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MYCORRHIZATION OF OAK AND HAZEL TREES WITH DIFFERENT SPECIES OF THE GENUS *TUBER*

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Abstract - The root system of oak (*Quercus cerris* L., *Q. robur* L., *Q. petrea,* Lieblein and *Q. borealis* L.) and hazel trees (*Corylus avellana* L.) raised in greenhouse conditions was inoculated with a suspension of ascospores of: *Tuber aestivum* Vitt., *T. melanosporum* Vitt., *T. macrosporum* Vitt. (black truffles) and *T. magnatum* Pico ex Vitt. (white truffle). Roots were examined after 4, 8, 10 and 36 months. After 4 months symbiosis could be observed at ultrastructural level. Mycelium from isolated mycoclenes was applied to oak tree root systems with an aim of proving its identity and viability. Mycorrhiza was obtained and the mycelium was reisolated.

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INTRODUCTION

During the mycorrhizal association of fungal hyphae and root system of trees or vascular plants, a balanced relationship is obtained, in which usually each partner benefits. In the past 50 years, the use of mycorrhizae has been very intensive in agriculture, forestry and other branches (Klironomos and Kendrick 1993).

Fungi from different fungal taxa, 5-6000 species, are involved in mycorrhizal association with a great variety of plant species. A fungus can bind itself with the host root in several morphologically different ways. Ectomycorrhizal fungi envelop the root tips with their mycelium in the form of a compact sheath, the mantle. Root growth becomes modified, root hairs undergo atrophy and mycorrhizal apices branch and develop under an already existing hyphal net. Hyphae continue to penetrate the epidermal and cortical intercellular spaces, forming a netlike intercellular system, where metabolites are exchanged (Agerer 1991). The morphology of ectomycorrhiza can vary in color or form depending on fungal species and plant genus, which is often illustrated in atlases of ectomycorrhiza (Agerer 1987-1991).

Truffles are hypogeous fruiting bodies of the *Tuber* genus. The biology and ecology of truffle species remains insufficiently understood (Lanfranco *et al.* 1995). The life cycle of truffles relies on a long term symbiotic association with a tree and because of that, the species which are gastronomic specialities, such as *Tuber magnatum* (white truffle), *T. aestivum* (summer truffle), *T. melanosporum* (black truffle) are very expensive. Biotechnologial tools for *in vitro* production of mycelium of truffles are specific and depend on different fungal and plant species used. All steps used in production of mycorrhizal trees are known but insufficiently explained.

Truffles in Serbia have been extensively investigated during the past eight years. Collected material, fruit bodies of *Tuber* species found and identified in Serbia (Glamočlija *et al.* 1997), were used for mycorrhization of oak and hazel trees. The inoculation of appropriate plant species and their placing in the ecologically suitable substrate could lead to the production of friut bodies after a certain period of time.

The aim of this study was focused at determination of different parameters optimal for the initiation of ectomycorrhiza in controlled conditions. In the aim to prove successfull mycorrhization, roots were observed at the structural and ultrastuctural level; the mycelium of truffles was isolated from mycoclenes and used for inoculation of new seedlings.

MATERIAL AND METHODS

In experiments of controlled mycorrhization, oak (*Quercus* sp.) and hazel (*Corylus avellana*) 3-5 months-old sprouts were inoculated with the spores of 4 species of the genus *Tuber*. The following *Tuber* species were used: *T. melanosporum, T. aestivum, T. macrosporum* and *T. magnatum*. To raise the sprouts, the oak acorns, without pericarp, were exposed to specific pre-treatments with the aim to initiate germination: - acorns of *Q. cerris* were cultured for 10 days at 4 °C in the dark; - those of *Q. robur* and *Q. petrea* were cultured for 1-2 weeks at 25 °C, on a white light regime (16 *h* light, 8 *h* dark); - acorns of *Q. borealis* were stored at 0 °C for 30 min and then incubated for 1-2 weeks at 25 °C under constant white light (65 W 4500 K, light flux density 47 $\mu mol\ 1s^{-1}m^{-2}$). Young hazel seedlings were obtained from hazelnuts that germinated without any pre-treatment. Before applying spores, the apex of the main root of the plant was shortened by chopping the tip, so as to favor superficial root system growth.

Ascospores of the *Tuber* species were isolated from fruiting bodies stored for 4 months at 4 °C; 1-2 mL spore suspension in sterile water ($1\text{-}4 \times 10^3$ spores per mL) were applied to the root. After the treatment, the plants were placed in soil mixture, composed of peat : manure : send : gypsum, 67% : 17% : 8% : 8% with alkaline reaction (pH 7.7) favorable for truffled seedling growth. The plants were grown in a greenhouse under semisterile conditions. After 4, 8, 10, and 36 months the root systems were examined. Four years after the start of experiments, the plants were transferred to an experimental field.

The root tips in symboisis with the fungus were morphologically changed. Root hairs atrophied and mycorrhizal apices branched and formed the mycoclenes (Meotto *et al.* 1995). After detection and excision mycoclenes were rinsed with tap water and treated with the following series of solutions: - 0.7 % CaOCl until the beginning of color change, followed by 3 rinses with sterile water (Chu-Chou 1979); - 3 % NaOCl for 30-60 s, and two rinses with sterile water (Chevalier 1972); - 0.25 % NaOCl for 210 s rinsed 20 times with sterile water (Mischiati and Fontana 1993).

The mycoclenes were placed in Petri dishes (90 *mm*) containing 25 mL of medium. The following culture media (Mischiati and Fontana 1993) were tested for growth of all examined *Tuber* species: Murashige and Skoog (MS), casein hydrolysate (CH), malt agar (M), potato dextrose agar (PDA), modified Hagem-Modess (MOD) and modified Melin-Norkrans (MMN). Modified Modes-Micolo (MM) medium was tested too (Poitou *et al.* 1983). All media were prepared within the range of pH values (6.0 - 8.5). The antibiotic chloramphenicol was added to the media at a concentration of 500 mgL^{-1}, but omitted when axenic cultures were being established. Following the results obtained from this preliminary experiment, growth trials were carried out using only three of the above substrates on which the mycelial growth was the best, MM pH 7.9 (for mycoclenes isolated from seedlings inoculated with black truffles), MOD pH 6.0 and MMN pH 6.0 (for mycoclenes isolated from seedlings inoculated with white truffle). The cultures were grown under laboratory conditions (16 *h* daylight, 8 *h* darkness, temperature 20-23 °C).

For ultrastructural analyses, isolated mycoclenes were fixed in 3 % glutaraldehyde. Postfixation was conducted in 2 % OsO_4, after which the samples were mounted in Araldite. Ultrathin sections (60-70 nm) were stained with uranylacetate and lead citrate (Reynolds 1963) and analyzed using a Philips CM-12 Eindhoven microscope.

RESULTS

Control of mycorrhizal plants

The inspection of the roots of all plant species inoculated with a suspension of tuber ascospores (*Tuber aestivum, T. melanosporum, T. macrosporum* and *T. magnutum*) showed that each of the 4 species of oak and hazel trees were sucessfully mycorrhized. In this paper we present only the most representative results.

Mycoclenes were noticed on the roots of *C. avellana* after 10 months in plants inoculated with spores of *T. melanosporum*. Short root tips were swollen, pyramidally and fingerlike branched (Fig. 1). Trunks were branched and the root system was dense and very interlaced. Light brown mycoclenes were seen along the whole root system.

The first observation of oak seedlings was done 4 months after inoculation. Primary changes were noticed on the roots of *Q. cerris* inoculated with spores of *T. melanosporum* and *T. aestivum*. Under a binocular microscope (Carl Zeiss Jena, 16-25 X), a number of branched, very broad root tips, significantly broader than the control, were seen. Modified root tips formed coralloid light brown structures. Six months later, further mycorrhizal development was confirmed on the same plants and observed as root hair atrophy. Root tips became broader, more slimy and multiply branched comparing to the previous phase. The above mentioned changes were reported only on certain segments very close to places where ascospores were deposited. After three years, the inoculated roots were considerably more branched than the controls. The lighter mycorrhizal root tips were arranged on the whole surface of the root system. Mycorrhizal plants produced a denser tree crown, with a higher number of leaves than control plants.

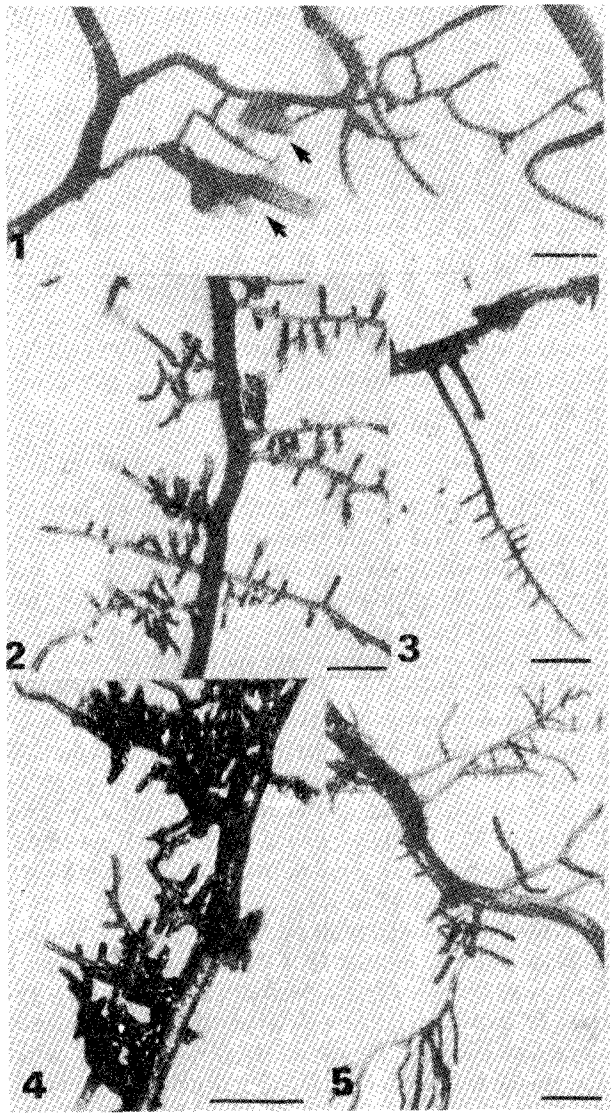

Fig. 1. Root of *Corylus avellana* 10 months after being inoculated with *Tuber melanosporum*. Mycorrhizzed root tips are light in color and pyramidally branched (arrow). (Bar 1000 μm)

Fig. 2. Root of *Q. borealis* 8 months after inoculation with *T. melanosporum*. (Bar 1000 μm)

Fig. 3. Uninfected control roots *Q. borealis*. (Bar 1000 μm)

Fig. 4. Mycorrhizae of *T. magnatum* on *Q. robur* after 8 months. (Bar 1000 μm)

Fig. 5. Root tips of control plant *Q. robur*. (Bar 1000 μm)

Roots of *Q. borealis* plant, 8 months after inoculation with black truffle, *T. melanosporum*, spores contained mace-like, broadened root tips of a lighter color (Fig. 2), which differed from both the inoculated roots of the same plant and the roots of control plants (Fig. 3). Similar modifications were also noticed on the roots of *Q. borealis* inoculated with other black truffle spores (*T. aestivum* and *T. macrosporum*) and white truffle spores (*T. magnatum*). After 8 months of inoculation root changes were noticed in *Q. robur* inoculated with *T. magnatum* spores. Modified root tips were branched once or twice and very mucous (Fig. 4). Along with the increase in the number of branches a change in root tip color also occurred. Mycorrhizal parts of root tips had a darker color which differed from the roots of control plants (Fig. 5). Similar modifications were seen in roots of the same plant species inoculated with the summer truffle, *T. aestivum,* as well as in a smaller number of plants inoculated with *T. macrosporum* and *T. melanosporum*.

The root system of *Q. petrea* seedlings was inoculated with spores of all four truffle species. The best realization of mycorrhiza was archieved in plants inoculated with spores of *T. melanosporum* and *T. magnatum.* Two years after the inoculation the root system of the control plants was visibly less developed (less branched and more truncated) comparing to the ones inoculated with truffle spores.

Ultrastructural modifications

Structural modifications were observed on ultrathin sections of *Q. cerris* roots 4 months after inoculation with spores of *T. aestivum.* The hyphae were observed at the epidermal surface and they also penetrated into intercellular spaces. Cells of the root layers moved apart, partly underwent atrophy and became lyzed. The hyphae penetrated into epidermal and cortical intercellular spaces, enveloping plant cells and forming a network. Host cells changed their shape, became elongated and their walls slightly wrinkled. A large quantity of an electron dense material was situated at the periphery of the cell. Dense aggregates are thought to contain tannins (polyphenols). They can appear as a single aggregate in the vacuole or in the form of a broader layer which envelops the vacuolar tonoplast. The mantle of hyphae in this early phase of ectomycorrhiza was fibrous. These mantles provide a connection between the two organisms. The hyphae were short and of irregular shape, septate and enlarged in places (Fig. 6).

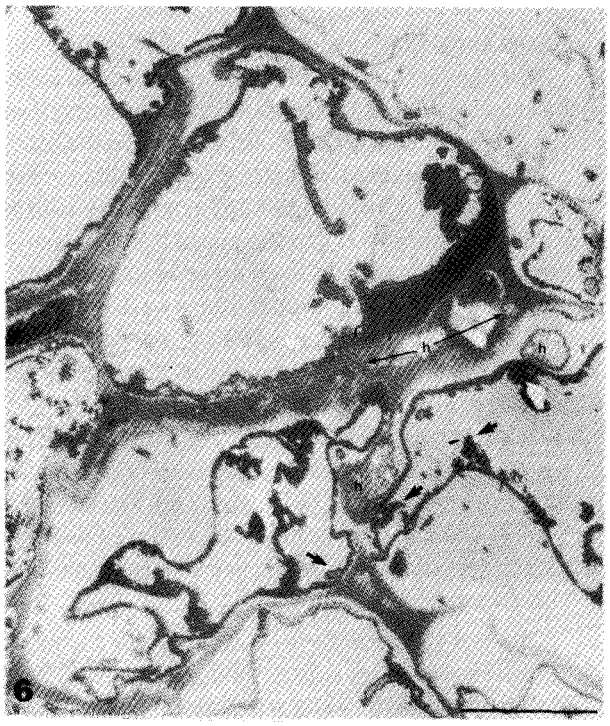

Fig. 6. Ultrathin sections *Q. cerris* roots inoculated with *T. aestivum* spores, after 4 months. Hyphae (h) penetrate into epidermal and cortical intercellular spaces and envelope plant cells forming a network. Host cells change their shapes, being elongated and their walls become slightly wrinkled. A large amount of an electron dense material is situated at the cell periphery (arrow). (Bar 5 μm)

Reisolation of mycelia

The mycoclenes were placed in different culture media. Depending on the truffle species the medium type and pH value were chosen. Mycoclenes were isolated after 4 months from *Q. cerris* roots inoculated with black truffle, *T. aestivum,* and placed in MM medium, pH 7.9. A mycelium, named Q_3, developed from the isolated root tips after 2 months. The mycelium grew slowly, partly in the substrate, and partly aerially. It appeared dense on the inoculum and more sparce at the periphery, so the culture was cotton-like in the center and then progressively flaky. Mycelium color changed from whitish yellow, brown to reddish, which is characteristic for mycorrhizal sheath. The change of color into brown started in the center of the colony and was spreading with hyphal senescence. Mycoclenes from *Q. robur* which was inoculated with white truffle, *T. magnatum,* were isolated after 8 months. Mycelium, named Q_{38}, which grew in solid MOD medium pH 6.0 during 30 days, and after that was transferred to solid and liquid MMN media pH 6.0, was similar in appearance to the previous mycelium.

On the microscopic preparations of the obtained Q_3 and Q_{38} mycelia, two hyphal types were observed, thin hyaline hyphae, and thicker light yellow hyphae, septate with pear-like swellings.

To verify the ability of the isolated mycelium to form mycorrhizae again, the roots of the new seedlings of *Q. cerris* were inoculated with Q_3 mycelium and seedlings of *Q. robur* were inoculated with Q_{38} mycelium. Modifications occurred after 8 months. Modified root tip swelling and color changes were observed in most plants. The obtained mycoclenes were transfered to adequate nutritient media; *Q. cerris* - Q_3 to MM medium and *Q. robur* - Q_{38} to MOD and MMN media. The mycelium that emerged from the myco-clenes was similar to Q_3 and Q_{38}, respectively.

DISCUSSION

Intensive research on truffles in Serbia were initiated since 1991. After two years, a great number of fruit bodies were collected and further used in various experiments, mostly for mycorrhization of oak and hazel trees. In order to establish a mycorrhization and to find the best procedure for our plants and truffles, we tried the methodes recommended in the literature. Root inoculation in this work was done using macerated ascocarps containing mature spores. This method of inoculation, "sporal synthesis" was used before for other plant species (Chevalier *et al.* 1973; Chevalier and Desmas 1977; Giovannetti and Fontana 1982). A high number of inoculated oaks and hazels successfully formed mycorrhizae.

Disinfection methods previously applied by other authors (Chevalier 1972; Chu-Chou 1979) were not successful with our material. According to Mischiati and Fontana (1993) disinfection of mycoclenes was successful and bacterial growth in the cultures avoided. Mycelia Q_3 and Q_{38} obtained by reisolation in our experiments showed that culture appearance, color, growth dynamics and hyphal characteristics are in accordance with data previously published for truffle mycelium obtained by reisolation from mycorrhizal tips (Chevalier 1972, 1973; Grente *et al.* 1972; Mischiati and Fontana 1993). The second reisolation was done with the aim of confirming the data on previously obtained culture. In this case, cultures identical to Q_3 and Q_{38} were obtained. Thus, it was confirmed that Q_3 and Q_{38} mycelia caused the mycorrhization. The validity of mycelium isolated from mycoclenes "mycelial synthesis" was confirmed by Chevalier *et al.* (1973), Boutekrabt *et al.* (1990).

In 1996, the plants were transferred to an experimental field. Long-term monitoring of the development of the fungal species in host root systems is

difficult because of the persisting difficulties in characterizing *Tuber* species ectomycorrhizae. As truffles do not develop until 8-10 years after the inoculation, the control of mycorrhization was neccessery.

The processes that happen during the development of the symbiosis when the fine interactions between suitable mycorrhizal fungi and host plants are established are complex. Dark aggregates at the periphery of vacuolated host cells of mycoclenes in *Quercus* were seen, similar to those reported by Fontana *et al.* (1988) at the ectomycorrhiza between the roots of white poplar *Populus alba* and truffles *T. magnatum*. According to the literature, (Fortin *et al.* 1980; Willis and Cole 1987) the same was observed for the other ectomycorrhizal fungal species, too.

In our experiments we observed a changed shape in the external cortical cell layer, caused by fungal penetration. The very active hyphae could be seen, submerged in fibrous material which probably made the connection between host and fungal cells. In recent literature (Peterson and Farquhar 1994) it was suggested that polysaccharides, as well as mucilaginous substances, composed mostly of glycoproteins, are very important in the first contact and fungal attraction.

In the central and southern European countries, especially in France, Italy and Spain, as well as in the Southern Hemisphere and New Zealand, programs of using the mycorrhizal plants with black truffles are well developed (Hall *et al.* 1994). A high price of fruiting bodies of *T. magnatum* and *T. melanosporum* led to attempts to maximize truffle production on natural or inoculated truffieres (Rouch and Vercesi 1988; Giovannetti *et al.* 1994; Hall *et al.* 1994; Shaw *et al.* 1996). Making private truffieres is very promising (Olivier *et al.* 1996) and perheps in our country this should be stimulated too.

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МИКОРИЗАЦИЈА САДНИЦА ХРАСТА И ЛЕСКЕ РАЗЛИЧИТИМ ВРСТАМА РОДА *TUBER*

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Коренов систем садница храста (*Quercus cerris* L., *Q. robur L., Q. petrea,* Lieblein и *Q. borealis* L.) и леске (*Corylus avellana* L.) одгајаних у условима стакларе инокулисан је са суспензијом аскоспора *Tuber aestivum* Vitt., *T. melanosporum* Vitt., *T. macrosporum* Vitt. (црни тартуфи) и *T. magnatum* Pico ex Vitt. (бели тартуф). Коренови су посматрани после 4, 8, 10 и 36 месеци. Након 4 месеца симбиоза је доказана на ултраструктурном нивоу. Мицелија изолована са микоклена апликована је на коренов систем садница храста са циљем доказивања њеног идентитета и вијабилитета. Микориза је добијена и мицелија је реизолована.