medicinal plants, especially in tropical regions, are still poorly explored though they could represent a source of valuable new and bioactive compounds (Li et al. 2001). Recently, endophytic fungi isolated from a Brazilian medicinal plant were shown to produce guignardic acid (Rodrigues et al. 2001). Likewise, new bioactive metabolites were produced by a *Colletotrichum* sp., an endophytic fungus in *Artemisia annua*, a traditional Chinese medicinal herb (Lu et al. 2000).

Centella asiatica (L.) Urban (Apiaceae, Umbelliferae), also known as Gotu kola or Indian pennywort, is a small, annual, slender, creeping entwined herb that grows near swamps on damp ground. It is mainly found in tropical regions of India, Madagascar, Sri Lanka, China, Indonesia, Australia and South Africa. It is used for various medical treatments (Boiteau and Ratsimamanga 1956; Pointel et al. 1987).

Reports have shown that terpenoid-producing plants such as *Sequoia sempervirens* develop differential activity on foliar endophyte growth (Espinosa-Garcia et al. 1996). Moreover, a recent study using an improved HPLC method has revealed the presence of six triterpenes in *C. asiatica* extracts (Schaneberg et al. 2003). However, the endophytic community of this potentially important pharmaceutical medicinal herb has not yet been investigated. Here, we describe the diversity of endophytic fungi in leaves of *C. asiatica*. We also assess the within-leaf distribution of and possible interactions between major fungal endophytes. Our research is part of an ongoing project on the conservation of medicinal plants and their associated microorganisms in Madagascar. It may contribute to the discovery of novel bioactive compounds probably deriving from endophytes of these medical plants.

Materials and methods

Centella asiatica plants were collected in Mangoro (18°52′20″ South, 48°06′25″ East, 823 m a s l, middle eastern region of Madagascar, 200 km from Antananarivo), a collection site of IMRA (*Institut Malgache de Recherches Appliquées*) in May 2003 after the rainy season. Five apparently healthy about 5 months old and 10–15 cm long shoots were randomly selected, pulled up with their rhizomes, placed into a cooled bag and sent immediately to BCCM/MUCL (Belgium). On arrival, 2 days later, the samples were dipped in a container filled with tap water to refresh them and processed within 4 days.

Prior to surface sterilization, leaves were individually removed from shoots and thoroughly washed in running tap water to remove dust particles on leaf surfaces, then shaken in a flask containing 200 ml of sterile distilled water with the addition of two drops of Tween 80 (Sigma Chemical Co., St. Louis, Mo, USA). Thereafter, the samples were soaked in 75% ethanol for 1 min, then in 3% calcium hypochlorite (VWR international, Leuven) for 10 min and again in 75% ethanol for 30 s. Finally, the leaves were rinsed three times in sterile distilled water and dried with sterile paper toweling.

In order to test the effectiveness of surface sterilization, 20 ml of the last rinsing water were centrifuged for 10 min at 3000 rpm. The supernatant

was removed, leaving 500 µl in the centrifugal tube, which were homogenized with a vortex; 100 µl of this volume were then plated onto a medium containing 2% malt extract agar supplemented with chloramphenicol to suppress growth of bacteria (MEAC: 20 g malt extract, 20 g glucose, 1 g peptone and 15 g agar in 1 l distilled water and 200 mg/l chloramphenicol). In addition, sterilized leaves were imprinted onto MEAC before incubating the plates at room temperature for 3 weeks. The test was validated if no mycelial growth occurred (Schulz et al. 1998).

After surface sterilization, five leaves were cut up into very small segments of approximately 2 × 2 mm using a flame-sterilized scalpel. About 600 leaf pieces were placed in 30 Petri dishes (90 mm) containing MEAC, with an average of 20 pieces per dish. The scalpel was flame-sterilized anew for each of the 600 pieces to avoid the contamination of the following piece of leaf by the endophyte of its predecessor. The plates were sealed with cellophane and incubated at room temperature. Fungi growing out from the plant tissue fragments were subcultured onto 2% malt extract agar without antibiotics by transferring a plug of agar with the piece of leaf under which hyphal tips emerged. The emerging hyphae were then transferred with the plant material to avoid that the growing mycelium masked the growth of the others and to ensure to yield the whole endophytic communities, because such a minute plant square could still contain another type of hyphae of slow-growing fungi (Gamboa and Bayman 2001).

The overall colonization rate (CR) was calculated as described by Petrini et al. (1982):

$$\frac{\text{total number of leaf segments in a sample yielding} \geq 1 \text{ isolate}}{\text{total number of leaf segments}} \times 100$$

The isolation frequency (IF) was determined by the following formula:

$$\frac{\text{the sum of isolation number per leaf of a given taxon}}{\text{total number of leaf segments}} \times 100$$

Pure isolates were subcultured onto different media for morphological identification. After 3 weeks of incubation at 25°C in two 12-h cycles, one of cool white fluorescent light followed by one of darkness, most isolates sporulated on either 2% malt agar (MA2: 20 g malt extract, 15 g agar and 1 l distilled water), oatmeal agar (OA: 10 g rolled oats, 15 g agar in 1 l distilled water) or potato dextrose agar (PDA: 4 g potato extract, 20 g glucose, 15 g agar and 1 l distilled water) and could be identified at genus level. The remaining isolates that failed to produce reproductive structures on PDA, MA and OA after two months were again subcultured onto 2% water agar in a Petri dish containing a strip of autoclaved *C. asiatica* leaf and the cultures were subjected to near-UV light (366 nm). Cultures that did not sporulate after four months or more after these treatments were considered as sterile mycelium and separated into taxonomic groups according to cultural characteristics (Fröhlich et al. 2000). Representative strains were deposited in the BCCM/ MUCL collection.

The main endophytic fungi encountered were identified by using molecular tools. Genomic DNA was extracted from ground mycelium by the method of Lee et al. (1988) and purified from solution using the GENECLEAN® III Kit (Qbiogene, Inc., Carlsbad, California) according to the manufacturer's instructions, to yield a total volume of 50 µl purified DNA. The regions of ribosomal DNA (rDNA) were amplified using the primers NS7 and ITS4 (White et al. 1990). The amount of purified DNA acquired was adjusted to 10 ng/µl using a BioPhotometre (Eppendorf) at 260 nm. First, 10 µl of fungal DNA containing 10 ng/µl were transferred to the reaction tube. Then, 40 µl of master mix consisting of 5 µl buffer (100 mM Tris HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% Bovine serum albumin), 3 µl $MgCl_2$ (50 mM) (Invitrogen SA, Merelbeke, Belgium), 1 µl deoxynucleotide triphosphates dNTP mix (10 mM) (Fermentas GMBH, Germany), 2 µl primers (10 µM) (Invitrogen

SA, Merelbeke, Belgium), 0.5 µl Taq DNA polymerase (5 U/µl) (Invitrogen SA, Merelbeke, Belgium) and 28.5 µl of sterile distilled water were added. Tubes were placed in a DNA Thermal Cycler 480 (Perkin Elmer Life and Analytical Sciences, Inc., Milano, Italy) and PCR was performed applying the following programme: initial denaturation for 5 min at 94°C followed by 30 cycles, each consisting of 90 s denaturation at 94°C, 90 s annealing at 55°C and 3 min extension at 72°C. At the end, a final extension step of 7 min at 72°C was included. The PCR products were cooled down to 4°C before purification, using QIAquick® PCR Purification Kit (QUIAGEN Inc., Hilden, Germany), according to the manufacturer's protocols. The purified PCR products were processed for reverse and forward sequencing, using a CEQ DTCS Quick Start kit (Beckman Coulter, Inc., Fullerton, CA). Reaction products were run on CEQ 2000XL capillary automated sequencer (Beckman Coulter). Sequence similarities were obtained from GenBank using the BLAST® programme.

Bioassays were performed using the fungal disk technique (Fuhrmann 1994) to survey the interactions between endophytes. Pairs of fungal disks were placed 5 cm from each other on opposite sides of a Petri dish containing malt extract agar. Plates were incubated at room temperature and the radius of the centripetally spreading parts of the two colonies were measured every 2 days, starting 7 days after incubation. The control dishes contained only a disk of each strain in the centre. Assays were stopped when mycelial control reached the edge of the plates. The experiments were performed in triplicate. The ratios between mycelium growth radius on tested strains were calculated with the following formula:

$$\text{Growth reduction } (\%) = 100 - \frac{\text{T x } 100}{\text{C}}$$

C: control growth radius
T: tested strains growth radius

Results

No mycelium growth was observed in the test on effectiveness of surface sterilization.

About 600 4 mm^2 segments were generated from five leaves collected at random and cut up completely. Mycelium emerged from 468 out of 600 segments, yielding an overall colonization rate of 78%. In most cases (465 leaf segments out of 468), a single fungal strain emerged from a leaf segment. Overall, 475 isolates were recovered and were classified into 45 different taxa according to morphological characters (Table 1). All isolates belonged to the Ascomycetes. Teleomorphs were produced in 151 isolates in culture and represented five distinct taxonomic groups belonging to five genera: *Glomerella* Spauld & H. Schrenk, *Guignardia* Viala & Ravaz, an unidentified ascomycete (ASCO1), *Leptosphaerulina* McAlpine and *Physalospora* Niessl. Anamorphs only were encountered in 125 isolates, representing eleven different taxa in nine genera (six coelomycetous, five hyphomycetous): *Colletotrichum* Corda (three species), *Phomopsis* (Sacc.) Bubák, *Phoma* Sacc., *Pestalotiopsis* Steyaert, *Phialophora* Medlar, *Penicillium* Link, *Nodulisporum* Preuss, *Curvularia* Boedijn and *Cladosporium* Link. 199 isolates remained sterile and were grouped into twenty-nine distinct morphologies (NSS1-29).

In a decreasing order of frequency, the non-sporulating species NSS1 (IF 19.2%), *Colletotrichum* sp.1 (IF 13.2%), *Guignardia* sp. (IF 8.5%), *Glomerella* sp. (IF 7.7%), an unidentified ascomycete ASCO1 (IF 7.2%), the non-sporulating species NSS2 (IF 3.7%) and *Phialophora* sp. (IF 3.5%) represented the most frequently isolated species (Table 2). Other species occurring with an IF of less than 1% belonged to the genera *Phoma*, *Nodulisporium*, *Physalospora*, *Pestalotiopsis*, *Colletotrichum*, *Phomopsis*, *Curvularia* and *Cladosporium* (Table 2).

ITS sequences of the four main endophytes (NSS1, *Colletotrichum* sp.1, *Glomerella* sp. and *Guignardia* sp.), submitted to EMBL-EBI, were compared with published sequences using BLAST searches at GenBank. Isolates used for sequencing analysis and their MUCL strain and GenBank accession numbers are listed in Table 3. The search using the 579 bp fragment of NSS1 (accession number AM403716) demonstrated homology with members of the Xylariaceae. *Xylaria longipes* (GenBank accession number AF163038) appeared to be most similar. It differed by 46 bp from the sequence of NSS1 (91% similarity), followed by various other xylariaceous taxa of the genera *Xylaria* Hill ex Schrank, *Rosellinia* De Not.,

Table 1 Number of isolates per leaf

Taxon	Group*	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Leaf 5	Total
Xylariaceae sp.1 (NSS1)[a]	NSS_1	27	24	8	32	24	115
Non-sporulating species N°2 (NSS2)	NSS_2	3	2	7	4	6	22
Other non-sporulating species	$NSS_{3–29}$	21	9	14	6	12	62
Colletotrichum higginsianum[a]	AC_1	10	28	11	12	18	79
Colletotrichum sp.2	AC_2	1	1	1	–	–	3
Colletotrichum sp.3	AC_3	1	–	–	–	–	1
Pestalotiopsis sp.	AC_4	1	–	1	–	1	3
Phomopsis sp.	AC_5	1	–	–	–	–	1
Glomerella cingulata[a]	T_1	15	2	12	10	7	46
Guignardia mangiferae[a]	T_2	4	9	18	8	12	51
Unidentified ascomycete (ASCO1)	T_3	2	4	20	5	12	43
Leptosphaerulina sp.	T_4	4	4	–	–	–	8
Physalospora sp.	T_5	–	2	1	–	–	3
Phialophora sp.	AH_1	4	5	6	3	3	21
Penicillium sp.	AH_2	–	2	–	3	2	7
Phoma sp.	AH_3	–	–	2	2	1	5
Nodulisporium sp.	AH_4	2	1	–	–	–	3
Curvularia sp.	AH_5	–	–	–	1	–	1
Cladosporium sp.	AH_6	–	–	–	–	1	1
Total of isolates		96	93	101	86	99	475
Total of leaf segments[b]		120	120	120	120	120	600

* Forty-five different morphologies grouped in 29 distinct non-sporulating species (NSS_m), 5 anamorph coelomycetous (AC_n), 5 teleomorphs (T_p) and 6 anamorph hyphomycetous (AH_x). Footnotes in each group specify each individual species

[a] ITS sequence analysis of the representative isolate of the taxon was performed

[b] Leaf segments: a square of 4 mm^2 piece of leaf

Table 2 Frequency of endophytic fungi isolates from *Centella asiatica* leaves

Taxon	Isolation frequency (%)
Xylariaceae sp.1 (NSS1)	19.2
Colletotrichum higginsianum	13.2
Guignardia mangiferae	8.5
Glomerella cingulata	7.7
Unidentified ascomycete (ASCO1)	7.2
Non-sporulating species N°2 (NSS2)	3.7
Phialophora sp.	3.5
Leptosphaerulina sp.	1.3
Penicillium sp.	1.2
Other sterile mycelium	10.3
Rare isolates[a]	3.5

[a] Less than 1% of isolation frequency for each species: *Phoma* sp., *Nodulisporium* sp., *Physalospora* sp., *Pestalotiopsis* sp., *Colletotrichum* sp.2, *Colletotrichum* sp.3, *Phomopsis* sp., *Curvularia* sp. and *Cladosporium* sp

Nemania Gray and *Kretzschmaria* Fr. Sequence similarities of 500 bp of the ITS regions of *Colletotrichum* sp.1 (accession number AM411029) revealed an identity score of 99% (498/500) with *Colletotrichum higginsianum* (GenBank accession number AB0422303) and with *Colletotrichum destructivum* (GenBank accession number AB0105959), the first taxa proposed by the Blast search. Morphological identification of *Colletotrichum* sp.1 showed immersed, light brown mycelium on PDA. Setae were present. Appressoria were pale to medium brown and ovoid. Conidia formed in whitish masses were hyaline, aseptate, straight or slightly curved, guttulate, measuring 13–18 µm × 3–4 µm (measurements were performed on 50 conidia). These cultural characters closely resembled those of *C. higginsianum* according to Sutton (1992) and the species is therefore identified as such. Complete ITS sequences of *Glomerella* sp. (accession number AM403718) and *Guignardia* sp.

Table 3 Main endophytes newly sequenced for this study with MUCL and GenBank numbers

Scientific name	Voucher specimen no.	MUCL no.	GenBank accession no.
Xylariaceae sp.1	MS1(=NSS1)	MUCL45004	AM403716
Guignardia mangiferae	WFCO008I	MUCL44939	AM403717
Glomerella cingulata	WFCO006I	MUCL44931	AM403718
Colletotrichum higginsianum	COL1	MUCL44942	AM411029

(accession number AM403717) showed an identity score of 100% to *Glomerella cingulata* (GenBank accession number AY423474) and *Guignardia mangiferae* (GenBank accession number AY277717).

The within-leaf distribution patterns of the most commonly isolated endophytes (IF > 7%) are presented in Fig. 1. The distribution showed a disjointed pattern. NSS1 was mainly isolated from the distal part of leaf 1 (23 out of 27 isolated) while *G. cingulata* (11/15) and *C. higginsianum* (8/10) were mostly recorded nearer to the leaf base. In leaf 2, NSS1 (20/24) mostly occupied the right side, while *C. higginsianum* (25/28) occupied the left. In leaf 3, *G. mangiferae* (17/18) was found on the right side, while the unidentified ascomycete ASCO1 (18/20) was observed on the opposite side. In leaf 4, no particular pattern was noticed. In leaf 5, ASCO1 (8/12) was mostly scored on the left side, while *C. higginsianum* (12/18) and *G. mangiferae* (8/12) were located on the right side. These observations led us to check for interactive relationships between these major leaf endophytes of *C. asiatica* by using a fungal disk technique.

Several tested fungi showed inhibitory effects on the growth of the others (Fig. 2). NSS1 and *C. higginsianum* repressed the growth of ASCO1 by 27.3% and 9.1%, respectively (Fig. 2A). NSS1 and *C. higginsianum* markedly reduced the growth of *G. mangiferae* by 33.3% and 70.8%, respectively (Fig. 2B). *Colletotrichum higginsianum* was clearly inhibited by NSS1 and ASCO1 by 35% and 31.2%, respectively (Fig. 2C). The growth of NSS1was slowed down by ASCO1 and *G. mangiferae* by 17.5% (Fig. 2D). *G. mangiferae* slightly stimulated the growth of ASCO1 by 9%.

Discussion

We preferred the use of calcium hypochlorite (Rodrigues 1994, Arnold et al. 2001) as a surface disinfectant because it damages the tissue less than sodium hypochlorite (Boccon-Gibod 1982). We showed that our surface sterilization procedure is sufficiently effective. Therefore, we conclude that all isolated fungi are endophytes.

The colonization rate of *C. asiatica* leaves by endophytes reached 78%. High colonization rates ranging from 81% to 89% were reported in palms in Brunei and Australia (Fröhlich et al. 2000) and up to 95–98% in leaf fragments of *Guarea guidonia* (Meliaceae) in Puerto Rico (Gamboa and Bayman 2001). On the other hand, lower rates (29–67%) were recorded in other tropical hosts such as banana (Brown et al. 1998) and 21–30% in the palm *Euterpe oleracea*, in Brazil (Rodrigues 1994). Our results were near the upper range. A possible explanation of the relatively high overall colonization rate noted in the present study could be due to the position of *C. asiatica* leaves close to the soil, facilitating the penetration and the colonization of endophytes. Moreover, samples were collected after rainy season, offering favorable spore dispersal.

All 45 distinct morphological taxa that we isolated were ascomycetes. About 58% produced either a teleomorph or an anamorph but 42% of isolates remained sterile. This confirms Petrini's findings (1986) that fungal endophytes mainly belong to the ascomycetes. Fifteen morphologically distinct taxa could be identified to genus according to microscopic examinations of any sporulation and reproductive structures including asci, ascospores, conidia, conidiophores and conidiomata. Twenty nine taxonomic groups remained sterile among which NSS1 was the most common taxon. In order to capture maximum diversity among non-sporulating isolates, minor variations in culture were taken into account to distinguish near-identical strains and assign them to different taxonomic groups.

Molecular characterization of NSS1 showed that this species most likely belongs to the Xylariaceae.

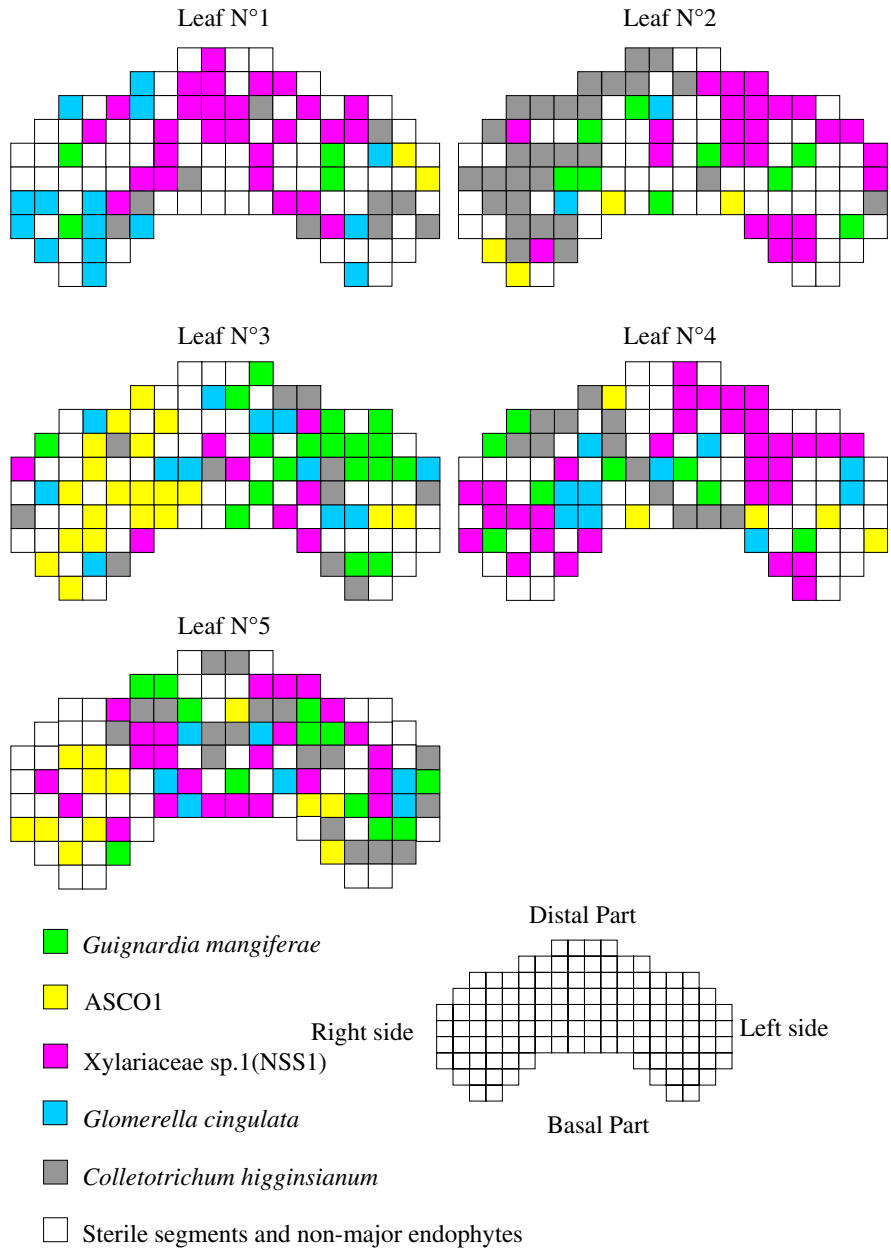

Fig. 1 Diagram showing the within-leaf distribution patterns of selected endophytes of *Centella asiatica* leaves

Although we did not observe any xylariaceous teleomorph in culture during this survey, endophytic community in *C. asiatica* was dominated by the xylariaceous non sporulating species NSS1. In addition, *Nodulisporium*, a Xylariaceae anamorph, was isolated from two leaves with less than 1% of isolation frequency. This is in agreement with earlier studies showing that xylariaceous fungi were the most commonly isolated endophytes in tropical regions but very seldom form teleomorph in culture (Rodrigues 1994; Rodrigues and Petrini 1997). Also, various proportions of mycelia sterilia have been reported from investigations of endophytes from different hosts and an attempt to identify them have shown that they belonged mainly to the Xylariaceae (Guo et al. 2003).

The second most frequently isolated endophyte of *C. asiatica* was *C. higginsianum*. This species can cause an anthracnose leaf spot disease on a wide range of cruciferous plants (Higgins 1917). It displays

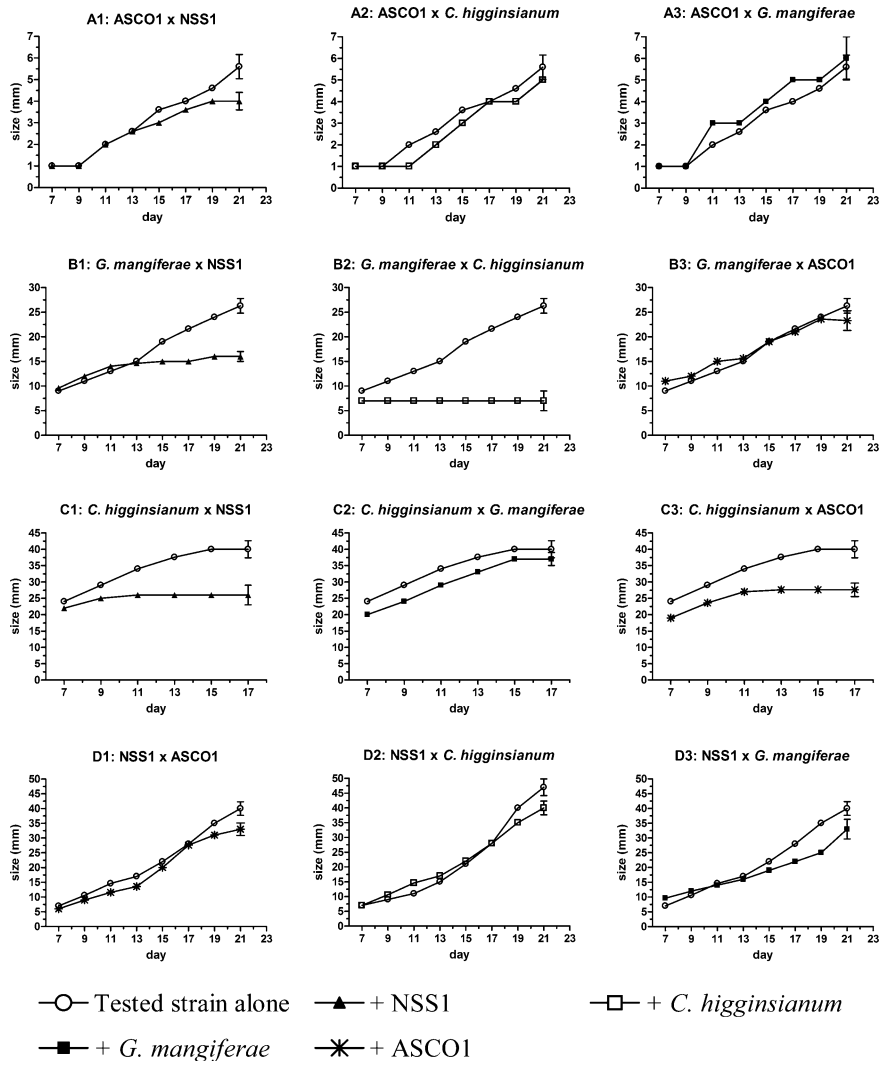

Fig. 2 Growth curves of major endophytes in the presence of other endophytes. **A**: ASCO1 in the presence of NSS1 (**A1**), *C. higginsianum* (**A2**) and *G. mangiferae* (**A3**). **B**: *G. mangiferae* in the presence of NSS1 (**B1**), *C. higginsianum* (**B2**) and ASCO1 (**B3**). **C**: *C. higginsianum* in the presence of NSS1 (**C1**), *G. mangiferae* (**C2**) and ASCO1 (**C3**). **D**: NSS1 in the presence of ASCO1 (**D1**), *C. higginsianum* (**D2**) and *G. mangiferae* (**D3**). —○—: Tested strain alone; —▲—+NSS1; —□—+*C. higginsianum*; —■—+*G. mangiferae;* +ASCO1

intracellular hemibiotrophic colonization strategies with a transient asymptomatic biotrophy followed by a rapid necrotrophic phase (Narusaka et al. 2004). *Colletotrichum higginsianum* has never been described as an endophyte. It is possible that we have encountered this species during its symptomless, latent infection stage.

The endophytic community in *C. asiatica* was characterized by two most frequently isolated endophytes (the xylariaceous NSS1 and *C. higginsianum*) and a numerous species isolated with rare occurrence (Table 1). Typical distribution has also been reported from studies on endophytic mycobiota in plants (Petrini 1991; Rodrigues and Petrini 1997; Fröhlich et al. 2000). For species with less than 1% of isolation frequency were cosmopolitan species such as *Phoma* sp., *Phomopsis* sp., *Pestalotiopsis* sp., *Curvularia* sp., *Physalospora* sp. and *Cladosporium* sp. and have been isolated as endophytes from a wide range of different host plants (Kumaresan and Suryanarayanan 2001; Cannon and Simmons 2002).

The observation that the main endophytes were found on opposite areas on *C. asiatica* leaves suggested antagonistic effects between those endophytic fungi. The results of our dual culture tests clearly indicate that the differential distribution patterns could be the result of competition or any other interactions between fungi. Similar patterns have been attributed to competitive interactions (Hata et al. 2002). These observations suggest that some endophytic species in *C. asiatica* could synthesize compounds that inhibit the growth of the others and were responsible for the observed distribution patterns. Xylariaceous fungi are a rich source of diverse secondary metabolites (Whalley and Edwards 1995). The competitive ability of xylariaceous NSS1 and specifically its strong inhibitory effect on other tested fungi may be attributed to production of inhibitory compounds. Xylariaceous NSS1 may be beneficial to the host for protection against pathogens and should be considered for further investigation as potential source of bioactive compounds.

Conclusions

Centella asiatica leaves harboured a high diversity of fungal species with 45 different morphological taxa, of which the xylariaceous non-sporulating species NSS1 and *C. higginsianum* were the most frequently isolated species. Within leaf distribution of most frequently encountered endophytes showed an asymmetric pattern that may be ascribed to interaction or competition between fungi as shown by *in vitro* dual cultures.

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