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Diversity of endophytic fungi in *Glycine max*

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ABSTRACT

Endophytic fungi are microorganisms that live within plant tissues without causing disease during part of their life cycle. With the isolation and identification of these fungi, new species are being discovered, and ecological relationships with their hosts have also been studied. In *Glycine max*, limited studies have investigated the isolation and distribution of endophytic fungi throughout leaves and roots. The distribution of these fungi in various plant organs differs in diversity and abundance, even when analyzed using molecular techniques that can evaluate fungal communities in different parts of the plants, such as denaturing gradient gel electrophoresis (DGGE). Our results show there is greater species richness of culturable endophytic filamentous fungi in the leaves *G. max* as compared to roots. Additionally, the leaves had high values for diversity indices, i.e. Simpsons, Shannon and Equitability. Conversely, dominance index was higher in roots as compared to leaves. The fungi *Ampelomyces* sp., *Cladosporium cladosporioides*, *Colletotrichum gloeosporioides*, *Diaporthe helianthi*, *Guignardia mangiferae* and *Phoma* sp. were more frequently isolated from the leaves, whereas the fungi *Fusarium oxysporum*, *Fusarium solani* and *Fusarium* sp. were prevalent in the roots. However, by evaluating the two communities by DGGE, we concluded that the species richness was higher in the roots than in the leaves. UPGMA analysis showed consistent clustering of isolates; however, the fungus *Leptospora rubella*, which belongs to the order Dothideales, was grouped among species of the order Pleosporales. The presence of endophytic *Fusarium* species in *G. max* roots is unsurprising, since *Fusarium* spp. isolates have been previously described as endophyte in other reports. However, it remains to be determined whether the *G. max Fusarium* endophytes are latent pathogens or non-pathogenic forms that benefit the plant. This study provides a broader knowledge of the distribution of the fungal community in *G. max* leaves and roots, and identifies the genetic relationships among the isolated species.

1. Introduction

Endophytic fungi are microorganisms found inside plant species (Stone et al., 2000) and may play a key role in plant development by controlling phytopathogens and herbivorous insects or producing growth-promoting substances (Neto et al., 2002). Endophytic fungi also have functions related to saprophytic microorganisms, and many species are involved in the process of maturation and leaf decomposition (Promputtha et al., 2007; Sieber, 2007). In addition, these fungi produce several enzymes responsible for the decomposition of plant organic matter (Sunitha et al., 2013).

The endophytic organisms live in protected environments that provide a competitive advantage over the microorganisms present in the rhizosphere and phyllosphere and are beneficial for nutrient flow, pH and humidity (Backman and Sikora, 2008). In turn, endophytes can guarantee greater host resistance to biotic and abiotic stresses, including water deficits, salinity, and elevated heavy metal concentrations in the soil. Furthermore these mutualists can protect plants against the effects of herbicides, herbivory and phytopathogens, and they may also act to stimulate growth through induction of morphological, physiological and biochemical changes in their hosts (Bayat et al., 2009; Gundel et al., 2010).

Endophytic microorganisms found in plants include fungi (Larran et al., 2002; Leite et al., 2013; Tenguria and Firodiya, 2013; Nalini et al., 2014) and bacteria (Zinniel et al., 2002; Rosenblueth and Martínez-Romero, 2006; Jasim et al., 2014; Ji et al., 2014). For example, the density of endophytic bacteria in *Phaseolus vulgaris* leaves varied from 4.5×10^2 to 2.8×10^3 colony-forming units (CFU)/g^{-1} of fresh weight plant material (Costa et al., 2012) and, for endophytic fungi, colonization rates in leaves can vary up to

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90% in tropical forest trees (Lodge et al., 1996). These microorganisms penetrate into the plant tissue through the stomata, roots, and wounds and directly by secretion of hydrolytic enzymes (Esposito and Azevedo, 2004). Another method of endophytic propagation is by vertical transmission through seeds (Saikkonen et al., 2010; White et al., 1993) or horizontal transmission through spores (Rodriguez et al., 2009). Endophytic fungi and plants have been in association for over 400 million years (Krings et al., 2007).

Endophytic fungi are distinctly distributed throughout the organs and tissues of plants and are associated with various plant structures, such as leaves, branches, stems, roots, shoots (Porras-Alfaro and Bayman, 2011). Among the endophytic fungi isolated from different cultures, notoriously pathogenic species are commonly isolated. Alternatively, in some cases, the endophytes are not pathogenic and their presence can in fact, benefit the host plant as described for *Fusarium verticillioides* on maize (Lee et al., 2009) and non-pathogenic strains of *Fusarium oxysporum* on banana (Athman et al., 2006). In other cases, it was observed that some endophytic fungi are able to cause disease symptoms in the plant after long incubation period, indicating that they were a latent form of the pathogen, as observed by Photita et al. (2004) on banana plants infected by the fungus *Deightoniella torulosa*.

Soybean is a plant belonging to the genus *Glycine* that constitutes several species originating from the regions of Africa, East Asia and Australia, including the cultivated soybean species *Glycine max* (Sediyama, 2009). The reproductive stages in soybean consist of eight stages in four development phases: flowering (R1 and R2), pod development (R3 and R4), seed development (R5 and R6) and plant maturation (R7 and R8). The period represented by R9 corresponds to soybean harvest (Sediyama, 2009). In the 2014/2015 harvest, the world production of soybean reached 315.1 million tons with Brazil soybean production estimated at 94.4 million tons (USDA, 2015).

Dalal and Kulkarni (2014) found that the diversity and distribution of endophytic fungi in the soybean plant are influenced by the vegetative and reproductive stages. The age of the plant and type of cultivation environment, such as open fields and greenhouses, also contribute to the diversity of endophytic fungi in soybean (Pimentel et al., 2006). Studies on the fungal community in *G. max* showed that the method used for fungal isolation influences the abundance and richness of isolated species (Impullitti and Malvick, 2013; Leite et al., 2013).

An understanding of the endophytic fungi present in *Glycine max*, through direct isolation techniques or molecular measurement is important because it broadens knowledge of the distribution of these microorganisms in the plant. Importantly, this may reveal potential biological control agents against pathogens or help to source novel antimicrobial compounds. In this study, our objectives were therefore to (1) to isolate and identify genetically and morphologically filamentous endophytic fungi of *G. max* leaves and roots, (2) perform the grouping among species using molecular techniques, (3) investigate variation in the diversity and distribution of the endophytic fungi population of *G. max* leaves and roots through culture-dependent and culture-independent approaches.

2. Materials and methods

2.1. Source of the G. max plant samples

Ten whole plants of the cultivar Monarca® in the R2 stage of development were collected for removal of leaves and roots at the experimental field Diogo Alves de Mello at the Universidade Federal de Viçosa – UFV, Brazil (20°46′0.47″ S and 42°52′10.8W) from random points covering the entire cultivated area of 10.000 m^2, which had not previously been exposed to any pesticide.

2.2. Isolation of endophytic fungi through dilution-to-extinction culturing

In total, 50 leaves were selected randomly while all root systems were harvested. These were washed to remove soil residue and dust, then, 10 g each of leaves and roots were disinfected by soaking in 70% ethanol for 1 min followed by 3% sodium hypochlorite (NaOCl) for 2 min. After rinsing to remove excess NaOCl, the samples were pressed on potato dextrose agar (PDA-Himedia) culture medium for 5 s to assess the efficacy of surface disinfection (Schulz et al., 1993, 1998, 1999). Samples that did not incur any growth on the culture medium after 10 days (photoperiod of 12 h, 27 °C) were considered surface-sterile and used for the isolation of endophytic fungi.

Each plant sample type was pooled for the isolation of endophytic fungi according to the protocols developed by Paulus et al. (2003), Collado et al. (2007), Unterseher and Schnittler (2009) and Leite et al. (2013) with modifications. Ten grams of each leaves and roots were added to a 0.85% NaCl solution (w/v) and homogenized in a blender for 60 s. The resulting suspension was filtered through 500 and 106 μm sterile sieves. The plant pieces retained on the 106 μm sieve were collected and added to a test tube containing 30 mL 0.85% saline solution. Then, 300 μL of the supernatant was plated onto PDA, and 15 Petri dishes with culture medium and each plant sample were established. The culture medium was supplemented with 50 mg/L streptomycin sulfate (Sigma) and 50 mg/L tetracycline (Sigma) to avoid isolating endophytic bacteria. Next, the Petri dishes were placed in a growth chamber with a photoperiod of 12 h at 27 °C for 10 days. The fungi were collected from fifth to tenth days and transferred to corn meal agar (Himedia) for sporulation and PDA medium for DNA extraction.

2.3. DNA extraction, amplification and sequencing of the rDNA ITS region of the filamentous fungi

After seven days of fungal growth on PDA, the DNA of each isolate was extracted using the UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories) as per manufacturer instruction.

Fungal DNA was analyzed by sequencing the internal transcribed spacer (ITS) region of the rDNA, which utilized the universal primers ITS 1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990; Gardes and Bruns, 1993), and the fragment corresponding to the ITS-1, 5.8S and ITS-2 region was amplified by PCR using an Eppendorf Mastercycler® thermal cycler (Eppendorf, Germany) programmed to perform an 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, with a final extension at 72 °C for 7 min.

The PCR mixture consisted of 8.75 ng of total DNA, 1× Colorless Go Taq®Flexi Buffer, 1 mM $MgCl_2$, 0.1 mM dNTPs, 0.2 μM ITS1F primer, 0.2 μM ITS4 primer, 0.2 units Go Taq® DNA Polymerase (Promega, Madison, USA) to a total reaction volume of 25 μL. The negative controls replaced DNA with MilliQ water to evaluate the presence of contaminants. All materials used for the preparation of reactions were sterile and nuclease free. After amplification, the PCR products were analyzed by electrophoresis on a 1.2% agarose gel stained with ethidium bromide (0.5 ng/mL agarose) by stirring gently for 15 min; the products were then visualized under UV light. PCR reactions were sent to Macrogen (Seoul/South Korea) for sequencing.

2.4. Identification, clustering analysis and diversity indexes of the filamentous fungi

All the nucleotide sequences of the ITS region (ITS1, 5.8S and ITS2) of each isolate were aligned using the software DNA Baser®

3.5.3. Nucleotide sequences obtained from the fungal isolates were identified by comparison against the GenBank database using a local alignment algorithm for nucleotide sequences (BlastN) (Altschul et al., 1990). The database sequences that exhibited the highest identity, query and score and the lowest *e*-values relative to our sequences allowed us to identify fungi to genus or species level. Identifications were confirmed by morphological taxonomy. The clustering analysis was constructed by importing the sequences of each fungal species into the MEGA 6.06® software and aligning them with reference sequences from GenBank using the ClustalW software. The Tamura-Nei substitution model and Unweighted Pair Group Method using Arithmetic Averages (UPGMA) analysis were used. The robustness of the clustering analysis was tested by bootstrap analysis (≥50%) using a total of 1000 interactions (Tejesvi et al., 2010).

The species diversity of the endophytic fungi was measured using the diversity indices whish parameters are species richness and relative species abundance. The species evenness, which assesses the contribution of the individuals to the community, was also calculated.

The diversity indices used for the isolates were the Shannon–Wiener index (H') (formula: $\sum[(n_i/n)\ \ln(n_i/n)]$), Simpson's diversity index ($1-D$) (formula: $1-[D=\sum(n_i/n)^2]$) and Simpson's dominance index (D), where n_i is the number of distinct species (i) and (n) is the abundance of each species in the community. The equitability was calculated by the following formula: $E = H'/H_{max}$, where H' is the Shannon–Wiener index and H_{max} is the ln of n_i. The diversity analyses were performed using the software Past® version 2.15 (Hammer et al., 2001).

2.5. Morphological taxonomy

The identification of different fungi was primarily by molecular taxonomy. However, when there were uncertainties regarding identification, morphological taxonomy was also used. For this, morphological characteristics of the isolate were examined on corn meal agar (Himedia). Then, colony fragments were removed from the margin of developing cultures, transferred to slides containing lactophenol and visualized under a light microscope (Dialux 22EB, Leitz). Morphological characteristics of the isolate were then compared with previous descriptions available in the literature and taxonomic keys (Barnett and Hunter, 1972; Sutton, 1980; Hanlin, 1998; Seifert et al., 2011).

2.6. Preparation of plant samples and extraction of total DNA for diversity analysis using DGGE

The samples and surface disinfection was performed as described for the isolation of endophytic fungi.

The total DNA was extracted from leaves and roots with the commercial kit Nucleospin Soil® (Macherey-Nagel) according to the manufacturer's recommendations. The DNA was then quantified using a Qubit 2.0® Fluorometer (Invitrogen) and standardized to 10 ng/μL DNA.

2.7. Partial amplification of the SSU rDNA region for DGGE analysis

The specific fungal primers FF390 (5′-CGA TAA CGA ACG AGA CCT-3′) and FR1 GC (5′-CCC CCG CGC GCG GGC GGG GCG GGG GCA CGG GCC GAI CCA TTC AAT CGG TAI T-3′) (I = Inosine) described by Vainio and Hantula (2000) were used to amplify part of the SSU (small subunit or 18S) rDNA region. Three reactions of each sample (leaf and root) were conducted separately with the proper modifications required for each reaction. The amplifications were performed directly with the primer containing the GC clamp (nested) using the Eppendorf Mastercycler® thermal cycler (Eppendorf, Germany) programmed to perform an initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The 50 μL amplification reaction contained: 1× Colorless Go Taq® Flexi Buffer, 0.25 mM $MgCl_2$, 0.1 mM dNTPs, 0.2 μM FF390 primer, 0.2 μM FR1 GC primer, 0.5 mg mL^{-1} of bovine serum albumin (BSA), 2.0 μL DMSO, 1.0 μL formamide, Go Taq® DNA polymerase 1.25 units (Promega, Madison, USA), 10 ng of total genomic DNA for each sample. The negative controls consisted of PCR reaction without genomic DNA to evaluate the presence of contaminants. All materials used in the preparation of the reactions were sterile and nuclease free. After amplification, the PCR products were analyzed by gel electrophoresis on a 1.2% agarose gel.

2.8. Comparative analysis between leaves and roots by DGGE

DGGE was performed in a C.B.S. Scientific® model 240 DGGE system for access the endophytic fungal communities from leaves and roots. The electrophoresis was performed on a 1-mm thick 6% polyacrylamide gel, with an acrylamide–bisacrylamide ratio of 37.5:1, and the vertical denaturing gradient of urea and formamide from 35% to 55%. The running buffer was 1× TAE (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.4). Approximately, 50 ng of PCR product was used for the DGGE analysis and mixed with the same volume of staining buffer (2% bromophenol blue, 2% xylene cyanol, 100% glycerol). The samples were separated at a constant temperature of 60 °C for 16 h at 100 V, stained with SYBR Gold (50 μg/mL) (Invitrogen) by stirring gently for 15 min, and visualized under UV light.

The DGGE profiles were analyzes using the software Bionumerics® 5.1 (Applied Maths, Kortrijk, Belgium). Similarities in the banding patterns among the three replicates of leaves and roots were calculated based on the presence and absence of bands and expressed as a coefficient of similarity. The Dice similarity coefficient was used to compare the DGGE profiles. This similarity coefficient was calculated using the following formula: $D_{sc} = [2j/(a+b)]$, where a = number of bands or OTUs (operational taxonomic units) in DGGE on lane 1, b = number of bands on lane 2, j = number of common bands in lanes 1 and 2, and $D_{sc} = 1$ indicates an identical profile. Dendrograms showing a cluster were constructed using the UPGMA. Diversity of the fungal communities was analyzed using the Richness index (S). Variable richness was estimated based on the binary matrix that was generated by software BioNumerics® version 5.1. BioNumerics® analyzes a scanned image of the gel where the presence of a band is coded as one (1) and absence as zero (0).

3. Results

3.1. Identification and characterization of the filamentous endophytic fungi from G. max

A total of 229 isolates were obtained, with the majority (187 isolates) originating from the leaves and 42 isolated from the root (Table 1). Amplification of ITS rDNA region generated DNA fragments ranging from 530 to 908 bp in size. A list of the endophytic fungi isolated with their accession number (NCBI), genetic similarity to the sequences deposited (identity), accuracy (query) and origin can be found in Supplementary Material 1.

The genetic identity was considered co-specific for the majority of the isolates when 99% and 100% identities were reached; the only exceptions were *Peyronellaea prosopidis* (98% identity) and *Saccharicola bicolor* (96% identity). All the isolate characterizations were confirmed morphologically whenever reproductive

Table 1
Endophytic fungi isolated from the leaves and roots of *G. max*.

Isolated species	Leaves (%)*	Roots (%)*	Total (%)**	Order
Alternaria arborescens	4(2.13)	–	4(1.47)	Pleosporales
Alternaria sp.	–	1(2.38)	1(0.43)	Pleosporales
Alternaria tenuissima	–	1(2.38)	1(0.43)	Pleosporales
Ampelomyces sp.	17(9.09)	–	17(7.42)	Pleosporales
Cercospora apii	1(0.53)	–	1(0.43)	Capnodiales
Cercospora beticola	1(0.53)	–	1(0.43)	Capnodiales
Cercospora guatemalensis	2(1.06)	1(2.38)	3(1.31)	Capnodiales
Cercospora piaropi	4(2.13)	–	4(1.47)	Capnodiales
Cercospora sp.	2(1.06)	–	2(0.87)	Capnodiales
Cercospora zebrina	1(0.53)	–	1(0.43)	Capnodiales
Chaetomium funicola	–	1(2.38)	1(0.43)	Sordariales
Chaetomium sp.	–	1(2.38)	1(0.43)	Sordariales
Cladosporium cladosporioides	10(5.34)	–	10(4.36)	Capnodiales
Cladosporium gossypiicola	1(0.53)	–	1(0.43)	Capnodiales
Cladosporium sp.	1(0.53)	–	1(0.43)	Capnodiales
Cochliobolus geniculatus	1(0.53)	1(2.38)	2(0.87)	Pleosporales
Colletotrichum boninense	6(3.20)	–	6(2.62)	Hypocreales
Colletotrichum fruticola	2(1.06)	–	2(0.87)	Hypocreales
Colletotricum gloeosporioides	23(12.29)	–	23(10.04)	Hypocreales
Colletotrichum karstii	1(0.53)	–	1(0.43)	Hypocreales
Coniothyrium sp.	1(0.53)	–	1(0.43)	Pleosporales
Coprinellus radians	2(1.06)	–	2(0.87)	Agaricales
Curvularia sp.	2(1.06)	–	2(0.87)	Pleosporales
Curvularia trifolii	–	1(2.38)	1(0.43)	Pleosporales
Diaporthe heveae	1(0.53)	–	1(0.43)	Diaporthales
Diaporthe helianthi	13(6.95)	–	13(5.67)	Diaporthales
Diaporthe phaseolorum	3(1.60)	–	3(1.31)	Diaporthales
Fusarium oxysporum	2(1.06)	21(50.00)	23(10.04)	Hypocreales
Fusarium solani	–	8(19.04)	8(3.49)	Hypocreales
Fusarium sp.	–	3(7.14)	3(1.31)	Hypocreales
Gibberella zeae	1(0.53)	–	1(0.43)	Hypocreales
Guignardia mangiferae	6(3.20)	–	6(2.62)	Botryosphaeriales
Leptosphaeria sp.	2(1.06)	–	2(0.87)	Pleosporales
Leptospora rubella	1(0.53)	–	1(0.43)	Dothideales
Macrophomina phaseolina	–	1(2.38)	1(0.43)	Botryosphaeriales
Myrothecium inundatum	1(0.53)	–	1(0.43)	Hypocreales
Myrothecium sp.	1(0.53)	–	1(0.43)	Hypocreales
Neofusicoccum parvum	2(1.06)	–	2(0.87)	Botryosphaeriales
Nigrospora oryzae	1(0.53)	–	1(0.43)	Trichosphaeriales
Peyronellaea prosopidis	1(0.53)	–	1(0.43)	Pleosporales
Pestalotiopsis sp.	1(0.53)	–	1(0.43)	Xylariales
Pestalotiopsis vismiae	1(0.53)	1(2.38)	2(0.87)	Xylariales
Phoma glomerata	5(2.67)	–	5(2.18)	Pleosporales
Phoma pomorum	1(0.53)	–	1(0.43)	Pleosporales
Phoma sp.	55(29.41)	1(2.38)	56(24.45)	Pleosporales
Phomopsis sp.	4(2.13)	–	4(1.47)	Diaporthales
Saccharicola bicolor	1(0.53)	–	1(0.43)	Pleosporales
Stagonosporopsis cucurbitacearum	2(1.06)	–	2(0.87)	Pleosporales
Total	187(81.65%)	42(18.34%)	229	

* Isolated fungi followed by the specified percentage of each environment (leaf or root).
** Sum total of isolated fungi in both environments followed by the total percentage in both environments.

structures could be confirmed. Six isolates were identified as *Phoma* sp. by ITS sequencing and did not produce reproductive structures when cultured on corn meal agar. In this case, identification was based solely on ribosomal sequences. All the fungal sequences corresponding to the rDNA-ITS regions obtained in this study and their taxonomic identifications were deposited in GenBank (Table 1 and Supplementary Material 1).

3.2. Analysis of diversity and clustering

All 229 isolates (Table 1) belonged to species of the phyla Ascomycota and Basidiomycota, and the isolates were represented by three classes, namely, Agaricomycetes (2 isolates, 0.87%), Dothideomycetes (132 isolates, 57.64%) and Sordariomycetes (95 isolates, 41.48%), and were distributed over 11 orders, including Agaricales (2 isolates), Botryosphaeriales (9 isolates), Capnodiales (24 isolates), Diaporthales (21 isolates), Dothideales (1 isolate), Hypocreales (70 isolates), Pleosporales (92 isolates), Sordariales (2 isolates), Trichosphaeriales (1 isolates), and Xylariales (3 isolate). In the leaves, the most prevalent order of fungi was Pleosporales, whereas in the roots, Hypocreales predominated. Members of orders of fungi listed above were present in the leaves, with the exception of the Sordariales, which was only found in the roots (Fig. 1). In leaves, the most abundant genera and species were *Phoma* sp. (29.41%), followed by *Colletotrichum gloeosporioides* (12.29%), *Ampelomyces* sp. (9.09%) and *Diaporthe helianthi* (6.95%). In the roots, the most abundant fungi were *F. oxysporum* (50%), followed by *Fusarium solani* (19.04%) (Table 1).

The diversity of endophytic fungi was observed at the level of their richness (number of taxa = *S*) and abundance (number of isolates or individuals), and there were differ between the tissues analyzed. There was a greater species richness and abundance in the leaves as compared to roots (*S* = 40 vs. 13, individuals = 187 vs. 42). The Simpson's, Shannon–Wiener, Dominance, and Equitability of fungi isolated in the leaves and roots were calculated. Highest diversity values were found in leaves compared to roots: Simpson

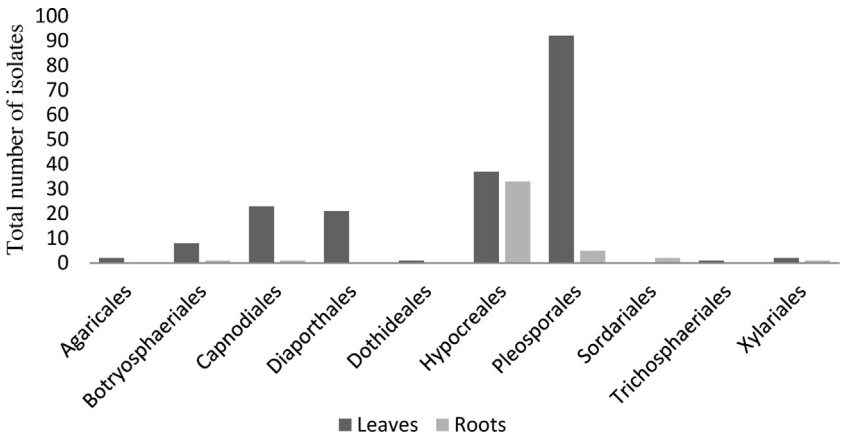

Fig. 1. Distribution of the endophytic fungal orders isolated from the leaves and roots of *Glycine max*.

Table 2
Analysis of the diversity of fungal isolates from *G. max*.

	Leaves	Roots
Taxa *S*	40	13
Individuals	187	42
Dominance *D*	0.123	0.297
Simpson 1 – *D*	0.876	0.702
Shannon *H*	2.777	1.741
Equitability *J*	0.752	0.678

Diversity indices calculated by software PAST version 2.5.

Table 3
Richness from *G. max* leave and roots analysis by DGGE profile.

	Leaves			Roots		
	L1	L2	L3	R3	R2	R1
Richness	6	6	5	13	12	14

The richness was analyzed by the presence of bands in the electrophoretic profile on DGGE gel and was obtained using binary matrix generated by gel analysis in BioNumerics 5.1 software. L1 to L3 are repetitions of the leaves and R1 to R3 are repetitions of the roots.

index (0.876 and 0.702), Shannon–Wiener index (2.777 and 1.741) and Equitability (0.752 and 0.678). Only the Dominance index had a value where roots were higher than leaves (0.297 and 1.123) (Table 2).

The clustering analysis shows the relationship between the different species isolated from the leaves and roots of *G. max* (Fig. 2). The clustering analysis was constructed using as out-group a fungus belonging to the Basidiomycota (*Psilocybe cubensis*) because most endophyte isolates obtained from *G. max* (99.12%) belonged to the Ascomycota. The values listed from left to right next to each cluster node correspond to the bootstrap values performed using 1000 replications, and the branches shown correspond to values ≥70%, being fungi grouped according to their Order. In this clustering analysis, the fungus *Leptospora rubella* (Dothideales order) was clustered among the species of the order Pleosporales, showing a strong genetic closeness to this order.

3.3. Analysis of the DGGE profiles

The molecular profiles of the *G. max* endophytic fungi obtained by amplification of the SSU ribosomal region are shown in Fig. 3. Richness analysis was performed by evaluating the number of bands in each sample (leaves or roots) by DGGE to indicate variability in the population of fungi between the leaves and roots of the plant. In total 17 bands were found in the leaf samples and 39 bands in the roots. However, four abundant bands were identified among replicates from leaves and twelve abundant bands from roots (Fig. 3 and Table 3). The cluster analysis performed by the UPGMA method showed low similarity between the two clades (leaves and roots), although in the leaves, there was greater similarity and uniformity than in the roots (Fig. 3).

4. Discussion

To study and identify endophytic representatives of this diverse group fungal, it is important to use molecular techniques and classical taxonomy (Garro et al., 1999; Sun and Guo, 2012). The use of molecular techniques alone, such as a sequence analysis of the ITS region, can be hampered by the presence of ambiguous sequences that were deposited in genomic databases, as observed by Yamamoto and Bibby (2014), who used Genbank sequences to show that 15.3% of the fungal rDNA ITS sequences analyzed were ambiguous as to the fungal species. However, the rDNA ITS region is regarded as the best region for identification at the fungal species level and was designated as a fungal barcode (Schoch et al., 2012). In turn, identification using morphological taxonomy only is hampered by the presence of a vast diversity of endophytic fungi. Many of the isolated endophytic fungi do not sporulate in culture and are therefore designated as *Mycelia sterilia*.

In our study, we used morphological taxonomy to confirm the molecular taxonomy and distinguish situations where the same sequence showed high levels of identity with sequences described for both sexual and asexual stages of some fungal species, including for asexual stage *C. gloeosporioides* from the sexual stage *Glomerella cingulata*, asexual stage *Phyllosticta capitalensis* from the sexual stage *G. mangiferae*, and sexual stage *Botryosphaeria parva* from the asexual form *Fusicoccum parvum*. In another example, the sequences showed 100% identity with sequences belonging to different genera of fungi, such as *Curvularia* sp., *Bipolaris* sp. and *Drechslera* sp., and in these situations, it was necessary to use morphological taxonomy to accurately identify the genus and/or species isolated.

The successful isolation of endophytic fungi requires the correct disinfection of the surface of the leaves to eliminate epiphytic microorganisms (Sun and Guo, 2012). Disease-free plants were used and at the time of disinfection, it was ensured that no symptoms of microbial diseases were present in the leaves or roots. The disinfection process indicated that all fungal isolates were of endophytic origins. On another note, the size of the plant fragments and number of samples influence the isolation and diversity of the isolated fungal community (Sun and Guo, 2012); however, it is difficult to establish a relationship between the isolated fungi and those described in other studies because many factors can influence

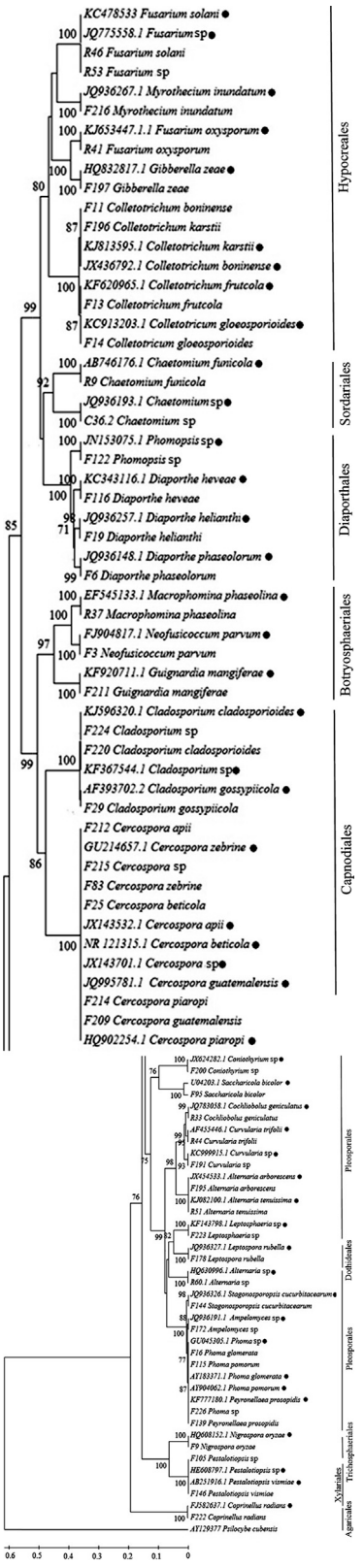

Fig. 2. UPGMA cluster analysis of endophytic fungi associated with *Glycine max*. Cluster analysis was inferred using the UPGMA method, showing the relationship between endophytic fungi based on the ITS1, 5.8S and ITS2 region of rDNA of all taxa isolated. The evolutionary distances were calculated using the Tamura-Nei model. An individual of each taxon isolated was used in the construction of the clustering together with a reference sequence retrieved from GenBank (●). The Bootstrap numbers shown on the left (≥70%) were obtained using 1000 replicates. The fungus *Psilocybe cubensis* was used as outgroup for the construction of the tree. The horizontal scale indicates the level of dissimilarity.

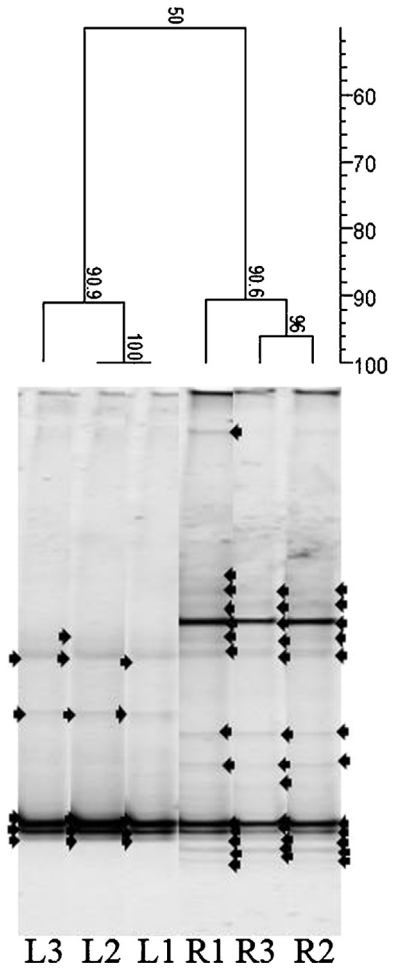

Fig. 3. Electrophoretic profile of ribosomal sequences corresponding to part of the small subunit of rRNA (SSU) of *G. max* leaves and roots obtained by Denaturing Gradient Gel Electrophoresis (DGGE). The profiles were obtained after amplification using oligonucleotides (FR1GC and FF390) specific to fungi (Vainio and Hantula, 2000). Were collected 10 whole plants, randomly, and a total of 50 leaves and all roots were randomly separated from plants and disinfected. Ten grams of each sample was used for extraction and amplification of DNA. All the process was repeated 3 times (L1, L2 and L3 for leaves and R1, R2 and R3 for roots. The dendrograms were generated based on the UPGMA method by Bionumerics 5.1 with denaturing gradient from 35% to 55%. The vertical scale indicates the level of similarity. The black arrows show bands.

isolation, such as the sample number, plant age and nutritional and plant water status.

In this study and previous studies on the isolation and identification of endophytic fungi from soybean leaves, genera such as *Alternaria* sp., *Cladosporium* sp., *Curvularia* sp., *Nigrospora* sp., *Phomopsis* sp. (Larran et al., 2002; Leite et al., 2013) and *Colletotrichum* sp. (Pimentel et al., 2006; Leite et al., 2013; Tenguria and Firodiya, 2013) were isolated. Among these genera, the species of the genus *Alternaria* were also present among the isolates of leaf endophytic fungi in several plant species including plants with medicinal qualities (Anitha et al., 2013; Goveas et al., 2011); *Pinus halepensis* (Botella and Diez, 2011); *Cannabis sativa* (Gautam et al., 2013); *Taxus globosa* (Rivera-Orduña et al., 2011); *Bletilla ochracea* (Tao et al., 2008) and algae (Zuccaro et al., 2008).

In the roots, there was a dominance of the order Hypocreales, and the species were represented by *F. oxysporum* (50%), *F. solani* (19.04%) and *Fusarium* sp. (7.14%) (Fig. 1). *F. oxysporum* was also isolated from leaves with low abundance, accounting for 1.06% of the total species isolated in the leaves (Table 1). Pathogenic fungi are commonly isolated following endophyte

extraction processes. The *Fusarium* case is particularly interesting because non-pathogenic isolates are frequently found as endophytes from different plants (Gotz et al., 2006; Sun et al., 2014). *Fusarium* species has been isolated from various parts of the soybean plant (Impullitti and Malvick, 2013; Kumar and Kaushik, 2013; Leite et al., 2013; Pimentel et al., 2006; Rathod and Pawar, 2013; Roy et al., 2000). However, it undetermined whether these isolates are non-pathogenic and beneficial to the plant or if they are latent pathogens.

Endophytic *Fusarium* strains obtained in our study should be further tested for their ability to produce disease symptoms in *G. max* and whether they protect the plant against pathogenic isolates. However, the use of non-pathogenic *Fusarium* endophytes to control pathogens must be treated with care. Ma et al. (2010) showed the transfer of just two chromosomes between *F. oxysporum* strains can create a pathogenic strain from a non-pathogen. The mechanism responsible for the transfer of these chromosomes is currently unclear. However, it is important to consider that exchange can occur between two *F. oxysporum* isolates colonizing the same plant.

The diversity indices calculated for endophytic fungi isolates of *G. max* leaves and roots are shown in Table 2. The Simpson's index estimates the probability that two randomly selected individuals from a community belong to different species (Simpson, 1949). In our results, the index calculated was greater for the leaves than that for the roots (0.876 and 0.702). The Shannon–Wiener index assesses species richness and relative species abundance, ranging from 0 in communities with only one species to high values in communities with many species, which comprise a few individuals each. The highest index value was found in the leaves rather than in roots (2.777 and 1.741), demonstrating the leaves have a more diverse fungal community compared the roots. Equitability reflects the contribution of an individual to the community, ranging between 0 and 1, where 1 represents maximum equitability (i.e. when the proportion of all species is similar). Again, the highest value was found in the leaves (0.752) than in roots (0.678). This suggests that there is greater uniformity in the leaves than in roots. For the Dominance index, which refers to the dominance of one or more species in a given community, the roots had a higher calculated index than the leaves (0.297 and 0.123, respectively). This is likely due to the isolation of *F. oxysporum* and *F. solani* in the roots (Table 1).

The DGGE technique can be can be widely used, including for studying changes in microbial communities in natural environments and environments subjected to stress conditions or for monitoring specific microorganisms in a natural environment (Rosado and Duarte, 2002). In addition to its use in characterizing complex communities, DGGE is also applied to the monitoring of microorganisms from environmental samples to track the dynamics of specific populations according to environmental changes or operational conditions of a system (Duong et al., 2006; Kirk et al., 2004; Vainio and Hantula, 2000; Xiaoxu and Fuqiang, 2011). The use of the FF390 and FRCG primers generated fragments of approximately 400 bp of the SSU region, which is a desirable size for good resolution on a DGGE gel, beyond these primers are specific for fungi (Vainio and Hantula, 2000). Unlike the observation of greater fungal richness and diversity in the leaves than in the roots for the cultivable fungi isolated in this study, the analysis of the DGGE results through the evaluation of bands suggests the opposite, i.e. greater fungal species richness in the roots compared to the leaves (17 bands in the leaves and 39 bands in the roots) (Table 3). The identification of these bands (or OTUs—operational taxonomic units) would be necessary to determine the species that are present in these communities as well as their distribution.

As the roots are in an environment where populations of fungi are numerous, is possible that this interferes with the increased richness values obtained when roots are compared to the leaves by DGGE. According to Asan et al. (2010), high concentrations of fungi were isolated from soil samples, i.e., 170.6×10^4 CFU/g, compared to just 737 CFU from air samples. Additionally, this study isolated 33 species belonging to 16 genera in soil with the most isolated genus being *Penicillium*, followed by *Fusarium* and *Aspergillus*. Comparatively, in air samples, the most isolated genus was *Alternaria*, followed by *Cladosporium* and *Phoma*.

A comparison between the endophytic fungi populations isolated from the leaves and roots of the soybean plant revealed a tendency of certain species of fungi to colonize different parts of the plant. *Phoma* sp. (29.41%), *C. gloeosporioides* (12.29%) and *Ampelomyces* sp. (9.09%) were the most predominant fungi in leaves, whereas *F. oxysporum* (50%) and *F. solani* (19.04%) were the most predominant in the roots. The leaves had the highest species richness for the isolated fungi compared to the roots ($S = 40$ and 13), however, the richness between the leaves and roots analyzed through the isolation of endophytic fungi and molecular profiles generated by DGGE showed inverse values. For example, the richness index generated by the number of bands showed greater value in the roots than in the leaves ($S = 39$ and 17). In addition, the UPGMA analysis of DGGE profile showed the formation of distinct clustering for endophytic fungi community of roots and leaves. Thus, the differences between fungal populations of the leaves and roots are obvious and are shown by both fungal isolation and molecular profiling analysis. Notably, according Bayman (2006), the best strategy for endophytic fungi sampling is to use a combination of both methods.

Genera such as *Alternaria*, *Cercospora*, *Fusarium* and *Phomopsis* are frequently isolated from soybean, as demonstrated by Roy et al. (2000) and in our study; however, the genera *Ampelomyces*, *Cladosporium*, *Colletotrichum*, *Diaporthe*, *Guignardia* and *Phoma* are also fairly representative of the isolates in this study. According to Azevedo (2014), studies on the biodiversity of endophytic fungi in their hosts help develop strategies for future biotechnological applications, such as the production of enzymes by *Phoma* sp. and *Phomopsis* sp. (Suryanarayanan et al., 2012), antibiotics by *G. mangiferae* (Mei et al., 2012) and antitumor drugs by *Pestalotiopsis* sp. and *Phomopsis* sp. (Baker and Satish, 2012). Saikkonen et al. (2010) performed a literature review on the relationship of endophytic fungi and their hosts with herbivory and reported that the presence of endophytic fungi can increase resistance to herbivory through the production of alkaloids and/or mycotoxins. According to Porras-Alfaro and Bayman (2011), certain genera have a preference for particular regions of the plant, which was also observed in our study, as indicated by the high rates of *Fusarium* isolates (which have phytopathogenic species in the roots). As shown by Promputtha et al. (2010), genera such as *Colletotrichum* sp., *Fusarium* sp., *Phomopsis*, and *Leptosphaeria* and species such as *C. gloeosporioides* and *G. mangiferae* are important endophytic fungi that start producing various enzymes after leaf senescence, and each species or genus produces specific enzymes at a particular decomposition stage of the plant material; together, these activities complete a sequence of stages culminating in the degradation of the plant.

5. Conclusion

We conclude that the diversity observed following isolation of endophytic fungi in *Glycine max* is greater in leaves compared to roots. Conversely, the fungal communities sampled by DGGE analysis showed that the endophytic fungi community of leaves is different of roots. Among the endophytic fungi isolated from *G. max*, a high dominance was observed in the roots of *Fusarium* species, which can be worrisome because this species is causing large outbreaks of disease in plant species. For the isolated fungal

species, we found that molecular identification techniques based on ITS region and classical taxonomy of fungi isolated are complementary and together, these techniques allow for accurate identification of endophytic fungi.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2015.05.010

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