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Diversity of endophytic fungi in *Glycine max*

Elio Gomes Fernandes[a], Olinto Liparini Pereira[b], Cynthia Cânedo da Silva[a], Claudia Braga Pereira Bento[a], Marisa Vieira de Queiroz[a,*]

[a] Departamento de Microbiologia - Bioagro, Universidade Federal de Viçosa, Av. PH Rolfs s/n, Campus Universitário, CEP 36570 900 Viçosa, MG, Brazil
[b] Departamento de Fitopatologia, Universidade Federal de Viçosa, Av. PH Rolfs s/n, Campus Universitário, CEP 36570 900 Viçosa, MG, Brazil

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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

* Corresponding author. Tel.: +55 3138992971; fax: +55 3138992573.
E-mail address: mvqueiro@ufv.br (M.V.d. Queiroz).

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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®