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THE ROOT COLONIZING FUNGI OF THE TERRESTRIAL ORCHID *CYPRIPEDIUM IRAPEANUM*

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ABSTRACT. This study investigated the mycorrhizal status and the identification of the fungi colonizing the roots of the terrestrial orchid *Cypripedium irapeanu* by restriction fragment length polymorphisms and by rDNA internal transcribed spacer sequencing. The orchid is endemic of different regions in Mexico, Guatemala and Honduras; usually at 1400–2250 m. It grows mainly in the remaining oak forests of the highlands and it is in the Mexican red list of plants in danger. The oak forests in Mexico are threatened constantly. The microscopic examination of stained root segments of the orchid revealed the presence of fungal structures of both orchidoid fungi (pelotons and coiled hyphae) and dark septate endophytes (DSE) (melinized hyphae and microsclerotia). Analysis of ITS1–5.8–ITS2 region sequences suggested that mycorrhizal tissue was dominated by Tulasneaceae: *Sistotrema* sp., *Rhizoctonia solani*, and *Epulorhiza* sp. Among the DSE one isolate revealed 100% similarity to *Phomopsis* sp. XJ-05, and another one 99% to the fungal endophyte MUT 885 which are both reported as dark septate endophytes. The putative dark septate endophyte *Phomopsis* sp. XJ-05 was isolated not only from the roots but also from the germinated seeds of *C. irapeanu*.

RESUMEN. Este estudio investigó el status micorrízico y la identificación de los hongos que colonizan la raíz de la orquídea terrestre *Cypripedium irapeanu* por medio de los polimorfismos de los fragmentos de la restricción y la secuenciación del espacio transcrito interno del rDNA. Esta orquídea es endémica de diferentes regiones de México, Guatemala y Honduras localizándose entre los 1400 y 2250 m de altitud. Crece principalmente en los remanentes de los bosques de encino del altiplano y está en la lista roja de México de las plantas amenazadas. Los bosques de encino en este país están constantemente amenazados. El examen al microscopio de los segmentos de raíces teñidas de las orquídeas nos mostró la presencia de estructuras fúngicas tanto de hongos de micorriza orquidoide (pelotones e hifas enrolladas) como de los llamados endófitos oscuros septados (DSE) (hifas melinizadas y microesclerios). El análisis de la secuenciación de la región ITS1–5.6–ITS2 sugiere que el tejido micorrizado está dominado por Tulasneaceae: *Sistotrema* sp., *Rhizoctonia solani*, and *Epulorhiza* sp. Entre los DSE, uno de los 10 aislados mostró 100% de similitud con *Phomopsis* sp. XJ-05, y otro 99% con el endófito MUT 885, ambos reportados como endófitos oscuros septados. El hipotético endófito septado oscuro *Phomopsis* sp. XJ-05 fue aislado tanto de las raíces como de las semillas que germinaron de *C. irapeanu* restriction fragment length polymorphisms and by rDNA internal transcribed spacer sequencing.

KEY WORDS: terrestrial orchid, orchidoid mycorrhiza, dark septate endophytes, microsclerotia

Introduction. Symbiosis are particularly important for plants resulting in significant nutritional advantage. Among these are the mycorrhizae from which most plants obtain the majority of their nutrients, including those limiting their growth. In general for the terrestrial orchids the mycorrhizal association is fundamental for the plant during germination and throughout all its life (Smith & Read 1997).

Of all orchids that have been studied, few have been

the object of mycorrhizal studies. Among the Mexican terrestrial orchids just 3% of the orchids have been studied (Ortega-Larrocea & Rangel-Villafranco 2007).

Many species of terrestrial orchids are threatened or in danger due to the habitat loss by anthropogenic activities and the attractive beauty of its flowers (Dearnaley 2007). *Cypripedium irapeanu* (Fig. 1) grows mainly in the remaining oak forests of the Mexican highlands. The oak forests in Mexico are



FIGURE 1. *Cypripedium irapeanum* flower from Puebla State, Mexico.

threatened constantly by the urban activities and by the development of recreational sites. The change of soil use of the oak forests has conducted to the degradation by soil erosion and loss of these forests and have allow not only to the soil loss, and cosequently to the loss of the symbiotic fungi, but also to the decrease of the number of pollinators and to the increase of pathogens, specially the attack of the capsules by screw worms; other problems that this plant has to face in the degraded habitat are cattle, and weed invasion (Valdés *et al.* 2005).

Cypripedium irapeanum was originally described based on a collection from the mountains of Irapeo near the present city of Morelia in Michoacan, Mexico (Cribb & Soto Arenas 1993). In this country it is known as *pichohuastle*, a native name for the plant. The orchid is endemic of differents regions in Mexico (Chiapas, Durango, Guerrero, Jalisco, Michoacán, Morelos, Nayarit, Oaxaca, Sinaloa, Querétaro, Puebla, Veracruz), Guatemala and Honduras; usually at 1400-2250 m of altitud. The orchid is in the red list of plants in danger of the Mexican Department of Natural Resources (SEMARNAT 1995).

In relation to the temperate lady's slipper orchids, *Cypripedium*, there are few studies on its associated fungi. According to Shefferson *et al.* (2005), the genus *Cypripedium* is characterized by high specificity mycorrhizal association, then the lack of these fungi

may limit their establishment and distribution. Upon infection of this orchid by a compatible mycorrhizal fungus the seed ("dust seed") germinates into a seedling that consumes the fungal sugars, processus known as myco-heterotrophy. The plants may retain the myco-heterotrophy into adulthood (Gill 1989).

The internal transcribed spacer (ITS) regions of ribosomal DNA, including both ITS1 and ITS2, have been used extensively used for environmental sampling as a target because several taxonomic group-specific primer sets exist for this gene region (Gardes & Bruns 1993). The ITS has been the region of choice for molecular analysis of fungal communities of this region has been useful since Gardes & Bruns (1996) used its restriction digests (RFLP) to differentiate species of mycorrhizal fungi colonizing individual roots. Furthermore, the DNA sequences of the ITS1 and ITS2 are highly variable being a good marker to identify fungi to the genera and/or species level (Gardes & Bruns 1993, Gardes & Bruns 1996, Henrion *et al.* 1992, Smith *et al.* 2007).

Objective of this paper was the isolation and identification of the fungal root and seed endophytes as well as mycorrhizal fungi of *C. irapeanum* by restriction fragment length polymorphisms and by rDNA internal transcribed spacer sequencing. The term "endophyte" refers to those fungi that can be detected at a particular moment within the tissues of apparently healthy plant hosts (Schultz & Boyle 2005).

Materials and methods. Collected *C. irapeanum* is surviving in a remaining patch of an oak forest which is located out of Puebla city in the State of Puebla, at 1840 m. We collected *C. irapeanum* growing close to an oak tree.

Due to the scarcity of the orchids we obtained a collect authorization (No. D00.02-3478) from SEMARNAT. *Cypripedium irapeanum* plants were collected including the rhizome and the surrounded soil to ensure that the root system was kept intact. We also sampled seeds. The soil core with the alive whole plants were maintained in the greenhouse before to be processed.

In order to observe the root fungal colonization *in situ* the roots were hydrolyzed and stain by the Philips and Hayman procedures (1970) resulting in 20 to 30 root samples per plant. Inter and intracellular

melanized hyphae with microsclerotia were recorded as Dark Septate Endophytes (DSE).

The isolation of the seed and root endophytes of *C. irapeanum* was done after a surface sterilization with a 5% sodium hypochlorite solution for 10 min, followed by a 0.1 % mercuric chloride solution for 2 min, and several washings with sterile distilled water. This drastic sterilization was done to prevent growth of root external microorganisms. Seeds were sowed in flasks containing Knudson (1946) culture medium, and the root fragments (1 cm long) in Petri plates containing Melin-Norkrans (Molina & Palmer, 1982) culture medium. After 4 days of incubation, plates with the root fragments having superficial contaminants were eliminated and those with no contaminants were incubated at 24°C for 3 months. Pure fungal isolates were propagated in Melin-Norkrans agar medium. Colonial and microscopic morphology was photographically documented (data not shown).

Genetic characterization of *C. irapeanum* endophytes involved 1) extraction of fungal DNA from isolated and purified fungi, 2) amplification of fungal genome region useful in determining fungal identity (ITS1-5.8S-ITS2), 3) restriction and RFLP analysis of the region, 4) DNA sequencing of the region, and 5) BLAST analysis for identification of endophytes.

DNA extraction of isolates was done utilizing the CTAB method (Gardes & Bruns 1993). Obtained DNA was purified with the Concert Nucleic Acid Purification (Gibco), according to the manufacturer instructions. Concentration and purity of the DNA was evaluated with a GeneQuant spectrophotometer. The ITS region of the rRNA operon was amplified according to Gardes & Bruns (1993) using the primers ITS1 (TCCGTAGGTGAACCTGCGC), ITS-1F (CTTGGTCATTTAGAGGAAGTAA), ITS 4 (TCCTCCGCTTATTGATATGC) and ITS4-B (CAGGAGACTTGTACACGGTCCAG). PCR was carried out in a Biometra-T personal thermocycler under 94°C for 85 s for the denaturation followed by 25 cycles of amplification and extension for 13 cycles at 95°C for 35s, 55°C for 55s and 72°C for 45s. This was followed by an incubation at 72°C for 10 more minutes. Obtained bands were visualized in an EtBr stained agarose gel.

PCR products were purified (Concert Nucleic Acid, Gibco) and restricted with enzymes *Hinf*I (at 37°C for

5 hours), *Alu*I (at 37°C for 1 hour), and *Taq*I (at 65°C for 3 hours). Fragments were analysed with the Kodak ID 3.6.1 program.

The amplicons were cloned and ligated using the TOPO XL PCR cloning (QIAGEN) according to the manufacturer's instructions. The recombinant vector was used for transforming cells of *E. coli* DH5α. Screening for recombinant cells was carried out by blue/white selection. Sequencing reactions were done in a Li-Cor 4202 G sequencer. Before sequencing, the amplicons were purified with the Pure Link Quick Gel Extraction kit (Invitrogen) following the manufacturer's instructions.

Sequences were subjected to BLAST analysis to determine their homology with other sequences available in the Gene Bank for the ITS1-5.8S-ITS2 region. The CLUSTAL package (Thompson *et al.* 1994) was used to align the sequences with the corresponding fungal ITS rDNA sequences.

Results and discussion. The microscopic examination of stained root segments of the orchid revealed the presence of fungal structures of both orchidioid fungi, pelotons and coiled hyphae (Fig. 2) and DSE, melinized hyphae and microsclerotia (Fig. 3). In 40% of the cortical cells pelotons were seen, and 30% of the cortical cells revealed the presence of microsclerotia inside the cells. We found partially digested pelotons in all *C. irapeanum* plants, suggesting that *C. irapeanum* may have mycoheterotrophic stages.

Table 1 shows the list of the endophytic fungi recovered from roots and germinated seeds of orchid *Cypripedium irapeanum*.

Dark Septate Endophytes were also observed in the germinated seeds. Two distinct types of microsclerotia were seen in the roots, one was of round shape and the other had irregular shapes (Fig. 3). A total of 10 different fungi were isolated, one from a germinated seed and 9 from the roots. Isolates 7, 9 and 10 were identified as DSE; Isolate 7 and 9 from the plants, and isolate 10 from the seeds. To our knowledge, this is the first report of the colonization of *Cypripedium irapeanum* by DSE fungi and the first report of the colonization and identification of both types of fungi in *C. irapeanum*.

As expected the amplified ITS1-5.8-ITS2 rDNA region resulted in a 650 bp product. Negative controls

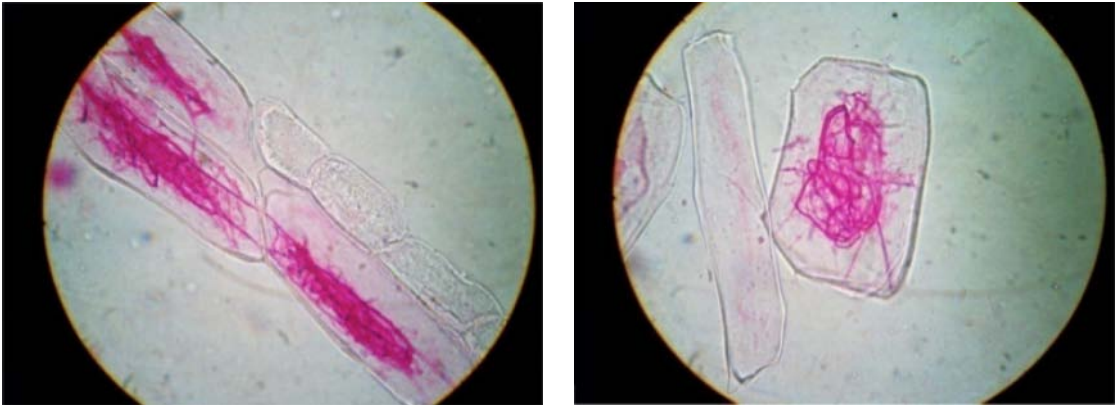


FIGURE 2. Squash preparation of a *C. irapeanum* stained root showing infection of cells, coiled hyphae and development of pelotons.

in PCRs (sterile milliporized water) consistently yielded no PCR product. The ITS 1F-ITS 4 combination yielded most of the PCR products except for the isolates 8, 9, and 10 which were amplified with the ITS 1-ITS 4B combination.

Restriction of the region yielded RFLPs different for all the analyzed fungi, except for the isolates 9 and 10 that were identical. Nine RFLP patterns were yielded with *AluI* and *TaqI* restriction enzymes and 7 patterns with *HindI* (Fig. 4), suggesting the diversity of the endophytes.

Analysis of ITS1-5.8-ITS2 region sequences

suggested that mycorrhizal tissue was dominated by Tulasnaceae: isolate 2 (GeneBank Accession No. JF313323) revealed 98% identity to *Sistotrema* sp., isolate 3 (GeneBank Accession No. JF313324) 99% to *Rhizoctonia solani*, and isolate 6 (GeneBank Accession No. JF313322) 97% to *Epulorhiza* sp., confirming Shefferson *et al.* (2005) and Shimura *et al.* (2009) results for the genus *Cypripedium*. Diverse Tulasnaceae form mycorrhiza also with epiphytic orchids (Suárez *et al.* 2006). Figure 5 shows a phylogenetic tree indicating the placement of the mycorrhizal fungi recovered from the roots of *C. irapeanum*.

TABLE 1. Endophytic fungi recovered from roots and germinated seeds of orchid *Cypripedium irapeanum*

Isolate	Molecular identification	Type of root colonizing fungus
C1	Fusarium	Fungal endophyte (Bayman & Otero, 2006)
C2	Sistotrema	Mycorrhizal (Currah et al, 1990)
C3	Rhizoctonia solani	Mycorrhizal (Warcup, 1971)
C4	Fusarium	Fungal endophyte (Bayman & Otero, 2006)
C5	Cylindrocarpon	Fungal endophyte (Fisher & Petrini, 1989)
C6	Epulorhiza	Mycorrhizal (Shan et al, 2002)
C7	MUT 885	Dark Septate Endophyte (Girlanda et al, 2002)
C8	Gliocladium catenulatum	Biological control fungus (Paavanen-Huhtala et al, 2004)
C9	Phomopsis	Dark Septate Endophyte (from plant) (Jumpponen, 2001)
C10	Phomopsis	Dark Septate Endophyte (from germinated seeds)

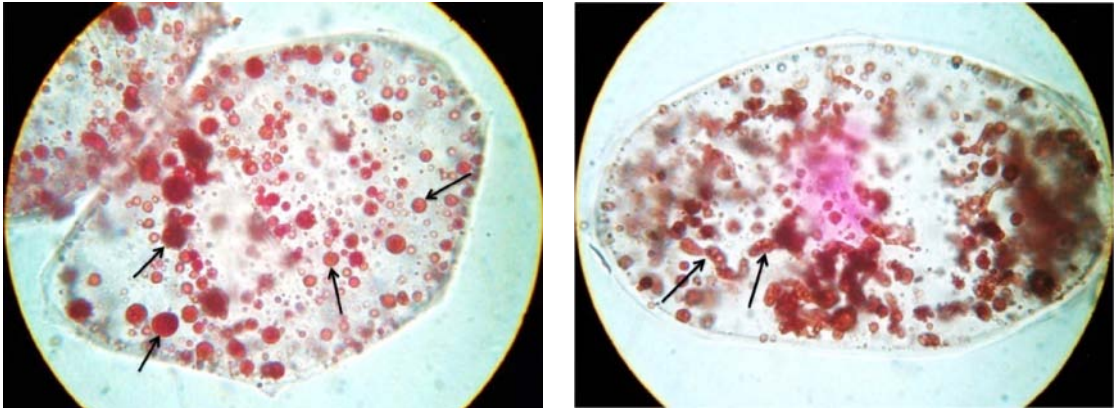


FIGURE 3. Two distinct microsclerotia (arrowheads) within the roots of *C. irapeanum*, round shape and irregular shape.

Isolates 9 and 10 revealed 100% identity to *Phomopsis* sp XJ-05, and isolate 7 revealed 99% identity to the fungal endophyte MUT 885 (a DSE fungus according to Girlanda *et al.*, 2002), corroborating the results of the RFLP analysis. *Phomopsis* sp XJ-05 was isolated not only from the roots of *C. irapeanum* plants but also from the germinated seeds, indicating a possible role of stimulation of germination.

Other endophytic fungi belonging to the Deuteromycetes were also isolated: isolate 1, identified as *Fusarium* sp 440 (99% identity); isolate 4 identified as *Fusarium* sp (97% identity); isolate 5 as *Cylindrocarpon* sp 4/97.1 (100% identity); and isolate 8 as *Gliocladium catenulatum* (99% identity).

The genus *Sistotrema* is defined by Currah *et al.* (1990) as a mycorrhizal fungi of boreal species. Moncalvo *et al.*, (2006) states that this fungus as a highly phylogenetic. However, analysis of ITS1-5.8-ITS2 region sequences of our isolate C2 showed a high identity to this fungus.

Rhizoctonia is known for its association with most other orchids (Rasmussen, 1995). This fungus is a genus based on asexual stages, is a polyphyletic fungus which includes fungi from the families Tulasnellaceae, Sebacinaceae and Ceratobasidiaceae. *Rhizoctonia solani* is a known anamorph of *Thanatephorus cucumeris*, has been isolated from absorbent tissues of orchids and confirmed as mycorrhizal endophytes because are able to stimulate the seed germination and development of the plant *in vitro* assays (Warcup, 1971).

The *Epulorrhiza* species are known as anamorphs of the genus *Rhizoctonia*. Shan *et al.*, 2002 mention

that certain species of this genus have been continually isolated of terrestrial orchids; by means of the RFLP and CAPS analysis of *Rhizoctonia* they were able to classify the genus and its anamorphs in 4 groups. Group II formed by *Epulorrhiza* showed a high ability to stimulate the germination and growth of several orchids. In contrast Group I stimulate a specific orchid. Other authors (Sharma *et al.* 2003) found that in advanced development of the plant the number of species of *Epulorrhiza* is low suggesting that the occurrence of the fungus may be less critical in this growth stage.

DSE have been reported for nearly 600 plants host

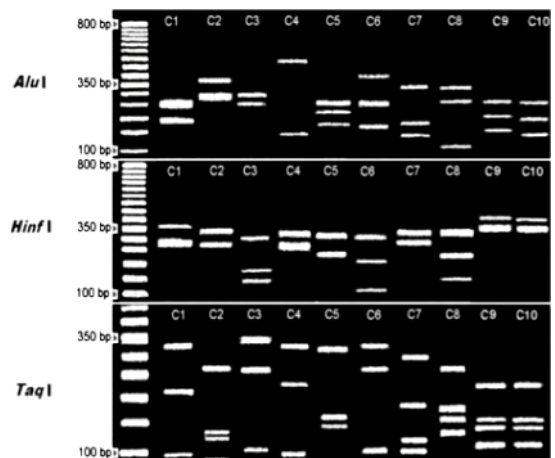


FIGURE 4. Restriction fragment length polymorphisms obtained by endonucleases of the internal transcribed spacer (ITS1-5.8S-ITS2) region of the different fungi isolated from *C. irapeanum*. Digestions were performed with *Alu* I, *Hinf* I, and *Taq* I. M=molecular weight marker.

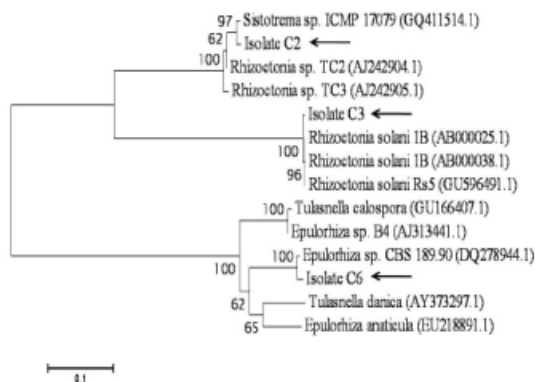


FIGURE 5. Neighbor-joining tree obtained from the internal transcribed spacer (ITS1-5.8S-ITS2) sequence alignment of isolates C2, C3 and C6 with sequences of Tulasnaceae. The Kimura two-parameter model was used for pairwise distance measurement. Bootstrap values above 50% are indicated (1000 replicates). Black arrows indicate the mycorrhizal fungal isolates recovered from the orchid *C. irapeanum*. Bar = Kimura distance.

species, including plants known to bear different types of mycorrhizae occurring in highly diverse habitats.

Their widespread occurrence and high abundance suggests lack of host specificity and an important role in the different ecosystems (Jumpponen & Trappe, 1988; Jumpponen, 1999). Jumpponen (2001) regards the DSE as nonconventional mycorrhizal symbiosis because some of them have found to enhance host mineral nutrition and growth (Fernando & Currah, 1996). The presence of DSE in the germinated seeds of *C. irapeanum* and their lack in the ungerminated seeds suggests its possible role for the germination of the orchid seed.

In relation of the occurrence of *Fusarium* as an endophyte of *Cypripedium*, Bayman & Otero (2006) have defined this genus and its telomorphs as a most interesting group of the orchids endophytes due to its ability to stimulate the seed germination of *C. reginae*.

Other found endophytes in *C. irapeanum* were *Cylindrocarpum* and *Gliocladium*. *Cylindrocarpum* sp. 4/97.1 was reported as an endophyte of roots of terrestrial orchids and mycoheterotrophic orchids (Bayman & Otero, 2006). *Gliocladium catenulatum* is well known as a biological control agent (Paavanen-Huhtala *et al.*, 2004) and parasite of other fungi (Tu & Vaartaja, 1980) suggesting an important role against pathogens in the orchid root.

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