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Establishment of an *in vivo* culture for mycorrhization of *Corylus avellana* with *Tuber melanosporum*

Abstract

Tuber melanosporum known as Black Truffle is a mycorrhizal fungus that grows in symbiosis with certain trees and shrubs. Due to its delicate mycorrhization process, the cultivation of this fungus represents a challenge for its large-scale production. The black truffle has a maturation time of 8 to 12 months from its inoculation and is totally dependent on the interaction it carries out with the host plant. Truffle characteristics such as color, odor and flavor can vary depending on the plant with which it is associated, the most commonly used for its cultivation being oaks (*Quercus ilex ssp. ilex, Q. ilex ssp. ballota*), oaks (*Quercus pubescens, Q. cerrioides, Q. petrae, Q. robur*), gall oak (*Q. faginea*), Kermes oak (*Q. coccifera*), and hazel (*Corylus avellana*), although associations with other plants have been found in the wild.

Similarly, *Tuber melanosporum* is influenced by the climate in which it grows, the ideal being the Mediterranean climate with hot summers and a high rainfall and winters and autumns with temperatures above -9°C with occasional rainfall. Favorable soils for the cultivation of *Tuber melanosporum* are limestone soils with clayey, loamy and sandy characteristics, not compacted, which allow the passage and filtration of water, essential for the truffle.

Tuber melanosporum has a great value in the gastronomic industry, reaching very high prices depending on its availability, being able to reach prices between 200 and 800 euros per kilogram, however, subsequent processes such as packaging, food preparation and shipping can raise these figures even higher.

This project seeks to design a methodology for the *in vivo* cultivation of *Tuber melanosporum*, which allows better results in its inoculation and mycorrhization through the use of *Tuber melanosporum* spores and roots of young hazelnut (*Corylus avellana*) seedlings, for subsequent transplantation in substrates with the appropriate characteristics for both organisms.

Keywords: Indole-3-butyric acid, *Tuber melanosporum*, *Mycelium*

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Introduction

Tuber melanosporum (Tm) is a mycorrhizal fungus that grows in symbiosis with certain plants. Due to its delicate mycorrhization process, the cultivation of this fungus represents a methodological challenge, since it is totally dependent on the interaction it carries out with the host plant. Similarly, Tm is influenced by the climate and substrate in which it develops. Recently, a new endemic truffle species was discovered in the state of Nuevo León, Mexico, called *Tuber regimontanum*, which opens the opportunity to establish Tm cultures in the country. This project sought to design a methodology for the *in vivo* cultivation of Tm, which allows better results in its inoculation and mycorrhization through the use of Tm spores and roots of young seedlings of *Corylus avellana* (Ca), for subsequent transplantation in substrates with the appropriate characteristics for both organisms.

Methods

Preparation of the inoculum of *Tuber melanosporum*. Tm samples were macerated with a previously sterilized mortar until 80 g of a uniform paste was obtained. The paste was divided into two beakers containing 40 g each. To one beaker 0.018 mg of indole-3-butyric acid powder was added. Microscopic observation. A sample of paste and thin pieces of Tm were taken and stained with lactophenol blue for observation under an optical microscope (ZEISS Primo Star BlueLine model). Quantification of spores and ascospores. 1g of previously prepared inoculum was taken and diluted in 1 ml of sterile distilled water. The sample was then placed in the Neubauer chamber and both spores and ascospores were quantified. Preparation of *Corylus avellana*. The substrate in which they were contained was removed, 16 seedlings were washed and disinfected with a solution of 2 liters of sterile distilled water, 10 ml of 10% dextran and 20 ml of 70% ethanol, submerged for 24 hours, then rinsed with sterile distilled water until the washing and disinfection solution was eliminated. Determination of pH. Solutions of 10 g clay, 10 g silt and 10 g sand were made with 100 ml of distilled water, mixed in vortex for 1 minute each; the potentiometer was calibrated with buffer solution and the pH measurements of each solution were made in a potentiometer (Hanna Instruments model HI2002-01). Preparation of substrates. Ten kg of sand, 6 kg of silt and 4 kg of clay were sterilized in an autoclave at a pressure of 15 psi and a temperature of 120°C for 40 minutes. Subsequently, the sterile substrates were mixed to obtain 20 kg of sterilized substrate with a proportion of 50% sand, 30% silt, and 20% clay. The same mixture was carried out with the same quantities and proportions with non-sterilized substrates. Inoculation of *Tuber melanosporum* in roots of *Corylus avellana*. Eight roots were inoculated with 5 g of paste added with indole-3-butyric acid

each, of which four were placed on sterile substrate (Group A) and four on unsterilized substrate (Group B). 8 roots were inoculated with unadded paste, of which 4 were placed on sterile substrate (Group C) and 4 on non-sterile substrate (Group D). In such a way that each group has 4 experimental units and distributed as shown in Table 1.

Table 1 Distribution of the experimental groups

	Sterilized substrate	Substrate not sterilized
With hormone	Group A	Group B
Without hormone	Group C	Group D (Control)

Incubation. Groups A, B and C were watered with 250ml of Murashige & Skoog (MS) medium, group D was watered with 250ml of sterile distilled water. The 16 seedlings in vases were placed in a plant cell culture incubator (Thermo Fisher Scientific model Plant Growth Chamber 818) at 32°C for 10 days, with intermittent light and dark cycles of 12 hours each.

Results and discussion

An approximate of $12x10^6$ spores and $3.8x10^6$ ascospores were estimated in the 5g of inoculum per root. Groups A and B, in contrast to groups C and D, which were not added, were the only ones that showed mycelial growth, suggesting that the indole-3-butyric acid present in the inoculum indirectly favors mycelial development, since this hormone does not act directly on Tm; its function is exercised in the roots of the plants, favoring an increase in their metabolic activity, which in turn can supply more and better nutrients to the mycelia associated with Tm. The results of Group A show a Tm mycelia formation of approximately 11cm in length, as can be seen in Figure 1. Group B presented a mycelia formation of approximately 4.5cm, as can be seen in Figure 2, which represents a difference of 59% greater growth in Group A, with respect to the mycelia formation presented by Group B. This could be due to the fact that Group B did not have a sterilized substrate, and could have less availability of nutrients due to competitiveness with microorganisms or other fungi present in the substrate.

Figure 1 Mycelia formation in Group A.

Figure 2 Mycelia formation in Group B.

Conclusion

The semi-solid preparation of spores proved to be effective for the inoculation of *Tuber melanosporum* in roots of *Corylus avellana* because it facilitates the control of the amount of inoculum, however, it is important to consider the variability of effectiveness of mycorrhization by means of spores. The temperature and pH of the substrate during the mycorrhization process play a very important role in promoting or retarding the germination of spores, the development of mycelia and later of *Tuber melanosporum* primordia. Therefore, a temperature of 32°C and a substrate pH of 7.5 to 8.5 are proposed as ideal parameters. With the results of groups A and B, we could deduce that in the mycorrhization process of *Corylus avellana* with *Tuber melanosporum*, indole-3-butyric acid favors root-mycelium interaction, accelerating their growth. The addition of MS medium and the sterilization of substrates, favors the supply of nutrients to *Tuber melanosporum*, thus promoting its development. Based on the results of group A, which had all the proposed factors, it is concluded that the designed methodology is viable to establish an *in vivo* culture for the mycorrhization of *Corylus avellana* with *Tuber melanosporum*.

Acknowledgments

None.

Conflicts of interest

Authors declare that there is no conflict of interest.

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