

Novel and Highly Diverse Fungal Endophytes in Soybean Revealed by the Consortium of Two Different Techniques[§]

Tiago de Souza Leite¹, Andréia Cnossen-Fassoni¹,
Olinto Liparini Pereira²,
Eduardo Seiti Gomide Mizubuti²,
Elza Fernandes de Araújo¹,
and Marisa Vieira de Queiroz^{1*}

¹Laboratory of Microorganism Molecular Genetics, Department of Microbiology/Institute of Microbiology Applied to Agriculture and Livestock Raising (BIOAGRO), ²Department of Phytopathology, Federal University of Viçosa, Viçosa-MG, Brazil

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Fungal endophytes were isolated from the leaves of soybean cultivars in Brazil using two different isolation techniques – fragment plating and the innovative dilution-to-extinction culturing – to increase the species richness, frequency of isolates and diversity. A total of 241 morphospecies were obtained corresponding to 62 taxa that were identified by analysis of the internal transcribed spacer (ITS) of the ribosomal DNA (rDNA). The Phylum Ascomycota predominated, representing 99% and 95.2% of isolates in the Monsoy and Conquista cultivars, respectively, whereas the Phylum Basidiomycota represented 1% and 4.8% of isolates, respectively. The genera *Ampelomyces*, *Annulohyphoxylon*, *Guignardia*, *Leptospora*, *Magnaporthe*, *Ophiognomonia*, *Paraconiothyrium*, *Phaeosphaeriopsis*, *Rhodotorula*, *Sporobolomyces*, and *Xylaria* for the first time were isolated from soybean; this suggests that soybean harbours novel and highly diverse fungi. The yeasts genera *Rhodotorula* and *Sporobolomyces* (subphylum Pucciniomycotina) represent the Phylum Basidiomycota. The species richness was greater when both isolation techniques were used. The diversity of fungal endophytes was similar in both cultivars when the same isolation technique was used except for Hill's index, N_1 . The use of ITS region sequences allowed the isolates to be grouped according to Order, Class and Phylum. *Ampelomyces*, *Chaetomium*, and *Phoma glomerata* are endophytic species that may play potential roles in the biological control of soybean pathogens. This study is one of the first to apply extinction-culturing to isolate fungal endophytes in plant leaves, thus contributing to the development and improvement of this technique for future studies.

Keywords: basidiomycota diversity extinction culturing fragment plating rDNA ITS

Introduction

The term “endophyte” was first introduced by De Bary (1866). However, the notion of fungal endophytes as it is most widely used at present was formulated by Petrini (1991), who defined these fungi as microorganisms that at some phase of their lifecycle colonize the internal part of a plant and do not cause disease symptoms. More definitions of the term “endophyte” have been commented by Hyde and Soyong (2008).

Fungal endophytes are ubiquitous organisms and, thus, may be found in a wide range of plant species inhabiting the leaves, petioles, reproductive structures, branches, barks and roots (Faeth and Fagan, 2002; Rodriguez *et al.*, 2009). Among the plant species inhabited by fungal endophytes, some are of economic interest, including corn (Fisher *et al.*, 1992; Orole and Adejumo, 2011), coffee (Santamaría and Bayman, 2005; Vega *et al.*, 2010), wheat (Larran *et al.*, 2002a, 2007), and soybean (Pimentel *et al.*, 2006). In addition to plants that are valuable in agriculture, arboreal plant species (Arnold *et al.*, 2003; Arnold and Lutzoni, 2007), the palm family (Frohlich *et al.*, 2000; Pinruan *et al.*, 2010) and grasses (Tanaka *et al.*, 2012), as well as pteridophytes (Fisher, 1996; Zubek *et al.*, 2010), algae (Zhang *et al.*, 2009b), mosses (U'Ren *et al.*, 2010), and lichens (Li *et al.*, 2007) have all been reported to harbor fungal endophytes. Most of the fungal endophytes that have been characterized belong to the Phylum Ascomycota and its anamorphs, whereas very few studies identified members of the Phylum Basidiomycota as endophytic (Arnold, 2008; Hyde and Soyong, 2008; Rungjindamai *et al.*, 2008; Pinruan *et al.*, 2010; Rivera-Orduna *et al.*, 2011).

The soybean (*Glycine max* (L.) Merrill) is one of the most widely grown leguminous crops worldwide due to the high oil and protein content of its grain. It is used in human and animal nutrition and in the production of biodiesel, disinfectants, lubricants, soap, and cosmetics, among other uses (Sediyama, 2009). Periodically, the CONAB - Companhia Nacional de Abastecimento [National Company of Food and Supply], conducts surveys of agricultural crops in order to quantify and monitor the Brazilian production. In the 2010/2011 harvest, Brazil was the second-largest producer of soybeans in the world with 75 million tons corresponding to 28% of the world production and resulting in net exports of 17.1 billion dollars. However, soybean production is threatened by several diseases that cause important losses to farmers (Embrapa, 2011). One strategy to increase sustainability in the production of various agriculture crops is to employ alternative measures for disease control, mainly biological control and the induction of resistance in

*For correspondence. E-mail: mvqueiro@ufv.br; Tel.: +55-31-3899-2975; Fax: +55-31-3899-2573

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hosts against pathogens (El-Ghaouth, 1997; Guetsky *et al.*, 2001; Martin, 2003; Nunes, 2012). In this regard, the role of fungal endophytes has proven to be significant, prompting increasing interest in the study of these organisms in recent years as an alternative means of disease control (Arnold *et al.*, 2003; Dingle and Mcgee, 2003; Bailey *et al.*, 2008; Cao *et al.*, 2009; Ownley *et al.*, 2010).

From this perspective several studies have pointed to the potential use of fungal endophytes in the biological control of diseases (Almeida *et al.*, 2007; Vazquez-Garciduenas *et al.*, 1998; Hanada *et al.*, 2008; Cao *et al.*, 2009) and pests (Carrol, 1988; Azevedo *et al.*, 2000; Zhang *et al.*, 2009a), in the induction of resistance in the host plant (Arnold *et al.*, 2003; Dingle and Mcgee, 2003) and in the promotion of plant growth (Maccheroni Jr and Azevedo, 1998; Sirrenberg *et al.*, 2007; Hamayun *et al.*, 2009). In addition to these uses, additional studies have demonstrated the importance of fungal endophytes in increasing the tolerance to drought-induced stress (Elbersen and West, 1996; Bayat *et al.*, 2009) and to heat in grasses (Redman *et al.*, 2002; Marquez *et al.*, 2007), in the production of antioxidant compounds (Schulz and Boyle, 2005; White Jr and Torres, 2010) and, mainly, as excellent producers of secondary metabolites of interest to the pharmaceutical industry (Stierle *et al.*, 1993; Strobel, 2003; Zhang *et al.*, 2006; Suryanarayanan *et al.*, 2009).

The diversity of fungal endophytes species isolated from the leaves of the same host varies significantly, and most of this variation is due to the different isolation techniques employed by each study (Hyde and Soyong, 2008). One of the most common techniques used to study the diversity of fungal endophytes is isolation using fragmented host tissues (Hata *et al.*, 2002; Wang *et al.*, 2007; Rakotoniriana *et al.*, 2008; Joshee *et al.*, 2009). However, the species richness obtained with this technique is affected by the size of the leaf fragments and the continuous surface of Petri dishes because both are factors that simultaneously facilitate the superposition of colonies and favor the isolation of the dominant and fast-growing species (Gamboa *et al.*, 2002; Rakotoniriana *et al.*, 2008).

Extinction culturing technique exploits the fact that culturable species diversity increases as inoculum density decreases (Collado *et al.*, 2007). The use of particle filtration associated with extinction culturing technique was adapted by Unterseher and Schnittler (2009) to isolate endophytic fungi. This technique employs multiwell plates with divided surfaces for cultivation that thereby decrease the interaction among colonies and consequently favor the isolation of slow-growing and rare (low occurrence) fungal species based on the extinction principle, while, at the same time, allows the isolation of ubiquitous and dominant taxa (Stone *et al.*, 2004).

In the specific instance of soybean fungal endophytes, some studies have sought to identify and study the diversity of these microorganisms in the main soybean producing countries. One of the first studies that attempted to isolate and identify fungal endophytes in soybean leaves was performed in the United States (Miller and Roy, 1982), followed by similar studies in Argentina (Larran *et al.*, 2002b) and Brazil (Pimentel *et al.*, 2006). However, in all such studies were used exclusively fragment plating and quantified diversity

in only one soybean cultivar or at only one developmental stage.

Among soybean cultivars, Conquista (MG/BR-46) is the most widely grown in Brazil due to its capacity to adapt to conditions that are unfavorable for other crops; it grows in low, medium or even high fertility soils (Sediyama, 2009; Embrapa, 2011). Cultivar Monsoy (M-SOY 6101), on the other hand, has a long sowing period and an excellent ability to adapt to climatic variations (Sediyama, 2009; Embrapa, 2011). To date, no study has been performed on the effect of soybean cultivars on the diversity of fungal endophytes.

Therefore, the aim of the present study was to isolate and identify fungal endophytes in the leaves of the soybean cultivars Conquista and Monsoy using two different isolation techniques, namely, fragment plating and the innovative extinction culturing technique, to establish the species richness and the frequency of isolates and to compare the diversity of fungal endophytes between the two cultivars using the same isolation technique.

Materials and Methods

Place of collection and processing of the plant material

Healthy leaf samples of the cultivars Conquista and Monsoy (M-SOY 6101) were collected from plants under field conditions between April and May 2010. The collection was performed at the Agronomy Experimental Field (20°45'59.8" South and 42°52'10.2" West, altitude above sea level: 631 m) located at the Federal University of Viçosa, Viçosa, Minas Gerais, Brazil.

A total of 40 plants from each cultivar that were randomly distributed in an experimental field were sampled (Arnold *et al.*, 2003). These plants were at the reproductive stage R2 (full bloom) and were under the same edaphoclimatic conditions. The number, age and size of these plants leaves were considered in the sampling process to ensure a reliable representation of the results (Lodge *et al.*, 1996; Gamboa *et al.*, 2002). Next, the samples were stored in plastic bags, immersed in ice (Stone *et al.*, 2004) and transported for immediate processing at the Laboratory of Microorganism Molecular Genetics located at the Institute of Microbiology Applied to Agriculture and Livestock Raising (BIOAGRO), Federal University of Viçosa, Viçosa, Minas Gerais, Brazil.

The samples were rinsed with tap water for 10 min to remove soil residues and dust. Next, they were immersed in a 70% ethanol solution containing two drops of Tween 80/100 ml. The leaves were then placed in a sodium hypochlorite (NaOCl) solution containing 2–2.5% active chlorine (commercial bleach brand Q-Boa) for 3.5 min and washed three times in sterile saline (NaCl 0.85% w/v) for 2 min per washing to remove the chlorine residue (Petrini, 1991).

After disinfection, the leaves were cut into approximately 0.25 cm² fragments. The resulting leaf fragments were used to test the efficiency of surface disinfection by pressing the adaxial side of leaves against the isolation medium (Schulz *et al.*, 1998). The Petri dishes were kept for 10 days at 22°C ± 2°C and a 12 h photoperiod, upon which no growth of fungal colonies was observed during the period assessed. The time of exposure to and concentration of the sodium hypo-

chlorite in contact with the leaves were tested to determine the optimal conditions for kill saprophytic and epiphytic, but keep alive the endophytic fungi (Pereira, 1993; Suryanarayanan *et al.*, 2005). In addition, aliquots of the final rinse water of the leaf fragments were also plated as a complementary test of surface disinfection (Pereira, 1993).

The remaining leaves were placed in 90 mm diameter Petri dishes for isolation purposes. Half of each cultivar's leaves were used for isolation by fragment plating and the other half were used for particle filtration and extinction culturing.

Isolation by fragment plating

For the isolation by fragment plating, approximately 20 leaves previously subjected to surface disinfection were cut into approximately 0.25 cm² fragments. Next, approximately 240 leaf fragments were added to five multiwell plates (Greiner Bio-One) instead of Petri dishes to reduce possible antagonistic effects. Each plate comprised 48 wells filled with 1 ml YMC medium (10 g malt extract, 2 g yeast extract, 3 g agar, and distilled water to reach 1 L) with a pH adjusted to 6.0 (Bills *et al.*, 2004; Stone *et al.*, 2004; Collado *et al.*, 2007), and the antibiotics tetracycline (50 mg/L) and streptomycin (50 mg/L) were added to inhibit bacterial growth. Next, the multiwell plates were kept for 16 days at 22°C±2°C and a 12 h photoperiod.

Particle filtration and extinction culturing

Twenty disinfected leaves were processed according to the extinction culturing technique following the protocol described by Paulus *et al.* (2003) and Collado *et al.* (2007) and later adapted by Unterseher and Schnittler (2009, 2010) for the isolation of fungal endophytes. The leaves were immersed in 300 ml of a 0.85% (w/v) NaCl sterile solution and homogenized in a blender for approximately 60 sec at high speed. The resulting particles were separated with three sieves of 500 µm, 212 µm, and 106 µm in mesh size; the particles in the size range of 107–211 µm were retained and resuspended in 20 ml of 0.85% (w/v) NaCl solution. The suspension was centrifuged at 2200×g for 5 min at room temperature.

The supernatant was discarded, 15 ml of 0.1% (w/v) carboxymethyl cellulose (CMC) were added and the suspension was centrifuged again at 2200×g for 15 min at room temperature. The supernatant was discarded and an additional 20 ml of 0.1% (w/v) CMC was added. The final suspension served as a 1× concentration of leaf particles and was the base for dilution in 0.1% (w/v) CMC to achieve a concentration of 1:2×. Thus, the concentrations of leaf particles used were 1× and 1:2×.

The concentrations of leaf particles used were previously determined by plating analysis with several particles concentrations (1×, 1:2×, 1:4×, 1:8×, 1:16×, and 1:64×) according to Collado *et al.* (2007).

The 40 µl volume of each 1× and 1:2× concentration was inoculated into the wells of 10 plates (five multiwell plates for each concentration), which were kept opens in a laminar-flow for 60 min before closing the lid to allow the evaporation of excess water from the wells. Next, the plates were closed and kept for four weeks at 22°C±2°C with a 12 h photoperiod.

Morphologic characterization of fungal endophytes

After the initial morphologic assessment of the fungal colonies inside the multiwell plates, the colonies were transferred to Petri dishes containing YMC medium supplemented with antibiotics and subjected to monosporic purification. Determination of the morphological species (morphospecies) of the colonies was performed based of fungal growth rate, colony shape, coloration and the effects on the culture medium (Lacap *et al.*, 2003). Then, the fungal colonies were allowed to grow in YMC medium without added antibiotics. As soon as the fungus has grown over the whole plate were added circles of filter papers and milk (10% w/v). Using a sterile forceps, mycelium and conidia were removed together with the filter papers and stored in sterile glass bottles containing silica gel. The glass bottles were kept at 4°C±2°C in the Laboratory of Microorganism Molecular Genetics.

DNA extraction, amplification, and sequencing of the rDNA ITS region

The fungal isolates were re-inoculated in YMC medium without added antibiotics and allowed to grow for seven days at 22°C±2°C with a 12 h photoperiod; next, approximately 200 mg of mycelia was collected, and the DNA was extracted using the UltraClean DNA Microbial Isolation Kit (MO BIO Laboratories).

For the molecular identification of fungal endophytes isolates, the primers ITS1F (5'-CTTGGTCATTTAGAGGAA GTAA-3') (Gardes and Bruns, 1993) and ITS4 (5'-TCCTC CGCTTATTGATATGC-3') (White *et al.*, 1990) were used, and the fragment corresponding to rDNA ITS region (ITS1-5,8s-ITS2) was amplified by PCR using the thermocycler Eppendorf Mastercycler (Eppendorf, Germany) programmed to perform the initial denaturation at 95°C for 2 min followed by 39 cycles at 95°C for 1 min, 50°C for 1 min and 72°C for 1 min, and a final elongation at 72°C for 7 min. Amplification was performed for a 25 µl final volume containing 5.0 µl of Colorless Go Taq® Flexi Buffer (5×) (Promega, USA); 2.5 µl of MgCl₂ (25 mM) (Promega); 1.0 µl of dNTPs (2.5 mM of each dNTP); 1.0 µl of the primer ITS1F (5 µM); 1.0 µl of the primer ITS4 (5 µM); 0.25 µl of the Go Taq® DNA Polymerase (5 U/µl) (Promega), 5.0 µl of genomic DNA (1.75 ng/µl) and 9.25 µl of autoclaved ultrapure water. After amplification, the PCR products were analyzed by electrophoresis in a 1.2% agarose gel.

The product of each PCR reaction (approximately 15 µl) was purified using the Kit Wizard® SV Gel and PCR Clean-Up System (Promega) and eluted to a final volume of 20 µl. The purified products were sent for sequencing at the Bioagro Genomic Laboratory. Samples were read in the automatic 96-capillary sequencer MegaBACETM 1000 (GE Healthcare); each strand of the amplification products was sequenced with the primers employed in the initial amplification. The products of the sequencing of both DNA strands were contiguously grouped, aligned and manually corrected using the software Sequencher version 4.7 (Genecodes Corporation, USA).

The nucleotide sequences obtained from the fungal isolates were identified by comparison against the GenBank database using a local alignment algorithm for nucleotide

Table 1. Number of fungal isolates from two soybean cultivars using two isolation techniques and the total isolates using both techniques

Species or Genera	Monsoy		Conquista		Monsoy Total	Conquista Total
	Fragment	Extinction	Fragment	Extinction		
<i>Alternaria alternata</i>	0	1	0	0	1	0
<i>Alternaria arborescens</i>	0	1	1	0	1	1
<i>Alternaria dauci</i>	0	0	1	0	0	1
<i>Alternaria macrospora</i>	0	0	1	0	0	1
<i>Alternaria solani</i>	0	0	1	0	0	1
<i>Alternaria</i> sp.	0	1	0	0	1	0
<i>Ampelomyces</i> sp.	1	0	2	0	1	2
<i>Annulohyphoxylon stygium</i>	3	0	0	0	3	0
<i>Arthrinium phaeospermum</i>	0	0	0	1	0	1
<i>Cercospora zebrinae</i>	0	0	0	1	0	1
<i>Chaetomium</i> sp.	0	0	1	0	0	1
<i>Cladosporium cladosporioides</i>	1	0	4	0	1	4
<i>Cladosporium colocasiae</i>	0	0	1	0	0	1
<i>Cochliobolus bicolor</i>	0	0	1	0	0	1
<i>Cochliobolus</i> sp.	0	0	1	0	0	1
<i>Cochliobolus lunatus</i>	0	0	3	0	0	3
<i>Cochliobolus sativus</i>	9	0	4	2	9	6
<i>Colletotrichum boninense</i>	5	7	2	2	12	4
<i>Colletotrichum capsici</i>	0	0	3	0	0	3
<i>Colletotrichum fragariae</i>	1	0	0	0	1	0
<i>Colletotrichum gloeosporioides</i> 1	8	0	5	0	8	5
<i>Colletotrichum gloeosporioides</i> 2	13	2	24	17	15	41
<i>Colletotrichum gloeosporioides</i> 3	1	0	0	0	1	0
<i>Colletotrichum lupini</i>	1	0	0	0	1	0
<i>Colletotrichum truncatum</i>	6	0	9	0	6	9
<i>Curvularia oryzae</i>	0	0	1	0	0	1
<i>Davidiella tassiana</i>	2	0	3	0	2	3
<i>Diaporthe helianthi</i>	2	0	3	0	2	3
<i>Diaporthe phaseolorum</i>	4	0	1	0	4	1
<i>Didymella bryoniae</i>	3	0	0	1	3	1
<i>Epicoccum nigrum</i>	0	1	0	0	1	0
<i>Eutypella scoparia</i>	0	1	0	0	1	0
Fungal endophyte	0	0	1	0	0	1
<i>Fusarium equiseti</i>	2	3	3	0	5	3
<i>Fusarium proliferatum</i>	1	0	1	0	1	1
<i>Gibberella moniliformes</i>	1	0	0	0	1	0
<i>Guignardia mangiferae</i>	1	0	0	0	1	0
<i>Guignardia vaccinii</i>	3	0	0	0	3	0
<i>Leptospora rubella</i>	0	0	0	1	0	1
<i>Magnaporthe grisea</i>	0	0	1	0	0	1
<i>Myrothecium gramineum</i>	0	0	2	0	0	2
<i>Myrothecium inundatum</i>	0	0	2	0	0	2
<i>Myrothecium</i> sp.	0	0	1	0	0	1
<i>Nectria mauritiicola</i>	1	0	0	0	1	0
<i>Neofusicoccum</i> sp.	2	0	0	0	2	0
<i>Nigrospora sphaerica</i>	0	2	0	0	2	0
<i>Ophiognomonia</i> sp.	0	0	0	1	0	1
<i>Paraconiothyrium brasiliense</i>	0	0	1	0	0	1
<i>Paraconiothyrium variabile</i>	0	0	1	0	0	1
<i>Phaeosphaeriopsis</i> sp. 1	0	1	1	1	1	2
<i>Phaeosphaeriopsis</i> sp. 2	0	0	0	1	0	1
<i>Phoma glomerata</i>	1	0	1	0	1	1
<i>Phoma herbarum</i> 1	0	0	1	1	0	2
<i>Phoma herbarum</i> 2	0	0	3	0	0	3
<i>Phoma</i> sp.	1	1	2	0	2	2
<i>Phomopsis</i> sp.	0	0	3	0	0	3
<i>Rhodotorula</i> sp.	1	0	1	0	1	1
<i>Sporobolomyces oryzaicola</i>	0	0	5	1	0	6
<i>Stemphylium solani</i>	0	0	2	0	0	2
<i>Xylaria berteri</i>	0	0	11	0	0	11
<i>Xylaria ianthinovelutina</i>	0	0	1	0	0	1

sequences (BlastN) (Altschul *et al.*, 1990). The database sequences that exhibited the highest identity, query and score and the lowest *e*-values relative to the sequences obtained in the present study allowed us to identify the fungi as belonging to a particular genus or species. The ITS region sequences obtained in the present study were deposited in GenBank and the name of isolates and respective accession numbers are listed in tables (Supplementary data Tables S1A to S2B).

Fungal isolates were considered as belonging to a particular species after a comparison of their nucleotide sequences (using BlastN algorithm) revealed an ITS region identity above 95%. The cut-off value of 95% was selected on the grounds of the studies by Nilsson *et al.* (2008), who reported intraspecific variations in the ITS region of 1.96% (SD=3.73) and 3.3% (SD=5.62) in the phyla Ascomycota and Basidiomycota, respectively. Other studies have also used the value of 95% identity as cut-off in the ITS region for the identification of isolated species (Arnold and Lutzoni, 2007). However, same species isolates that exhibited sequence homology below 95% were considered as different isolates (in calculating the diversity indexes) and were thus termed isolates 1, 2, 3, etc. while keeping the same name as that of the species in the GenBank database with the highest scoring sequence. This nomenclature was necessary because only the ITS region was used as a molecular parameter to identify isolates.

Phylogenetic analysis

Manual alignment of the nucleotide sequences of the rDNA ITS region of fungal isolates of the cultivars Conquista and Monsoy and of the reference sequences obtained from the GenBank database was performed using the software Mega 5.0 (Kumar *et al.*, 2008; Tamura *et al.*, 2011). The sequences were grouped by cultivar in performing the phylogenetic analysis.

The phylogenetic trees were constructed by Neighbor Joining (NJ) (Saitou and Nei, 1987), Maximum Parsimony (MP) (Huelsenbeck and Crandall, 1997) and Maximum Likelihood (ML) (Cavalli-Sforza and Edwards, 1967; Rogers and Swofford, 1998; Goldman *et al.*, 2000) methods. With the exception of NJ, which was performed with the software Mega 5.0, the phylogenetic trees were inferred with the software Paup 4b10 (Swofford *et al.*, 2001).

The software Modeltest 3.7 (Huelsenbeck and Crandall, 1997; Posada and Crandall, 1998) was used to establish the model of DNA evolution that best fit the data for the ML analysis. Next, a heuristic search using a tree bisection and reconnection algorithm (TBR) was performed, which was initialized with a NJ tree. This NJ tree was constructed using Kimura's two-parameter substitution and gamma distribution correction according to the ML model.

The MP tree was generated by a heuristic search initialized by stepwise addition of the sequences. The TBR algorithm was also employed to optimize this tree.

A nonparametric bootstrap test (Felsenstein, 1985) with 1,000 replications for each above mentioned method (NJ, MP, and ML) was performed to estimate the robustness of each internal branch of the trees because it provides statistical support indicating the reliability of the data. The heuristic algorithm NNI (nearest neighbor interchange) was used to

optimize the replications in MP and ML, and the bootstrap values were included using the TBR algorithm.

A phylogenetic reconstruction using Bayesian Inference (BI) (Yang and Rannala, 1997) was performed with the software MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) by applying the same model used to find the tree in ML, but in this case, it was selected using the software MrModeltest v2 (Nylander, 2004). Two independent runs with four Monte Carlo Markov chains (MCMC) were performed for 10,000,000 generations, and the trees were sampled and retained at every 1,000th generation. The first tree samples in this case, namely, 1,000,000th generation, were discarded in the burn-in phase, and the remaining trees were summarized to generate a majority rule consensus tree.

A tree was built using BI for each studied cultivar, and the bootstrap values of the NJ, MP, and ML methods were added to the corresponding trees in addition to a *posteriori* BI probability values.

The tree branches with bootstrap or a *posteriori* probability values below 80% were omitted. The reason for this approach is that values below 80% denote a low reliability of data with poor statistical support (Harada *et al.*, 1995).

Diversity indexes

The diversity of the fungal endophytes species was measured using diversity indexes that use species richness and relative species abundance as parameters.

The diversity indexes used were the Shannon-Wiener index $\{H' = - \sum [(ni/n) \ln (ni/n)]\}$ and Simpson's diversity index $\{1 - [D = \sum (ni/n)^2]\}$, Hill's index $N_1 = e^{H'}$, where *ni* is the number of different species (*i*), (*n*) is the abundance of each species in the community and *H'* is the Shannon-Wiener index (Hill, 1973). The equitability of the Shannon-Wiener index was calculated by $J' = H'/H'_{max}$, where *H'* is the Shannon-Wiener index and *H'_{max}* is the maximum diversity value for the number of species resulting from the ln of the *ni* value.

The Shannon-Wiener index assesses species richness and relative species abundance. It varies from 0 in communities with only one species to high values in communities with many species, which comprise a few individuals each. Hill's index (*N_i*) is an equity index, and it expresses the diversity of a community on a uniform scale (Hill, 1973).

Simpson's diversity index estimates the probability that two randomly selected individuals in a community belong to different species (Simpson, 1949).

Species equitability varies between 0 and 1 and reflects the contribution of individuals to the community, with 1 representing maximum equitability, i.e., when the proportion of all species is similar.

The frequency of isolation (FI) of the fungal endophytes was also calculated ($FI = N_i/N_t \times 100$), where *N_i* is the number of wells in the multiwell plates containing leaf fragments or particles colonized by fungal endophytes and *N_t* is the total number of assessed wells containing leaf fragments or particles.

A 95% confidence interval of the diversity indexes found was calculated using the lower and upper limits ($Lm = \text{diversity index value} \pm 1.96 \times \text{standard error}$); the standard error was calculated after 1,000 bootstrap replications. The

analysis of diversity indexes and calculations of the standard error were performed with the software R, version 2.14.1 (R Development Core Team, 2011).

Results

Fungal diversity

From 432 colony-forming units (CFUs) obtained by fragment plating and 110 CFUs with concentrations 1× and 1:2× of extinction culturing, 241 different morphospecies were analyzed and identified at the level of the genus or specie (taxa) using the GenBank BlastN tool; 146 individual morphospecies corresponded to the cultivar Conquista and 95 to Monsoy.

The FI of the fungal endophytes using fragment plating was 90% for both cultivars. Using extinction culturing, the FI was 22.8% in the cultivar Monsoy and 25% in Conquista.

Among the identified isolates, 48 different taxa were identified that were associated with the cultivar Conquista and 33 with Monsoy. Considering both cultivars together, a total of 62 different taxa were distinguished (Table 1).

The Phylum Ascomycota was represented in this study by the Class Dothideomycetes (Orders: Botryosphaeriales, Capnodiales, and Pleosporales) and by the Class Sordariomycetes (Orders: Diaporthales, Hypocreales, Magnaporthales, Sordariales, Trichosphaeriales, and Xylariales). Dothideomycetes and Sordariomycetes comprised to 31.5% and 67.3%, respectively, of the isolates obtained in the cultivar Monsoy and 29.4% and 65% in the cultivar Conquista, respectively. The Phylum Basidiomycota, represented by isolates of the subphylum Pucciniomycotina (Class: Uredioniomycetes and Order: Sporidiales), comprised 1% and 4.8% of the isolates from the cultivars Monsoy and Conquista, respectively (Supplementary data Table S3A).

The three most frequent genera isolated from the cultivar Monsoy were *Colletotrichum* (46.3%), *Cochliobolus* (9.5%), and *Fusarium* (6.3%). In the cultivar Conquista, the genus *Colletotrichum* was also the most abundant (42%), followed by *Xylaria* (8.2%) and *Cochliobolus* (7.5%). The remaining genera were rarely isolated, with frequencies varying between 0% and 6% according to the investigated cultivar. With regard to the 62 identified taxa, the distribution of isolates revealed that both common and rare taxa occur (Fig. 1).

There was no difference in the lower (left side) and upper

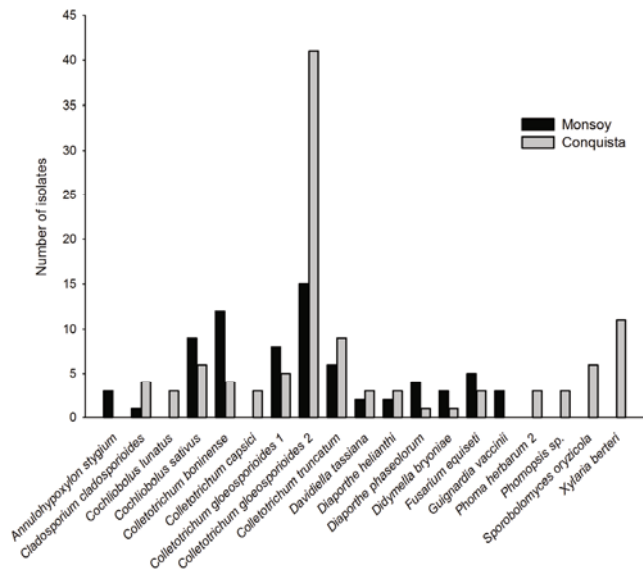


Fig. 1. Number of isolates and their respective taxa from the soybean cultivars Monsoy and Conquista. Only taxa exhibiting three or more isolates in any of the investigated cultivars were included. Numbers 1 and 2 added to species *Colletotrichum gloeosporioides* allude to the distinction between isolates that demonstrated less than 95% identity upon sequence comparison; nevertheless, the species name was kept based on the database sequence exhibiting the highest identity and score.

(right side) limits of the data (Lm_l-Lm_u) corresponding to the Shannon-Wiener diversity index between the cultivars Monsoy (2.64–3.08) and Conquista (3.03–3.43) using fragment plating and the 95% confidence interval (Table 2). A similar result was found between the cultivars Monsoy (1.73–2.47) and Conquista (1.23–2.17) using extinction culturing, thus demonstrating that the analyses were quite homogeneous with regard to the sampling and isolation procedures in the two investigated cultivars.

The lower and upper limits of Hill’s index (N_1) were higher for the cultivar Conquista (20.94–29.84) compared to Monsoy (14.37–20.57) using fragment plating. However, no difference was observed between the cultivars Conquista (3.31–7.67) and Monsoy (5.83–10.61) when the extinction culture and 95% confidence intervals were used (Table 2).

There was also no difference in the Simpson’s diversity index between the investigated cultivars using either the fragment plating (Monsoy = 0.06 – 0.10 and Conquista = 0.05 – 0.09) or the extinction culture (Monsoy = 0.06 – 0.26

Table 2. Number of morphospecies, equitability and lower and upper limits of diversity indexes using different isolation techniques for the cultivars Monsoy and Conquista

Isolation technique	Morphospecies (n)	Species richness (i)	Equitability ($J' = H'/H'_{max}$)	Shannon-Wiener index (H') (Lm_l-Lm_u)	Hill’s N_1 (Lm_l-Lm_u)	Simpson’s index (1-D) (Lm_l-Lm_u)
Fragment plating (M)	74	26	0.87	(2.64–3.08)	(14.37–20.57) ^a	(0.06–0.10)
Fragment plating (C)	116	42	0.86	(3.03–3.43)	(20.94–29.84) ^a	(0.05–0.10)
Extinction culturing (M)	21	11	0.87	(1.73–2.47)	(5.83–10.61)	(0.06–0.26)
Extinction culturing (C)	30	12	0.68	(1.23–2.17)	(3.31–7.67)	(0.16–0.52)
Monsoy Total	95	33	0.86	(2.82–3.22)	(16.95–24.61)	(0.05–0.09)
Conquista Total	146	48	0.81	(2.93–3.37)	(19.05–27.83)	(0.07–0.11)

The lower (left side) and upper limits (right side) of the diversity indexes (Lm_l-Lm_u) exhibit 95% confidence intervals ($Lm = index \pm 1.96X$ standard error) with 1,000 bootstrap replications for the standard error.
^a Significant variation of data.

and Conquista = 0.16 – 0.52) (Table 2).

The species equitability values for the cultivars Monsoy (fragment plating = 0.87 and extinction culturing = 0.87) and Conquista (fragment plating = 0.86) using a fixed value for the Shannon-Wiener index were relatively high, i.e., close to one. The one exception was the equitability value of the cultivar Conquista when extinction culturing was used (0.68) (Table 2).

The species richness (i) using fragment plating was 26 in the cultivar Monsoy and 42 in Conquista. When the extinction

culture was used, the i values for the cultivars Monsoy and Conquista were 11 and 12, respectively. The species richness was higher when both isolation techniques were used (Monsoy = 33 and Conquista = 48).

Singleton fungal isolates were the main contributors to the values for species richness in both investigated cultivars. When the fragment technique was used, singletons comprised 12 out of the 26 species in the cultivar Monsoy and 21 out of the 42 in Conquista.

When the extinction culturing was used, singletons repre-

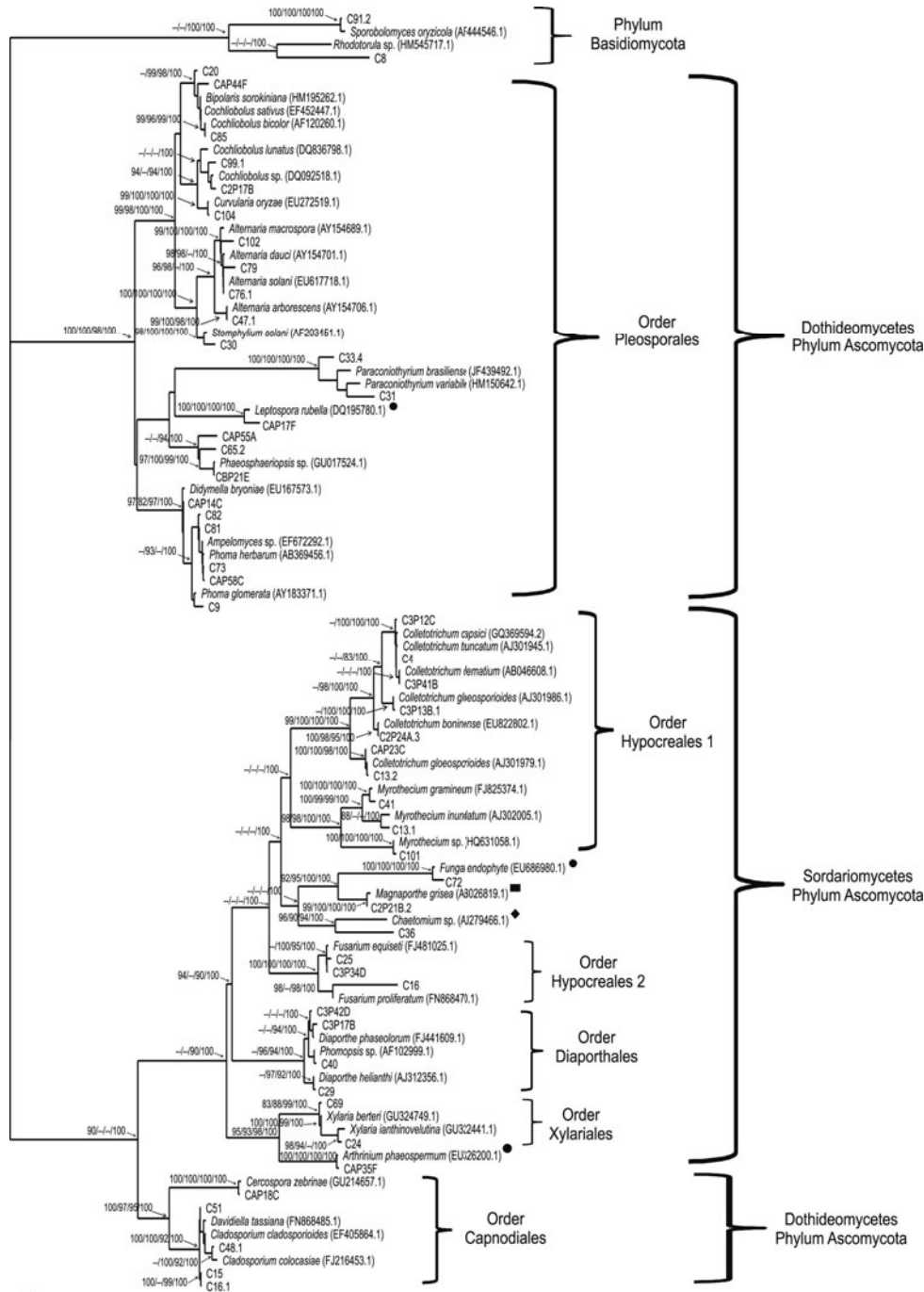


Fig. 2. Phylogenetic tree obtained by Bayesian Inference (BI) using the nucleotide sequence of the rDNA ITS region of 53 isolates from the cultivar Conquista and 45 fungi of GenBank. Left to right, the numbers next to each ancestral node represent the bootstrap values using the NJ, MP, and ML methods and the *a posteriori* probability values (converted to 100) using BI. Symbols represent different Orders present in the tree: (◆) Sordariales, (■) Magnaporthales, and (●) Indefinite Order. Bootstrap and probability values below 80 as represented by an interrupted line (–) were not included in the tree. Tree-Base accession number: 12687.

sented 7 out of the 11 species in the cultivar Monsoy and 9 out of the 12 in Conquista.

Phylogenetic analysis

In all phylogenetic trees that were constructed, the out-groups used were the isolates belonging to the Phylum Basidiomycota

because most isolates from the cultivars Monsoy (99%) and Conquista (95.2%) belonged to the Phylum Ascomycota.

The number of informative sites in the nucleotide sequence of the rDNA ITS region for the cultivar Monsoy in Maximum Parsimony (MP) was 316.0, with a tree length = 1506, consistency index = 0.4894, retention index = 0.8757, redimensioned consistency index = 0.4758 and homoplasy index =

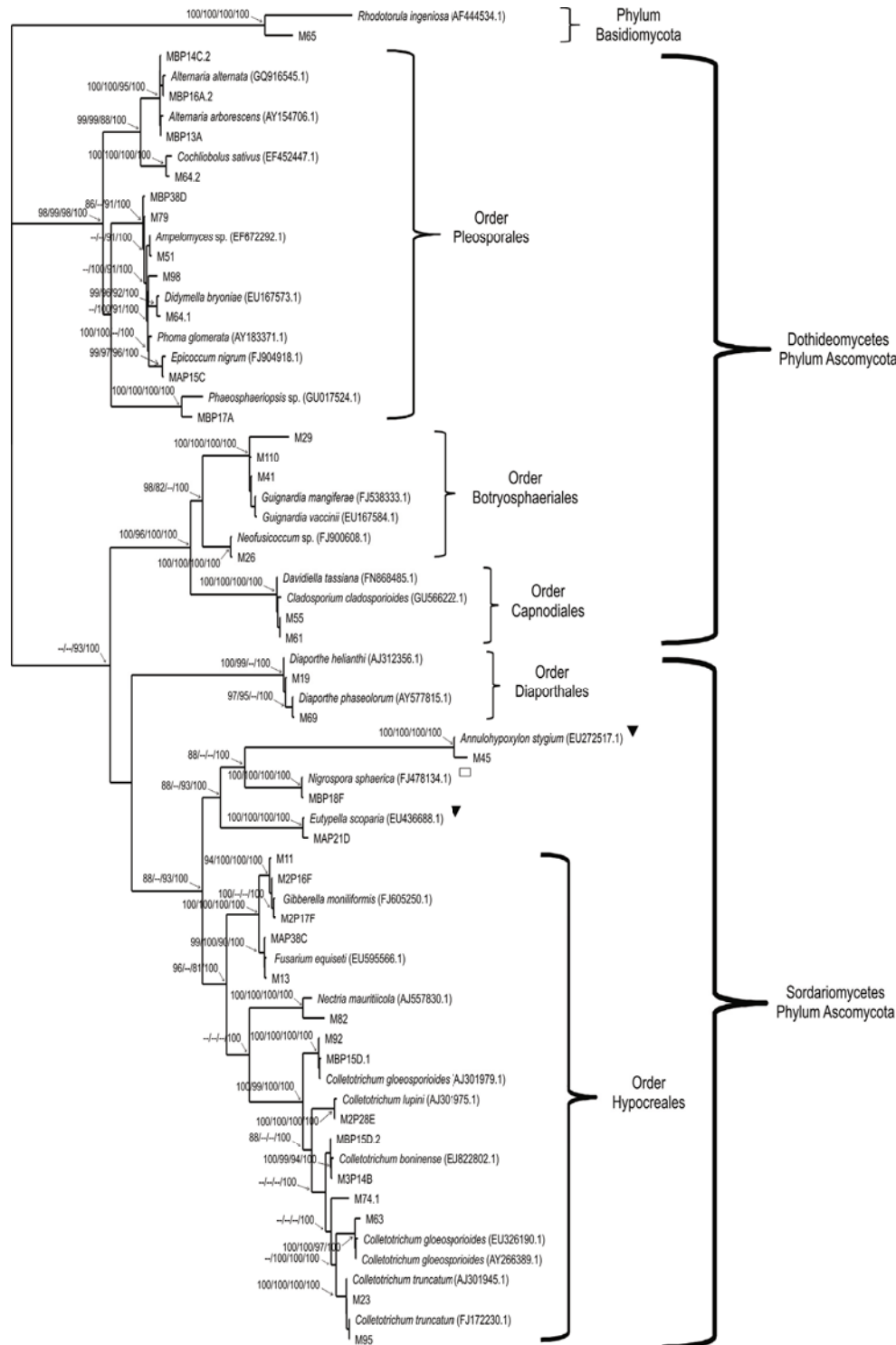


Fig. 3. Phylogenetic tree obtained by Bayesian Inference (BI) using the nucleotide sequence of the rDNA ITS region of 38 isolates from the cultivar Monsoy and 29 fungi of GenBank. Left to right, the numbers next to each ancestral node represent the bootstrap values using the NJ, MP, and ML methods and the *a posteriori* probability values (converted to 100) using BI. Symbols represent different orders present in the tree: (□) Trichosphaeriales and (▼) Xylariales. Bootstrap and probability values below 80 as represented by an interrupted line (-) were not included in the tree. TreeBase accession number: 12687.

0.5106. For the cultivar Conquista, the number of informative sites in MP was 320, with a tree length = 1977, consistency index = 0.3940, retention index = 0.8640, redimensioned consistency index = 0.3786 and homoplasy index = 0.6060.

The model selected for the Maximum Likelihood (ML) for both of the investigated cultivars was SYM+I+G. The proportion of invariable sites was 0.1586 in the cultivar Monsoy and 0.1519 in the cultivar Conquista, and the gamma distribution was 0.9362 and 0.8288, respectively. The NJ tree was built by correcting its gamma distribution based on the ML model.

The phylogenetic relationship of the endophytic isolates is represented by the majority consensus tree produced by Bayesian Inference (BI) for the cultivar Conquista (Fig. 2) and Monsoy (Fig. 3). The values listed from left to right next to each ancestral node respectively correspond to the bootstrap values obtained with the NJ, MP, and ML methods and of *a posteriori* probability (converted to 100) of most trees in BI. Most of the fungal endophytes isolates found in both of the investigated cultivars were grouped according to their Order (when defined), Class and Phylum.

Figs. 2 and 3 show that, after separation of the out-groups (species of the Phylum Basidiomycota), the species belonging to the Phylum Ascomycota diverge with regard to Order and Class. In Fig. 2, a first tree branch exhibiting robust support groups together fungal species belonging to the Order Pleosporales from the Class Dothideomycetes. A second tree branch with less support splits the fungal species belonging to the Class Dothideomycetes, represented by the Order Capnodiales, from the species belonging to the Class Sordariomycetes, represented by the Orders Diaporthales, Hypocreales 1 and 2, Magnaporthales, Sordariales and Xylariales. The organisms corresponding to the Order Hypocreales 1 and 2 were not grouped within the same Order, which might be due to the fact that the ITS region was the only region analyzed.

In Fig. 3, a first tree branch with robust support groups together the species from the Order Pleosporales, which belong to the Class Dothideomycetes. A second branch with less support splits the fungal species from the Class Dothideomycetes, represented by the Orders Capnodiales and Botryosphaerales, from the species belonging to the Class Sordariomycetes, represented by the Orders Hypocreales, Trichosphaerales, and Xylariales. Some branches are not statistically well supported (based on bootstrap or *a posteriori* probability values); however, the most recent common ancestor of the species belonging to a same Order is well supported (with some exceptions).

Discussion

The combination of the fragment plating and extinction culturing isolation techniques seems to be the most appropriate in order to isolate fungal species with a greater richness as demonstrated by the present study. Alone, none of these techniques were able to recover the total species richness realized in the cultivars Monsoy (33 species) and Conquista (48 species) (Table 2).

The isolation frequency of fungal endophytes using fragment plating was 90% for both of the investigated cultivars. This value indicates a density of endophytic colonization corresponding to the values of 90–95% reported by Lodge *et al.* (1996). However, values above 100% might sometimes be found due, for instance, to multiple fungal infections of the same leaf fragment.

However, the isolation frequency of fungal endophytes using extinction culturing was 22.83% in the cultivar Monsoy and 25% in Conquista, where singletons were the main contributors to the values found. These values correspond to the 20–30% range of total colonized wells proposed by Collado *et al.* (2007) as the optimal frequency because this range tends to favor the isolation of a more diverse community of fungal endophytes.

The present study represents one of the first to use extinction culturing to achieve fungal endophytes diversity. However, although extinction culturing is the best technique to derive most saprophytic fungi from *Elaeocarpus dentatus* soil litter (Collado *et al.*, 2007), further studies are clearly needed to standardize this technique. According to Unterseher and Schnittler (2009), such studies should focus on the modification of the culture medium (primary vs. selective and rich vs. low nutrient media) because species richness and relative species abundance are influenced by the type of culture medium and, thus, also by the interpretation of fungal endophytes biodiversity.

The use of multiwell plates associated with the extinction culture technique is considered to be advantageous for several reasons: 1) it requires only one single inspection to obtain colonies at the end of the incubation period; 2) it allows for the retention of colonies in small wells, thus extending the incubation time and, consequently, revealing slow-growing species; 3) it is time-saving because one single plate with several colonies permits rapid observation and facilitates the selection of morphospecies; and 4) it is more affordable because 1.0 L of culture medium is sufficient to fill approximately 20 multiwell plates (Unterseher and Schnittler, 2009).

A further advantage of using multiwell plates is the prevention of cross-contamination by fast-growing fungi. However, to avoid cross-contamination, the incubation time should be shorter or compounds such as cyclosporine A, dicloran or Rose Bengal must be used to retard the growth of fungi (Bills *et al.*, 2004; Collado *et al.*, 2007). The use of multiwell plates instead of conventional Petri dishes also reduces the expense of culture media in the fragment plating technique, whereas the retention of colonies in wells avoids cross-contamination.

The use of substances that retard fungal growth represents an alternative way to optimize the extinction culturing technique. However, the use of inhibitors might not only retard fungal growth but also select rare species and omit the dominant ones (Arnold and Lutzoni, 2007; Arnold, 2008).

Among the genera isolated, *Ampelomyces*, *Annulohyphoxylon*, *Guignardia*, *Leptospora*, *Magnaporthe*, *Ophiognomonia*, *Paraconiothyrium*, *Phaeosphaeriopsis*, *Rhodotorula*, *Sporobolomyces*, and *Xylaria* have never before been reported to occur endophytically in soybean leaves, stalks or seeds. The remaining isolated genera, however, were previously reported by studies that exclusively used the fragment plating

technique for isolation (Miller and Roy, 1982; Sinclair, 1991; Roy *et al.*, 2001; Larran *et al.*, 2002b; Pimentel *et al.*, 2006).

An interesting feature is the relative isolation of the same taxa in both cultivars. This finding confirms that preferential host colonization is much more common than is exclusivity or specificity in the fungal colonization of hosts (Lodge *et al.*, 1996; Zhou and Hyde, 2001).

The findings of the present study corroborate those of Arnold and Lutzoni (2007) because the Classes Dothideomycetes and Sordariomycetes from the Phylum Ascomycota were the most abundant. According to those authors, such Classes comprise more than 75% of the fungal endophytes found in environments ranging from the arctic to the tropics, although the relative abundance compared to other Classes varies as a function of the latitude.

Fungal endophytes isolated in this study may also include epiphytic components because the endophytic community may include species classified as epiphytic such as *Alternaria alternata*, *Cladosporium cladosporioides*, and *Epicoccum purpurascens*, among others, that enter the foliar tissue facultatively during senescence (Petrini, 1991; Larran, 2002b).

In both investigated cultivars, the dominant endophytic genus was *Colletotrichum*, followed by *Cochliobolus* and *Fusarium* in Monsoy and *Xylaria* and *Cochliobolus* in Conquista. The dominance of such genera is well supported by many studies in which the genera of fungal endophytes found in tropical plants were *Colletotrichum*, *Xylaria*, and *Fusarium* (Arnold and Lutzoni, 2007), which typically grow rapidly and competitively in non-selective or plant-based media as was the case with the YMC used in our study.

The genera *Rhodothorula* and *Sporobolomyces* from the subphylum Pucciniomycotina (Phylum Basidiomycota) were isolated for the first time as endophytic in the soybean. Although not frequent, some members of the Phylum Basidiomycota belonging to the subphyla Agaricomycotina, Pucciniomycotina and Ustilaginomycotina have also been reported to be endophytic (Arnold, 2008; Hyde and Soy-tong, 2008; Rungjindamai *et al.*, 2008; Pinruan *et al.*, 2010; Sakayaroj *et al.*, 2010; Rivera-Orduna *et al.*, 2011).

The diversity of fungal endophytes community isolated from leaves of the cultivars Monsoy and Conquista did not vary according to the Shannon-Wiener index (H') regardless of whether fragment plating or extinction culturing was used (Table 2). These results were unexpected because our working hypothesis anticipated that the use of two different soybean cultivars would be the main factor affecting the diversity of fungal endophytes, particularly as measured by the Shannon-Wiener index. Thus, it may be stated that the use of different soybean cultivars does not affect the diversity of fungal endophytes community when they are isolated from plants grown under the same edaphoclimatic conditions.

According to Gazis and Chaverri (2010), the values of the Shannon-Wiener index usually range between 1.5 and 3.5, where 1.5 represents low and 3.5 represents high diversity. The values in the present study ranged from low (Conquista in extinction culture: 1.23–2.17) to high diversity (Conquista in fragment plating: 3.03–3.43).

The Simpson's diversity index estimates the probability that two randomly selected individuals from a community

belong to different species (Simpson, 1949). However, the values found for Simpson's index in the present study (Table 2) clearly demonstrate no variation in the probability that the obtained isolates belong to different species using either fragment plating or extinction culture upon comparison of the investigated cultivars.

Hill's index (N_1) expresses the community diversity on a uniform scale (Hill, 1973). It tends to favor the presence of rare species for which the values directly affect the species richness. Therefore, the higher value found in the cultivar Conquista using the fragment plating allowed us to document a significant difference in Hill's index (N_1) compared to the cultivar Monsoy (Conquista = 25.39 ± 4.45 vs. Monsoy = 17.47 ± 3.1) when considering the 95% confidence interval data. This fact may be explained by the greater number of morphospecies found in the cultivar Conquista (146) compared to Monsoy (95), which might be related to the genetic and culture characteristics of each cultivar or even a random presence of singleton morphospecies. Nevertheless, this difference in Hill's index (N_1) documented using the fragment plating technique was not observed when the extinction culturing was used instead to compare the two cultivars.

The equitability was relatively high for both cultivars, with values close to 1.0 (Table 2), with the exception of the cultivar Conquista when the extinction culturing was used (0.68). This lower value can be explained by the presence of the dominant species *C. gloeosporioides* 2. Equitability values close to 1.0 are not very usual, whereas low equitability in the distribution of species is a common phenomenon across several biological systems and also observed in plant-microorganism interactions (Magurran, 1988; Frohlich *et al.*, 2000).

Alone, neither of the isolation techniques was able to recover the total species richness present in the cultivars Monsoy (33 species) and Conquista (48 species) (Table 2). This fact may be explained by the random presence of singletons independent of the isolation technique employed in this study.

Nucleotides corresponding to the fungal endophytes rDNA ITS region were used to identify the isolates found and to perform phylogenetic analysis. For both of the phylogenetic trees generated in this study, a majority of the ancestors nodes are robust and are supported by higher bootstrap and *a posteriori* probability values. Most isolates of the fungal endophytes from both cultivars were grouped according to Order (when defined), Class and Phylum, thus concurring with other studies that used the ITS region for phylogenetic analysis (Arnold and Lutzoni, 2007; Huang *et al.*, 2009; Gazis and Chaverri, 2010).

The ITS region has been extensively used in studies on fungal endophytes because it provides excellent resolution of fungal isolates below the level of species for some of the most common endophytic taxa (Arnold, 2007; Nilsson *et al.*, 2008). In addition, the ease of obtaining the sequence of the ITS region and the presence of a large database available regarding the ITS region of endophytic isolates from the arctic to the tropics reinforces the utility of the ITS region in providing an initial approximation to the genotypic difference among endophytic isolates taken from the most divergent families of plant species worldwide (Arnold, 2007; Arnold and Lutzoni, 2007; Nilsson *et al.*, 2008).

Most of the fungal endophytes species identified in the present study are either known plant pathogens, saprophytes or “potentially” mutualistic species. Independent of the ecological relationship, all may be tested in the future as biological control agents in the induction of systemic resistance in plants (Arnold *et al.*, 2003; Dingle and Mcgee, 2003) and in the promotion of plant growth (Sirrenberg *et al.*, 2007; Hamayun *et al.*, 2009).

As demonstrated by this and other studies (Miller and Roy, 1982; Larran *et al.*, 2002b; Pimentel *et al.*, 2006), the soybean exhibits high morphological and genotypic diversity of fungal endophytes, thus denoting the presence of quite complex ecological interactions.

The species of the genus *Colletotrichum* are usually considered to be common pathogens, although different species of this genus may express a mutualistic lifestyle with a wide scope of hosts in tropical areas (Guo *et al.*, 2000; Damm *et al.*, 2010; Phoulivong *et al.*, 2010). For hosts, the benefits of mutualistic association include the induction of systemic resistance to pathogens, the promotion of plant growth and a greater tolerance to drought (Redman *et al.*, 1999, 2001).

Xylaria is a common genus of wood-decomposing fungi distributed worldwide and is also commonly documented to be endophytic (Lodge *et al.*, 1996; Fröhlich *et al.*, 2000; Gamboa *et al.*, 2002; Santamaría and Bayman, 2005; Silva *et al.*, 2010).

Ampelomyces, *Chaetomium*, and *Phoma glomerata* are some of the endophytic fungi isolated in the present study that may play potential roles in the biological control of soybean pathogens.

The genus *Ampelomyces* contains species held to be mycoparasites of powdery mildew and already described as endophytic in plants (Aly *et al.*, 2008). The most common species is *Ampelomyces quisqualis*, for which an isolate known as M-10 has been commercially prepared as a mycofungicide that is predominantly used in the control of powdery mildew in cucumbers, carrots and mangoes (Sundheim, 1982; Paulitz and Bélanger, 2001; Kiss, 2003; Kaewchai *et al.*, 2009). Because the species *Microsphaera diffusa* (anamorph: *Oidium* sp.) of powdery mildew is an important soybean pathogen in Brazil (Kimati *et al.*, 1997; Embrapa, 2011), the genus *Ampelomyces* isolated in this study may be used as an excellent alternative as biological control agent.

The species of the genus *Chaetomium* are normally found in the soil and in organic compounds (Soytong *et al.*, 2001) and have been described as antagonist toward several plant pathogens, in particular those harbored in the soil and seeds (Dhingra *et al.*, 2003; Park *et al.*, 2005). Although they normally appear as free-living fungi, some species of the genus *Chaetomium* were isolated as endophytic of wheat (Syed *et al.*, 2009) and *Ginkgo biloba* (Qin *et al.*, 2009). In Brazil, the species *Chaetomium globosum* has been tested in the control of the soybean phytopathogen *Diaporthe phaseolorum* f. sp. *meriodionalis* (the etiologic agent of stem canker) and demonstrated excellent results (Dhingra *et al.*, 2003).

Similar to the species of the genus *Ampelomyces*, the species *Phoma glomerata* was also identified as a mycoparasite of powdery mildew in oak (Sullivan and White, 2000). In addition, *Phoma glomerata* produces epoxydon, which is a substance capable of controlling the soil pathogen *Plasmo-*

diophora brassicae, the etiologic agent of clubroot in species of the family Brassicaceae (Arie *et al.*, 1998).

All of the fungal isolates found in this study will be tested in the future with regard to their potential to serve as biological control agents of disease, as well as to produce secondary metabolites with potential interest for the pharmaceutical industry.

The present study isolated 62 taxa of fungal endophytes from soybean leaves. The genera *Colletotrichum*, *Cochliobolus*, and *Fusarium* were the most frequently isolated in the cultivar Monsoy, and *Colletotrichum*, *Xylaria*, and *Cochliobolus* were the more frequent in the cultivar Conquista. The species richness was higher when both isolation techniques were used. However, the fungal community diversity was similar in both cultivars using the same isolation technique with the exception of Hill's index (N_1), which was higher in the cultivar Conquista when the fragment plating was used. Singletons were the main contributors to the species richness independent of the isolation technique and sampling used.

The fragment plating technique tends to favor dominant and rapidly-growing species. The extinction culturing technique, which uses multiwell plates, allows for all rare and slow-growing as well as dominant and fast-growing species to grow; however, the latter technique exhibits a lower probability of isolation compared to the former. Thus, extinction culturing may be recommended as the better technique to isolate fungal endophytes. Nevertheless, caution is required, and the technique still needs to be improved in future studies. Therefore, we suggest that both techniques be used to obtain greater species richness, as demonstrated by the present study and also by that of Unterseher and Schnittler (2009).

This study is one of the first to use extinction culturing to isolate fungal endophytes in plant leaves, and thus it contributes to the development and optimization of this technique to be applied in future studies on the isolation of these microorganisms.

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References

- Almeida, F., Cerqueira, F.M., Silva, R.D.N., Ulhoa, C.J., and Lima, A.L. 2007. Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: evaluation of coiling and hydrolytic enzyme production. *Biotechnol. Lett.* **29**, 1189–1193.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

- Aly, A.H., Edrada-Ebel, R., Wray, V., Muller, W.E., Kozytska, S., Hentschel, U., Proksch, P., and Ebel, R. 2008. Bioactive metabolites from the endophytic fungus *Ampelomyces* sp. isolated from the medicinal plant *Urospermum picroides*. *Phytochemistry* **69**, 1716–1725.
- Arie, T., Kobayashi, Y., Okada, G., Kono, Y., and Yamaguchi, I. 1998. Control of soilborne clubroot disease of cruciferous plants by epoxydon from *Phoma glomerata*. *Plant Pathol.* **47**, 743–748.
- Arnold, A.E. 2007. Understanding the diversity of foliar fungal endophytes: progress, challenges, and frontiers. *Fungal Biol. Reviews* **21**, 51–66.
- Arnold, A.E. 2008. Endophytic fungi: Hidden components of tropical community ecology, pp. 254–271. In Schnitzer, S.A. and Carson, W.P. (eds.), *Tropical Forest Community Ecology* Blackwell Scientific.
- Arnold, A.E. and Lutzoni, F. 2007. Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? *Ecology* **88**, 541–549.
- Arnold, A.E., Mejia, L.C., Kylo, D.A., Rojas, E.I., Maynard, Z., Robbins, N., and Herre, E.A. 2003. Fungal endophytes limit pathogen damage in a tropical tree. *Proc. Natl. Acad. Sci. USA* **100**, 15649–15654.
- Azevedo, J.L., Maccheroni Jr., W., Pereira, J.O., and Araújo, W.L. 2000. Endophytic microorganisms: a review on insect control and recent advances on tropical plants. *Electron J. Biotechnol.* **3**, 40–65.
- Bailey, B.A., Bae, H., Strem, M.D., Crozier, J., Thomas, S.E., Samuels, G.J., Vinyard, B.T., and Holmes, K.A. 2008. Antibiosis, mycoparasitism, and colonization success for endophytic *Trichoderma* isolates with biological control potential in *Theobroma cacao*. *Biol. Control* **46**, 24–35.
- Bayat, F., Mirlohi, A., and Khodambashi, M. 2009. Effects of endophytic fungi on some drought tolerance mechanisms of tall fescue in a hydroponics culture. *Russ. J. Plant Physiol.* **56**, 510–516.
- Bills, G.F., Christensen, M., Powell, M., and Thorn, G. 2004. Saprobic soil fungi, pp. 271–302. In Mueller, G.M., Bills, G.F., and Foster, M.S. (eds.), *Biodiversity of fungi – Inventory and Monitoring Methods* Elsevier Academic Press, Oxford.
- Cao, R., Liu, X., Gao, K., Mendgen, K., Kang, Z., Gao, J., Dai, Y., and Wang, X. 2009. Mycoparasitism of endophytic fungi isolated from reed on soilborne phytopathogenic fungi and production of cell wall-degrading enzymes *in vitro*. *Curr. Microbiol.* **59**, 584–592.
- Carrol, G. 1988. Fungal endophytes in stems and leaves: from latent pathogen to mutualist symbiont. *Ecology* **69**, 2–9.
- Cavalli-Sforza, L.L. and Edwards, A.W.F. 1967. Phylogenetic analysis. Models and estimation procedures. *Am. J. Hum. Genet.* **19**, 233–257.
- Collado, J., Platas, G., Paulus, B., and Bills, G.F. 2007. High-throughput culturing of fungi from plant litter by a dilution-to-extinction technique. *FEMS Microbiol. Ecol.* **60**, 521–533.
- Damm, U., Baroncelli, R., Cai, L., Kubo, Y., O’Connell, R., Weir, B., Yoshino, K., and Cannon, P.F. 2010. *Colletotrichum*: species, ecology and interactions. *IMA Fungus* **1**, 161–165.
- De Bary, A. 1866. Morphologie und Physiologie der Pilze, Frechten und Myxomyceten. In H. s. H. o. P. Botany (ed.), vol. 2. Engelmann, Leipzig, Germany.
- Dhingra, O.D., Mizubuti, E.S.G., and Santana, F.M. 2003. *Chaetomium globosum* for reducing primary inoculum of *Diaporthe phaseolorum* f. sp. meridionalis in soil–surface soybean stubble in field conditions. *Biol. Control* **26**, 302–310.
- Dingle, J. and Mcgee, P.A. 2003. Some endophytic fungi reduce the density of pustules of *Puccinia recondita* f. sp. tritici in wheat. *Mycol. Res.* **107**, 310–316.
- El-Ghaouth, A. 1997. Biologically-based alternatives to synthetic fungicides for the control of postharvest diseases. *J. Ind. Microbiol. Biotechnol.* **19**, 160–162.
- Elbersen, H.W. and West, C.P. 1996. Growth and water relations of field-grown tall fescue as influenced by drought and endophyte. *Grass Forage Sci.* **51**, 333–342.
- Embrapa. 2011. Tecnologias de Produção de Soja – Região Central do Brasil 2012 e 2013, p. 263. Embrapa Soja, Londrina.
- Faeth, S.H. and Fagan, W.F. 2002. Fungal endophytes: common host plant symbionts but uncommon mutualists. *Integr. Comp. Biol.* **42**, 360–368.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783–791.
- Fisher, P.J. 1996. Survival and spread of the endophyte *Stagonospora pteridiicola* in *Pteridium aquilinum*, other ferns and some flowering plants. *New Phytol.* **132**, 119–122.
- Fisher, P.J., Petrini, O., and Scott, H.M.L. 1992. The distribution of some fungal and bacterial endophytes in Maize (*Zea mays* L). *New Phytol.* **122**, 299–305.
- Frohlich, J., Hyde, K.D., and Petrini, O. 2000. Endophytic fungi associated with palms. *Mycol. Res.* **104**, 1202–1212.
- Gamboa, M.A., Laureano, S., and Bayman, P. 2002. Measuring diversity of endophytic fungi in leaf fragments: does size matter? *Mycopathologia* **156**, 41–45.
- Gardes, M. and Bruns, T.D. 1993. ITS primers enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Mol. Ecol.* **2**, 113–118.
- Gazis, R. and Chaverri, P. 2010. Diversity of fungal endophytes in leaves and stems of wild rubber trees (*Hevea brasiliensis*) in Peru. *Fungal Ecol.* **3**, 240–254.
- Goldman, N., Anderson, J.P., and Rodrigo, A.G. 2000. Likelihood-based tests of topologies in phylogenetics. *Syst. Biol.* **49**, 652–670.
- Guetsky, R., Shtienberg, D., Elad, Y., and Dinoor, A. 2001. Combining biocontrol agents to reduce the variability of biological control. *Phytopathology* **91**, 621–627.
- Guo, L.D., Hyde, K.D., and Liew, E.C.Y. 2000. Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. *New Phytol.* **147**, 617–630.
- Hamayun, M., Khan, S.A., Kim, H.Y., Chaudhary, M.F., Hwang, Y.H., Shin, D.H., Kim, I.K., Lee, B.H., and Lee, I.J. 2009. Gibberellin production and plant growth enhancement by newly isolated strain of *Scolecobasidium tshawytschae*. *J. Microbiol. Biotechnol.* **19**, 560–565.
- Hanada, R.E., de Jorge Souza, T., Pomella, A.W.V., Hebbar, K.P., Pereira, J.O., Ismaiel, A., and Samuels, G.J. 2008. *Trichoderma martiale* sp. nov., a new endophyte from sapwood of *Theobroma cacao* with a potential for biological control. *Mycol. Res.* **112**, 1335–1343.
- Harada, M.L., Schneider, H., Schneider, M.P., Sampaio, I., Czelusniak, J., and Goodman, M. 1995. DNA evidence on the phylogenetic systematics of New World monkeys: support for the sister-grouping of Cebus and Saimiri from two unlinked nuclear genes. *Mol. Phylogenet. Evol.* **4**, 331–349.
- Hata, K., Atari, R., and Sone, K. 2002. Isolation of endophytic fungi from leaves of *Pasania edulis* and their within-leaf distributions. *Mycoscience* **43**, 369–373.
- Hill, M.O. 1973. Diversity and Evenness – Unifying notation and its consequences. *Ecology* **54**, 427–432.
- Huang, W.J., Cai, Y.Z., Surveswaran, S., Hyde, K.D., Corke, H., and Sun, M. 2009. Molecular phylogenetic identification of endophytic fungi isolated from three *Artemisia* species. *Fungal Divers.* **36**, 69–88.
- Huelsenbeck, J.P. and Crandall, K.A. 1997. Phylogeny estimation and hypothesis testing using maximum likelihood. *Annu. Rev. Ecol. Syst.* **28**, 437–466.
- Huelsenbeck, J.P. and Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**, 754–755.
- Hyde, K.D. and Soyong, K. 2008. The fungal endophyte dilemma.

- Fungal Divers.* **33**, 163–173.
- Joshee, S., Paulus, B.C., Park, D., and Johnston, P.R. 2009. Diversity and distribution of fungal foliar endophytes in New Zealand Podocarpaceae. *Mycol. Res.* **113**, 1003–1015.
- Kaewchai, S., Soyong, K., and Hyde, K.D. 2009. Mycofungicides and fungal biofertilizers. *Fungal Divers.* **38**, 25–50.
- Kimati, H., Amorim, L., Bergamin Filho, A., Camargo, L.E.A., and Rezende, J.A.M. 1997. Manual de Fitopatologia Vol. 2: Doenças das plantas cultivadas, pp. 705. 3th ed. Editora Agronômica Ceres., São Paulo–SP, Brazil.
- Kiss, L. 2003. A review of fungal antagonists of powdery mildews and their potential as biocontrol agents. *Pest Manage. Sci.* **59**, 475–483.
- Kumar, S., Nei, M., Dudley, J., and Tamura, K. 2008. MEGA: a biologist–centric software for evolutionary analysis of DNA and protein sequences. *Brief. Bioinform.* **9**, 299–306.
- Lacap, D.C., Hyde, K.D., and Liew, E.C.Y. 2003. An evaluation of the fungal ‘morphotype’ concept based on ribosomal DNA sequences. *Fungal Divers.* **12**, 53–66.
- Larran, S., Perello, A., Simon, M.R., and Moreno, V. 2002a. Isolation and analysis of endophytic microorganisms in wheat *Triticum aestivum* L.) leaves. *World J. Microbiol. Biotechnol.* **18**, 683–686.
- Larran, S., Perello, A., Simon, M.R., and Moreno, V. 2007. The endophytic fungi from wheat (*Triticum aestivum* L.). *World J. Microbiol. Biotechnol.* **23**, 565–572.
- Larran, S., Rollán, C., Bruno Ángeles, H., Alippi, H.E., and Urrutia, M.I. 2002b. Nota corta: Endophytic fungi in healthy soybean leaves. *Invest. Agr. Prod. Veg.* **17**, 173–178.
- Li, W.C., Zhou, J., Guo, S.Y., and Guo, L.D. 2007. Endophytic fungi associated with lichens in *Baihua mountain* of Beijing, China. *Fungal Divers.* **25**, 69–80.
- Lodge, D.J., Fisher, P.J., and Sutton, B.C. 1996. Endophytic fungi of *Manilkara bidentata* leaves in Puerto Rico. *Mycologia* **88**, 733–738.
- Maccheroni, Jr., W. and Azevedo, J.L. 1998. Synthesis and secretion of phosphatases by endophytic isolates of *Colletotrichum musae* grown under conditions of nutritional starvation. *J. Gen. Appl. Microbiol.* **44**, 381–387.
- Magurran, A.E. 1988. Ecological diversity and its measurement. p. 179. Princeton University Press, Princeton, USA.
- Marquez, L.M., Redman, R.S., Rodriguez, R.J., and Roossinck, M.J. 2007. A virus in a fungus in a plant: three–way symbiosis required for thermal tolerance. *Science* **315**, 513–515.
- Martin, F.N. 2003. Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. *Annu. Rev. Phytopathol.* **41**, 325–350.
- Miller, W.A. and Roy, K.W. 1982. Mycoflora of soybean leaves, pods and seeds in Mississippi. *Can. J. Bot.* **60**, 2716–2723.
- Nilsson, R.H., Kristiansson, E., Ryberg, M., Hallenberg, N., and Larsson, K.H. 2008. Intraspecific ITS variability in the kingdom fungi as expressed in the International Sequence Databases and its implications for molecular species identification. *Evol. Bioinform.* **4**, 193–201.
- Nunes, C.A. 2012. Biological control of postharvest diseases of fruit. *Eur. J. Plant. Pathol.* **133**, 181–196.
- Nylander, J.A.A. 2004. MrModeltestv2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Sweden.
- Orole, O.O. and Adejumo, T.O. 2011. Bacterial and fungal endophytes associated with grains and roots of maize. *J. Ecol. Nat. Environ.* **3**, 298–303.
- Ownley, B.H., Gwinn, K.D., and Vega, F.E. 2010. Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. *BioControl* **55**, 113–128.
- Park, J-H., Choi, G.J., Jang, K.S., Lim, H.K., Kim, H.T., Cho, K.Y., and Kim, J-C. 2005. Antifungal activity against plant pathogenic fungi of chaetoviridins isolated from *Chaetomium globosum*. *FEMS Microbiol. Lett.* **252**, 309–313.
- Paulitz, T.C. and Bélanger, R.R. 2001. Biological control in greenhouse systems. *Annu. Rev. Phytopathol.* **39**, 103–133.
- Paulus, B., Gadek, P., and Hyde, K.D. 2003. Estimation of micro-fungal diversity in tropical rainforest leaf litter using particle filtration: the effects of leaf storage and surface treatment. *Mycol. Res.* **107**, 748–756.
- Pereira, J.O. 1993. Ph. D. thesis. Fungos endofíticos de hospedeiros tropicais *Stylosanthes guianensis* e *Musa Cavendish*. Esalq/USP, Piracicaba, Brazil.
- Petrini, O. 1991. Fungal endophytes of tree leaves. In Andrews, J.H. and Hirano, S.S. (eds.), *Microbial Ecology of Leaves*. Springer Verlag, New York, USA.
- Phoulivong, S., Cai, L., Chen, H., McKenzie, E., Abdelsalam, K., Chukeatirote, E., and Hyde, K.D. 2010. *Colletotrichum gloeosporioides*: is not a common pathogen on tropical fruits. *Fungal Divers.* **44**, 33–43.
- Pimentel, I.C., Glienke-Blanco, C., Gabardo, J., Stuart, R.M., and Azevedo, J.L. 2006. Identification and colonization of endophytic fungi from soybean (*Glycine max* (L.) Merrill) under different environmental conditions. *Braz. Arch. Biol. Technol.* **49**, 705–711.
- Pinruan, U., Rungjindamai, N., Choeyklin, R., Lumyong, S., Hyde, K.D., and Jones, E.B.G. 2010. Occurrence and diversity of basidiomycetous endophytes from the oil palm, *Elaeis guineensis* in Thailand. *Fungal Divers.* **41**, 71–88.
- Posada, D. and Crandall, K.A. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.
- Qin, J.C., Zhang, Y.M., Gao, J.M., Bai, M.S., Yang, S.X., Laatsch, H., and Zhang, A.L. 2009. Bioactive metabolites produced by *Chaetomium globosum*, an endophytic fungus isolated from *Ginkgo biloba*. *Bioorg. Med. Chem. Lett.* **19**, 1572–1574.
- R Development Core Team. 2011. R: A language and environment for statistical computing. *R Foundation for Statistical Computing*, Vienna, Austria.
- Rakotoniriana, E.F., Munaut, F., Decock, C., Randriamampionona, D., Andriamboloniaina, M., Rakotomalala, T., Rakotonirina, E.J., Rabemanantsoa, C., Cheuk, K., Ratsimamanga, S.U., and et al. 2008. Endophytic fungi from leaves of *Centella asiatica*: occurrence and potential interactions within leaves. *Antonie van Leeuwenhoek* **93**, 27–36.
- Redman, R.S., Dunigan, D.D., and Rodriguez, R.J. 2001. Fungal symbiosis from mutualism to parasitism: who controls the outcome, host or invader? *New Phytol.* **151**, 705–716.
- Redman, R.S., Ranson, J.C., and Rodriguez, R.J. 1999. Conversion of the pathogenic fungus *Colletotrichum magna* to a nonpathogenic, endophytic mutualist by gene disruption. *Mol. Plant–Microbe Interact.* **12**, 969–975.
- Redman, R.S., Sheehan, K.B., Stout, R.G., Rodriguez, R.J., and Henson, J.M. 2002. Thermotolerance generated by plant/fungal symbiosis. *Science* **298**, 1581–1581.
- Rivera-Orduna, F.N., Suarez-Sanchez, R.A., Flores-Bustamante, Z.R., Gracida-Rodriguez, J.N., and Flores-Cotera, L.B. 2011. Diversity of endophytic fungi of *Taxus globosa* (Mexican yew). *Fungal Divers.* **47**, 65–74.
- Rodriguez, R.J., Freeman, D.C., McArthur, E.D., Kim, Y.O., and Redman, R.S. 2009. Symbiotic regulation of plant growth, development and reproduction. *Commun. Integr. Biol.* **2**, 141–143.
- Rogers, J.S. and Swofford, D.L. 1998. A fast method for approximating maximum likelihoods of phylogenetic trees from nucleotide sequences. *Syst. Biol.* **47**, 77–89.
- Roy, K.W., Baird, R.E., and Abney, T.S. 2001. A review of soybean (*Glycine max*) seed, pod, and flower mycofloras in North America, with methods and a key for identification of selected fungi. *Mycopathologia* **150**, 15–27.
- Rungjindamai, N., Pinruan, U., Choeyklin, R., Hattori, T., and Jones, E.B.G. 2008. Molecular characterization of basidiomycete-

- tous endophytes isolated from leaves, rachis and petioles of the oil palm, *Elaeis guineensis*, in Thailand. *Fungal Divers.* **33**, 133–161.
- Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.
- Sakayaroj, J., Preedanon, S., Supaphon, O., Jones, E.B.G., and Phongpaichit, S. 2010. Phylogenetic diversity of endophyte assemblages associated with the tropical seagrass *Enhalus acoroides* in Thailand. *Fungal Divers.* **42**, 27–45.
- Santamaría, J. and Bayman, P. 2005. Fungal epiphytes and endophytes of coffee leaves (*Coffea arabica*). *Microb. Ecol.* **50**, 1–8.
- Schulz, B. and Boyle, C. 2005. The endophytic continuum. *Mycol. Res.* **109**, 661–686.
- Schulz, B., Guske, S., Dammann, U., and Boyle, C. 1998. Endophyte host interactions II. Defining symbiosis of the endophyte host interaction. *Symbiosis* **25**, 213–227.
- Sediyama, T. 2009. Tecnologias de produção e usos da soja, p. 314. Editora Mecenas, Londrina.
- Silva, G.H., de Oliveira, C.M., Teles, H.L., Pauletti, P.M., Castro-Gamboa, I., Silva, D.H.S., Bolzani, V.S., Young, M.C.M., Costa-Neto, C.M., Pfenning, L.H., and *et al.* 2010. Sesquiterpenes from *Xylaria* sp., an endophytic fungus associated with *Piper aduncum* (Piperaceae). *Phytochem. Lett.* **3**, 164–167.
- Simpson, E.H. 1949. Measurement of diversity. *Nature* **163**, 688–688.
- Sinclair, J.B. 1991. Latent infection of soybean plants and seeds by fungi. *Plant Dis.* **75**, 220–224.
- Sirrenberg, A., Gobel, C., Grond, S., Czempinski, N., Ratzinger, A., Karlovsky, P., Santos, P., Feussner, I., and Pawlowski, K. 2007. *Piriformospora indica* affects plant growth by auxin production. *Physiol. Plant.* **131**, 581–589.
- Soytong, K., Kanokmedhakul, S., Kukongviriyapan, V., and Isobe, M. 2001. Application of *Chaetomium* species (Ketomium®) as a new broad spectrum biological fungicide for plant disease control: A review article. *Fungal Divers.* **7**, 1–15.
- Stierle, A., Strobel, G., and Stierle, D. 1993. Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Science* **260**, 214–216.
- Stone, J.K., Polishook, J.D., and White Jr., J.F. 2004. Endophytic fungi, pp. 241–270. In Mueller, G.M., Bills, G.F., and Foster, M.S. (eds.), *Biodiversity of Fungi – Inventory and Monitoring Methods*. Elsevier Academic Press, Oxford, UK.
- Strobel, G.A. 2003. Endophytes as sources of bioactive products. *Microbes Infect.* **5**, 535–544.
- Sullivan, R.F. and White Jr, J.F. 2000. *Phoma glomerata* as a mycoparasite of powdery mildew. *Appl. Environ. Microbiol.* **66**, 425–427.
- Sundheim, L. 1982. Control of cucumber powdery mildew by the hyperparasite *Ampelomyces quisqualis* and fungicides. *Plant Pathol.* **31**, 209–214.
- Suryanarayanan, T.S., Thirunavukkarasu, N., Govindarajulu, M.B., Sasse, F., Jansen, R., and Murali, T.S. 2009. Fungal endophytes and bioprospecting. *Fungal Biol. Reviews* **23**, 9–19.
- Suryanarayanan, T.S., Wittlinger, S.K., and Faeth, S.H. 2005. Endophytic fungi associated with cacti in Arizona. *Mycol. Res.* **109**, 635–639.
- Swofford, D.L., Waddell, P.J., Huelsenbeck, J.P., Foster, P.G., Lewis, P.O., and Rogers, J.S. 2001. Bias in phylogenetic estimation and its relevance to the choice between parsimony and likelihood methods. *Syst. Biol.* **50**, 525–539.
- Syed, N.A., Midgley, D.J., Ly, P.K.C., Saleeba, J.A., and McGee, P.A. 2009. Do plant endophytic and free-living *Chaetomium* species differ? *Aust. Mycol.* **28**, 51–55.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739.
- Tanaka, A., Takemoto, D., Chujo, T., and Scott, B. 2012. Fungal endophytes of grasses. *Curr. Opin. Plant. Biol.* **15**, 1–17.
- U'Ren, J., Lutzoni, F., Miadlikowska, J., and Arnold, A.E. 2010. Community analysis reveals close affinities between endophytic and endolithic fungi in mosses and lichens. *Microb. Ecol.* **60**, 340–353.
- Unterseher, M. and Schnittler, M. 2009. Dilution to extinction cultivation of leaf inhabiting endophytic fungi in beech (*Fagus sylvatica* L.) – Different cultivation techniques influence fungal biodiversity assessment. *Mycol. Res.* **113**, 645–654.
- Unterseher, M. and Schnittler, M. 2010. Species richness analysis and ITS rDNA phylogeny revealed the majority of cultivable foliar endophytes from beech (*Fagus sylvatica*). *Fungal Ecol.* **3**, 366–378.
- Vazquez-Garciduenas, S., Leal-Morales, C.A., and Herrera-Estrella, A. 1998. Analysis of the beta-1,3-glucanolytic system of the biocontrol agent *Trichoderma harzianum*. *Appl. Environ. Microbiol.* **64**, 1442–1446.
- Vega, F.E., Simpkins, A., Aime, M.C., Posada, F., Peterson, S.W., Rehner, S.A., Infante, F., Castillo, A., and Arnold, A.E. 2010. Fungal endophyte diversity in coffee plants from Colombia, Hawai'i, Mexico and Puerto Rico. *Fungal Ecol.* **3**, 122–138.
- Wang, B., Priest, M.J., Davidson, A., Brubaker, C.L., Woods, M.J., and Burdon, J.J. 2007. Fungal endophytes of native *Gossypium* species in Australia. *Mycol. Res.* **111**, 347–354.
- White, T.J., Bruns, T.D., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. (eds.), *PCR Protocols: a guide to methods and applications*. Academic Press, New York, N.Y., USA.
- White Jr, J.F. and Torres, M.S. 2010. Is plant endophyte-mediated defensive mutualism the result of oxidative stress protection? *Physiol. Plant.* **138**, 440–446.
- Yang, Z. and Rannala, B. 1997. Bayesian phylogenetic inference using DNA sequences: a Markov Chain Monte Carlo Method. *Mol. Biol. Evol.* **14**, 717–724.
- Zhang, Y., Mu, J., Feng, Y., Kang, Y., Zhang, J., Gu, P.J., Wang, Y., Ma, L.F., and Zhu, Y.H. 2009. Broad-spectrum antimicrobial epiphytic and endophytic fungi from marine organisms: isolation, bioassay and taxonomy. *Mar. Drugs* **7**, 97–112.
- Zhang, D.X., Nagabhyru, P., and Schardl, C.L. 2009. Regulation of a chemical defense against herbivory produced by symbiotic fungi in grass plants. *Plant Physiol.* **150**, 1072–1082.
- Zhang, H.W., Song, Y.C., and Tan, R.X. 2006. Biology and chemistry of endophytes. *Nat. Prod. Rep.* **23**, 753–771.
- Zhou, D. and Hyde, K.D. 2001. Host-specificity, host-exclusivity, and host-recurrence in saprobic fungi. *Mycol. Res.* **105**, 1449–1457.
- Zubek, S., Piatek, K., Naks, P., Heise, W., Wayda, M., and Mleczko, M. 2010. Fungal root endophyte colonization of fern and lycophyte species from the Celaque National Park in Honduras. *Am. Fern J.* **100**, 126–136.