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Short communication

Mycorrhizal synthesis of the edible mushroom *Turbinellus floccosus* with *Abies religiosa* from central Mexico

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ABSTRACT

The ectomycorrhizal (EM) synthesis between *Turbinellus floccosus* with *Abies religiosa* was accomplished under controlled conditions by using seedlings planted in a sterilized peat moss-vermiculite substrate and cultured mycelium. Yellow to brownish EM root tips were observed 3 mo after inoculation. We identified the mycobiont of the synthesized EM through sequence similarity of the nuclear large subunit ribosomal RNA gene (LSU) between three experimental sources: basidiomata, synthesized EM root tips and inoculated cultured mycelium. The morphological and anatomical characteristics of the synthesized EM root tips were presented and described.

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Members of *Turbinellus* Earle have a worldwide distribution in a variety of forest ecosystems and some species are considered to be wild edible mushrooms. The species of this genus are characterized by producing persistent, stalked, fleshy, funnel shaped, often scaled, cantharelloid-like basidiomata with wrinkled hymenophore bearing roughened basidiospores (Corner, 1966), and they establish ectomycorrhizal (EM) associations with various plant species (Trappe, 1960). Molecular systematic studies have revealed that members previously placed around *Gomphus floccosus* (Schwein.) Singer can be interpreted under *Turbinellus* in the order Gomphales (Giachini et al., 2010, 2012; Giachini and Castellano, 2011).

Turbinellus floccosus (Schwein.) Earle ex Giachini & Castellano occurs in stands with *Abies* and *Pinus* (Masui, 1926, 1927; Valdés, 1972), or *Pseudotsuga menziesii* and *Tsuga heterophylla* (Trappe, 1960; Kropp and Trappe, 1982) in different areas in the Northern Hemisphere. According to Giachini and Castellano (2011) this fungus, together with other *Turbinellus*

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species, has been listed as threatened in the 1994 Northwest Forest Plan because of its apparent close association with old-growth forests. In Mexico, *T. floccosus* is known under the name of *G. floccosus*, and its populations grow in association with *Abies* and *Pinus* trees, commonly collected at the end of the rainy season, either fruiting alone, scattered or gregariously (Guzmán and Villarreal, 1985; Aguilar and Villegas, 2010). However, its EM association has not been fully described and there is a lack of information regarding in its isolation in pure culture.

In the USA, this fungus is considered to be edible but not recommended because it can be indigestible (Arora, 1986; Phillips, 1991). This fungus and its related species are generally known to contain toxic compounds such as norcaperatic acid, which can cause stomach irritation (Henry and Sullivan, 1969; Ammirati et al., 1985; Cantrell et al., 2008; Imazeki et al., 2011). Nonetheless antimicrobial assays showed that mushroom extracts of *G. floccosus* presented antimicrobial activity against selected human pathogenic strains (Khaund and Joshi, 2014), and in Mexico, fruit bodies of *G. floccosus* were found to contain a certain degree of an alkaloid component (Bandala and Trigos, 1990). However, as part of the ancient traditions concerning natural resource usage, some rural communities in the country consume the fungal fructifications as food, and even receive economic income because it is commercialized in regional markets (Montoya et al., 2001; Pellicer et al., 2002).

In Central and Eastern Mexico, we have observed the species hereinafter referred to as *T. floccosus*, growing in mixed conifer forests but often near *A. religiosa*, suggesting the EM association with this tree species. *Abies* forests in Mexico are found principally on mountain slopes or in canyons around 2600–3660 m altitude and sometimes develop its pure stands with a great density. This altitudinal range is predominated by the temperate climate with frequent rain and high atmospheric humidity; it's upper and lower altitudinal ranges are drier climates, where *Abies* grows with lower density and forms mixed stands with *Pinus* species (Sánchez, 1969).

This study aimed to confirm the EM association between *T. floccossus* and *A. religiosa* in Mexico, by the mycorrhizal synthetic method under controlled conditions, and to describe its morpho-anatomical characteristics.

Mature and immature *T. floccosus* basidiomata were collected from Jul to Aug 2012, in a pure *A. religiosa* stand, at Agua Blanca, Estado de México (central Mexico). For the fungal species identification in the samples, we consulted Corner (1966), Giachini (2004), Giachini and Castellano (2011), Giachini et al. (2010) and Petersen (1971). Voucher specimens were deposited in the Universidad Autónoma del Estado de Mexico herbarium with the accession numbers Lamus 191, 194 and 195, and the established strains were deposited in the Mycology Laboratory with the numbers L194S and L195S. Small portions of tissues from inside of young basidiomata were inoculated on modified Melin-Norkrans agar culture medium (MMN, Marx, 1969) containing 10 g/l glucose. Isolates were kept at 23 °C in the dark, and 5 to 10 mycelial plugs were removed every month from the edge of colonies by a sterilized cork borer and inoculated on fresh MMN agar plates. The strain L195S was described in terms of colony color and hyphal morphology. To prepare fungal inoculum for the mycorrhizal synthesis, 40 g cultured mycelium (L194S) on agar was fragmented manually by a sterilized scalpel, submerged in 500 ml distilled water homogenized by mechanical agitation with a shaker.

We used seeds of *A. religiosa* collected in the State of Mexico Forest Protection Organization (PROBOSQUE), located in Toluca, México. They were rinsed twice with distilled water and submerged in 30% H_2O_2 for 30 min. Afterwards they were placed on plastic growth chambers with sterilized vermiculite, incubated at 23 °C, and moistened three times per wk with sterilized distilled water. Each of twenty germinated seedlings was transferred to a 200 cm^3 container previously filled with sterilized peat moss/vermiculite mixture at 1:1 (v/v), and fertilized according to Navarro et al. (2013). Then the seedling rootlets were inoculated with 50 ml of the mycelium suspension. In addition, a control group of 30 plants without fungal inoculation was used in order to detect air-contaminant fungi. This synthesis experiment was carried out in a growth chamber under fluorescent light with a 16 h photoperiod at 25 ± 2 °C for 7–8 mo. Seedlings were moistened with sterilized distilled water three times per wk.

The root systems were removed from the seedlings, washed with tap water and EM root tips were separated under a stereoscopic microscope. EM root tips were cut off in longitudinal and transverse cross sections, and the morphotype was characterized following Agerer (1987–2006). After these preparations, EM samples were stored at −20 °C for molecular analyses.

A total of eight samples were considered (two basidiomata, five EM root samples and one inoculated strain). For DNA extraction, DNeasy® Plant Mini Kit protocol (QIAGEN, Hilden) was used, with a step of crushing tissue by a pestle. Part of the nuclear large subunit ribosomal RNA gene (LSU) was amplified using a primer pair LROR/LR3 (Vilgalys and Hester, 1990; Rehner and Samuels, 1994). PCR amplification was done with a 25 μl reaction mixture including 1 × PCR buffer, 2.5 U of Taq DNA polymerase (Takara, Tsu), 12 μl of Mastermix, 0.5 μl of 1 μM primer and 2.5 μl of DNA extract. The thermal profile of the PCR consisted of an initial denaturation at 95 °C for 3 min, denaturation at 95 °C for 0.5 min, annealing at 60–50 °C for 0.5 min in the first, 10 cycles for touch down PCR with decreasing 1 °C/cycle, followed by 35 cycles at 55 °C and elongation at 72 °C for 1 min. A final cycle at 72 °C for 7 min followed to allow elongation of complete DNA strands, after which samples were stored at 4 °C. Amplified products were purified according to the QIAquick Purification protocol (QIAGEN, Hilden), and further sequenced using a Big Dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems®,Thermo Fisher Scientific Inc., Waltham), following manufacturer's instructions.

Once sequences of basidiomata (Lamus 191F and 194F), cultured strain (L194S) and EM root tips (Lamus 191ECMA1, 191ECMA2, 191ECMA3, 194ECMA1 and 194ECMA2) were obtained and edited, they were submitted to GenBank with accession numbers KJ159097, KJ159101, KJ159100, KJ159094, KJ159095, KJ159096, KJ159098 and KJ159099, respectively. To confirm the identity of the mycobiont of EM root tips, we performed a pairwise similarity comparison between sequences of basidiomata and EM root tips, using NCBI BLAST pairwise comparison (Tatusova and Madden 1999).

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