ABSTRACT: This study investigated the presence of mycorrhizae on seedlings from part of ten-year-old truffles plantation (about 3,000 m²) located in Eastern Serbia. This study is observation of the presence of ectomycorrhizal fungus from genus Tuber during its symbiotic stage on the roots of Corylus avellana L. Ten root samples were collected (randomly) and observed macroscopically and microscopically analyzed. There were changes in morphology and anatomy of the infected roots of C. avellana. Mantle was clearly observed to cover the roots and the mycelia formed the Harting net. Among arbitrary selected seedlings, there were found mature fruiting bodies on the surface of the soil. The truffles, identified as Tuber macrosporum Vittad, were found in the immediate vicinity of the hazelnut trees. There has been no previous information of mycorrhizae Tuber macrosporum/Corylus avellana on artificially created truffles plantation in Serbia.

KEYWORDS: Tuber spp., Corylus avellana L, ectomycorrhizae, morpho-anatomical characters, ascocarp, ascospore

INTRODUCTION

The mycorrhiza is a widespread symbiotic association between land plants (roots) and fungi. About 8,000 plant species form ectomycorrhiza, one type of mycorrhiza, were characterized by the presence of hyphal sheath or mantle on the surface of a root, Harting net (hyphae nets between cortex root cells) and
extraradical mycelium (Agerer, 1995). Ectomycorrhizal associations are usually mutual relationships in which a fungus provides water and nutrients for its plant partner and receives assimilates from its host (Finlay, 2008). In this type of mycorrhiza symbiosis, both higher plants from families such as Pinaceae, Betulaceae, Fabaceae, Dipterocarpaceae, Fagaceae and Myrtaceae are involved (Brundrett, 2009) and the fungi that belong mostly to the phyla Basidiomycota and Ascomycota. Ectomycorrhizal associations are formed by estimated 20,000–25,000 fungal species (Rinaldi et al. 2008).

The genus *Tuber* belongs to Ascomycotina, Pezizales, Tuberaceae family. These fungi establish an ectomycorrhizal symbiosis with trees and shrubs and as a result of this symbiosis hypogeous ascocarps – fruit body (truffles) is produced in order to accomplish their life cycle (Mello et al. 2006). The genus contains 180–230 species, subspecies and varieties distributed worldwide (Bonito et al. 2010). In Europe, around 32 species are considered to be valid (Ceruti et al. 2003). Truffles have been collected and consumed by humans for centuries. Because of their specific taste and smell, as well as the special conditions in which they can grow, these edible fungi are among the most expensive ones in the world. The most hunted and prized truffles species are the white species *Tuber magnatum* Pico and the black ones are *T. melanosporum* Vittad. and *T. aestivum* Vittad. (Wang and Marcone, 2011).

This first study on hypogeous fungi (truffles and truffle-like fungi) in Serbia started in 1992. Collected material, fruit bodies of *Tuber* species had been founded and identified and extensively studied during the last decade of the twentieth century (Milenković et al. 1992; Glamočlija, 1996; Glamočlija et al. 1997; Glamočlija, 1999a). According to the earlier reports and new investigations, Marjanović et al. (2010) provided expanded list of species of the genus *Tuber* founded in Serbia and showed the first molecular verification of the *Tuber* spp. samples originating from Balkan Peninsula. Recently, Milenković et al. (2015) founded new truffle species from Serbia.

Despite the current interest in truffles in Serbia, there is not much information about artificially established truffle plantations. In publications (Glamočlija, 1999b; 2000), given results represent a successful mycorrhization roots system of four species of oaks and hazel trees with ascospore suspension of different species of truffles. Plants had been grown in a greenhouse and after monitoring the development of mycorrhiza at the structural and ultrastructural level during 4, 8, 10 and 36 months, they were set in the experimental well. These presented data have no prior results relating to the period of observation of ectomycorrhizae after 36 months.

Information given in the daily press about the existence of 150 truffle plantations with a total area of 33 hectares have not been officially verified by government institutions. The owners of land that is suitable for the growth of artificially mycorrhized host plants with *Tuber* spp plant them, but there is no official company that deals with the control of production, transport and sales.
of inoculated plants in Serbia. The register orchards of truffles in Serbia have not been created yet.

Thus, the objective of this study was the morphological and anatomical characterization of an unknown ectomycorrhizas of host species Corylus avellana from cultivated seedlings from part of a ten-year-old truffles plantation and identification of valuable fruit body after that period.

MATERIALS AND METHODS

Identification of the truffle-ground, geographical data

In autumn 2016, we were invited to visit and determine what kind of mycorrhizae is situated in the plantation near the town of Požarevac (Eastern Serbia). The truffle-ground (about 3,000 m²) is placed in Kličevac 44°44′01.0″N 21°17′18.3″E where a plantation was established during the autumn 2007. According to the owner, he bought commercially produced seedlings of Corylus avellana inoculated with the black truffle.

Sample source

Twenty seedlings C. avellana from a small part of the plot were observed. Ten root samples were collected (randomly) from 10 cm depth increments of the root system, rinsed in water and observed under a stereo dissecting microscope (Leica WILD M3Z, Germany) in order to find ectomycorrhizal roots.

The three fresh mature ascocarps of truffles, each characterized by a gray-brown surface occur at about 1-5 cm depth. The fruit bodies were washed in tap water with a brush and air-dried afterwards. Each truffle was examined macroscopically and microscopically and identified by morphological methods, according to Montecchi and Lazzari (1993). The dried voucher specimens are deposited at the Fungal Collection Unit of the Mycological Laboratory, Department for Plant Physiology, Institute for Biological Research “Siniša Stanković”.

Light microscopy

Root tips 5–10 mm long and pieces of fruit body (10 mm x 5 mm) were fixed in FAA (formalin-acetic acid-ethanol 10:5:85), dehydrated in a graded ethanol series and embedded in paraffin wax at 58 °C. Sections (8 lm thick) were stained with haematoxylin (cross sections of fruit bodies), alcian blue and Schiff’s reagent (cross sections of root tips) and examined under a Zeiss Axiovert microscope (Carl Zeiss GmbH, Göttingen, Germany).
RESULTS AND DISCUSSION

Identification based on the morphological features of ascocarps collected from plantation of Corylus avellana

The fruit body has ascocarp: globose, regular shape, lobed, with a diameter of 2–5 cm. Peridium: brownish to black rigid with very short and flat warts. Gleba: grey brown to brown-lilac and purple-brown, with thick, branching and winding white veins (Figure 1). The globose to subglobose asci of 132–135 (130) x 75–82 (80) μm size contain 1-3 yellowish-brown spores (generally two) (Figure 2.). The ellipsoid spores were 58–62 (60) x 70–74 (70) μm, covered with reticulate-alveolate, polygonal, dense, closed and small meshes (Figure 2a). Odor of mature ascocarp was intensive and specific and had aromatic resemblance of garlic while its aroma was similar to the white truffle Tuber magnatum Pico (Benucci et al. 2016). The shape and size of all small fruit bodies found on parts of experimental orchards are in accordance with those reported for Tuber macrosporum Vitt.

“Garlic truffle” (T. macrosporum) is a common species in Serbia, collected from October to December in hilly terrains of low mountains. The hosts of T. macrosporum in Serbia are oak (Quercus robur L.), native poplars (Populus alba L., P. nigra L.), ash trees (Fraxinus angustifolia L., F. excelsior L.) and maple (Acer campestre L.) (Glamočlija et al. 1997; Marjanovic et al. 2010; Đurđević et al. 2015).

T. macrosporum has a wide distribution in Balkan Peninsula being common in Serbia but also occurs in Croatia and Slovenia. In Europe, harvested ascocarps of T. macrosporum are common in Hungary and Romania, less frequent in Italy and rare in France and Great Britain but occur in Switzerland, Germany and the Ukraine. Recently, it has been reported in Slovakia, Poland and Turkey (Benucci et al. 2016).

In contrast, the world’s most hunted and expensive truffles species (T. magnatum, T. melanosporum and T. aestivum) T. macrosporum has limited reputation and market (Zambonelli et al. 2015). This attractive species with small fruit bodies and specific organoleptic features can merit more attention. This is the first report of the valuable hypogeous fruit bodies T. macrosporum originated from truffle plantations in Serbia.

Identification based on the morphological features of mycorrhizal roots collected from plantation of Corylus avellana

Mycorrhizal roots were thick, cylindrical with rounded tips and developed a white mantle. Tuber macrosporum ectomycorrhiae were simple or ramified in monopodial-pinnate or monopodial-pyramidal pattern. Mycelia proliferate on the root surface and form the multi-layered mantle (Figure 3). According to Agerer (2001), mantles can be divided into two main groups depending on the hyphal distribution and organization: plectenchymatous and pseudoparenchymatous.
The mantle, formed on the surface of the *Corylus avellana* roots was psuedoparenchymatous, composed of angular/epidermoid cells that formed puzzle-like pattern and probably contained glycogen. Piche *et al.* (1981) using the PAS reaction (Schiff’s reaction) showed that the inner mantle and Harting net formed on short roots of *Pinus strobus* contained PAS-positive material, presumably glycogen.

**Figures 1-4.**

Legend of the figure:

*Figure 1.* Fruit body of *Tuber macrosporum*; *Figure 2.* Cross section of *T. macrosporum* fruit body; *Figure 2a.* Oval ascus with ascospores. Note reticular ornamented; *Figure 3.* Cross section of the ectomycorrhizal roots tip of *Corylus avellana.* Note mantle and Hartig net; *Figure 4.* Pseudoparenchymatous mantle; *Figure 4a.* Detail: pariepidermal Harting net

P-peridium; G-gleba; VE-venae externae; VF-venae fertilae; A-ascus; M-mantle; H- Harting net; RM-root apical meristem
Pseudoparenchymatous mantles have short-celled, inflated, compactly packed hyphae, that look like a true parenchyma. From a phylogenetic point of view, hyphal organization in pseudoparenchymatous mantles is considered more advanced (Agerer, 1995). Cells of ectomycorrhizal mantle of *T. macrosporum* varied in shape and size (Figure 4). Bennucci *et al.* (2012) showed significant differences in size and shape within the same ectomycorrhizas. Hyphae penetrate between epidermal cells to form a Hartig net (Figure 3, 4, 4a). The Hartig net, the zone of contact between the plant and the fungus, plays the key role in the transfer of nutrients between both partners (Corrêa *et al.* 2012).

The high economic value of truffles has stimulated researchers to find the most efficient methods for cultivating them. *T. macrosporum* ectomycorrhizae with hornbeam seedlings were published first by Giovannetti and Fontana (1980–1981). The other authors (Zambonelli *et al.* 1993; Granetti, 1995; Agerer and Rambold, 2004–2008) photographed and described ectomycorrhizae oaks and hazel seedlings inoculated by *T. macrosporum* spores. Later Benucci *et al.* (2012) described morphologically *T. macrosporum* on *Quercus robur* L., *Quercus cerris* L. and *Corylus avellana* L. and identified its DNA through the use of species-specific primers (Benucci *et al.* 2016).

Our results of morphological and anatomical characteristic of ectomycorrhizas structures from *C. avellana* roots are consistent with those presented in Benucci *et al.* (2012).

The successful cultivation of *T. macrosporum* on experimental orchards have been established in Italy (Vezzola, 2005). Among common host plants for *T. macrosporum* cultivation, hazelnut *C. avellana* with vigorous growth, with its tendency to form a well developed root system and its well-known capacity to form ectomycorrhizae is especially suitable. Previous studies carried out in Serbia demonstrated the feasibility of producing mycorrhizal plants of *C. avellana* inoculated with black truffle (Glamočlija, 1996).

In the European countries as well as in the Southern Hemisphere and New Zealand, programs of using the seedlings inoculated with truffle fungi are well developed. Over 40 years, an enormous progress has been made (Murat, 2015). In Serbia, there have been no scientific data on the state of private truffieres so far.

**CONCLUSION**

Root samples of hazel trees (*Corylus avellana* L.) and samples of fruiting bodies of truffles were collected from part of truffle-ground. The presence of *Tuber macrosporum* Vittad from natural habitats is well documented in Serbia while the existence of this fungus on an artificially established truffle plantation has not been documented.

We found that ectomycorrhizae of *Tuber macrosporum* were present on roots of seedlings *Corylus avellana* from part of ten-year-old plantation. Further research will be carried out on other parts of this private truffieres.
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МОРФО-АНАТОМСКА КАРАКТЕРИЗАЦИЈА МИКОРИЗЕ
_Tuber macrosporum/Corylus avellana_ ИЗОЛОВАНЕ СА ГАЈЕНИХ САДНИЦА: ПРИКАЗ СЛУЧАЈА

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РЕЗИМЕ: Испитивано је присуство микоризе на садницама из дела десет година старог тартуфишта (3.000 м²) у источној Србији. Овај рад представља први извештај о утврђивању присуства ектомикоризне гљиве из рода _Tuber_ током симбиотске фазе на кореновима _Corylus avellana_ L. Десет узорака коренова сакупљани су методом случајног узорка и макроскопским анализирани микро-сложним методама. Уочене су промене у морфологији и анатомији измењених коренова _C. avellana_. На површини корена јасно се уочава омотач од хифа – мантел, и мицелија која формира Хартингову мрежу. У непосредној близини насумично одабраних садница леске, на површини земљишта пронађена су зрела плодносна тела црног тартуфа – идентификована као _Tuber macrosporum_ Vittad. Према нашим сазнањима до сада нису постојале информације о микоризи _Tuber macrosporum/Corylus avellana_ на вештачки успостављеном тартуфишту у Србији.

КЉУЧНЕ РЕЧИ: _Tuber_ spp., _Corylus avellana_ L., ектомикориза, морфо-анатомска анализ, аскокарп, аскоспоре