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SHORT NOTE

First production of Italian white truffle (*Tuber magnatum* Pico) ascocarps in an orchard outside its natural range distribution in France

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Abstract

Truffles are ectomycorrhizal species forming edible ascocarps. The Italian white truffle (*Tuber magnatum* Pico) is the most famous and expensive species harvested to date; it comes exclusively from natural habitats in European countries. The annual production of *T. magnatum* is generally insufficient to respond to the high demands making its cultivation a research hotspot. The first attempt to cultivate *T. magnatum* started in the 1970s without success; only recently have mycorrhized plants been successfully produced. The aims of this study were (1) to assess the persistence of *T. magnatum* in the soil of plantations realized with mycorrhized plants and (2) to characterize the first *T. magnatum* orchard that produced ascocarps outside the known natural geographic range of this species. In 2018, five orchards were sampled in France, and *T. magnatum* was investigated in the soil. We confirmed that *T. magnatum* survived in the soil 3 to 8 years after planting. The key finding of this study was the harvest of *T. magnatum* ascocarps in 2019 and 2020 from one orchard. The production of ascocarps started 4.5 years after planting, and the ascocarps were harvested under different trees and during two consecutive seasons. A detailed analysis of the productive orchards (i.e., soil features, soil water availability, cultivation techniques) is presented. These results demonstrate the feasibility of *T. magnatum* cultivation worldwide by planting mycorrhized plants. The cultivation of *T. magnatum* could therefore become a real opportunity for farmers and could respond to the high demand of this high-priced food.

Keywords *Tuber magnatum* · Mycorrhized plants · Cultivation · Soil DNA

Introduction

The hypogeous ascocarp of the Italian white truffle (*Tuber magnatum* Pico) is produced by an ectomycorrhizal fungus living in symbiosis with trees such as oaks, willows, hornbeams, and poplars. *T. magnatum* is the most famous truffle presented in the menus of many high-quality restaurants worldwide. Its particular and attractive aroma makes this truffle unique. *T. magnatum* is commonly harvested in well-structured soils with high water content (Marjanović 2015; Bragato and Marjanović 2016; Iotti et al. 2018) in Italy, the Balkan peninsula, and marginally in Switzerland and in southeast France (Fig. 1) (Tabouret 2011; Riccioni et al. 2016; Büntgen et al. 2019). According the Köppen-Geiger climate classification (Beck et al. 2018), *T. magnatum* is mainly harvested in Csa (hot summer Mediterranean) and Dfb (warm-summer humid continental) climates. The annual production of *T. magnatum* is generally insufficient to respond to the high demands explaining its high price (up to 4000 €kg^{-1}) although it can reach even 100,000 €kg^{-1} in dedicated auctions (http://www.castellogrinzane.com/en/comunicato-stampa-2019). This makes white truffle one of the most expensive foods (Mello et al. 2006). *T. magnatum* is a crucial component of the local economy and cultural heritage in Italy (Pieroni et al. 2016), but its importance is wider since *T.*

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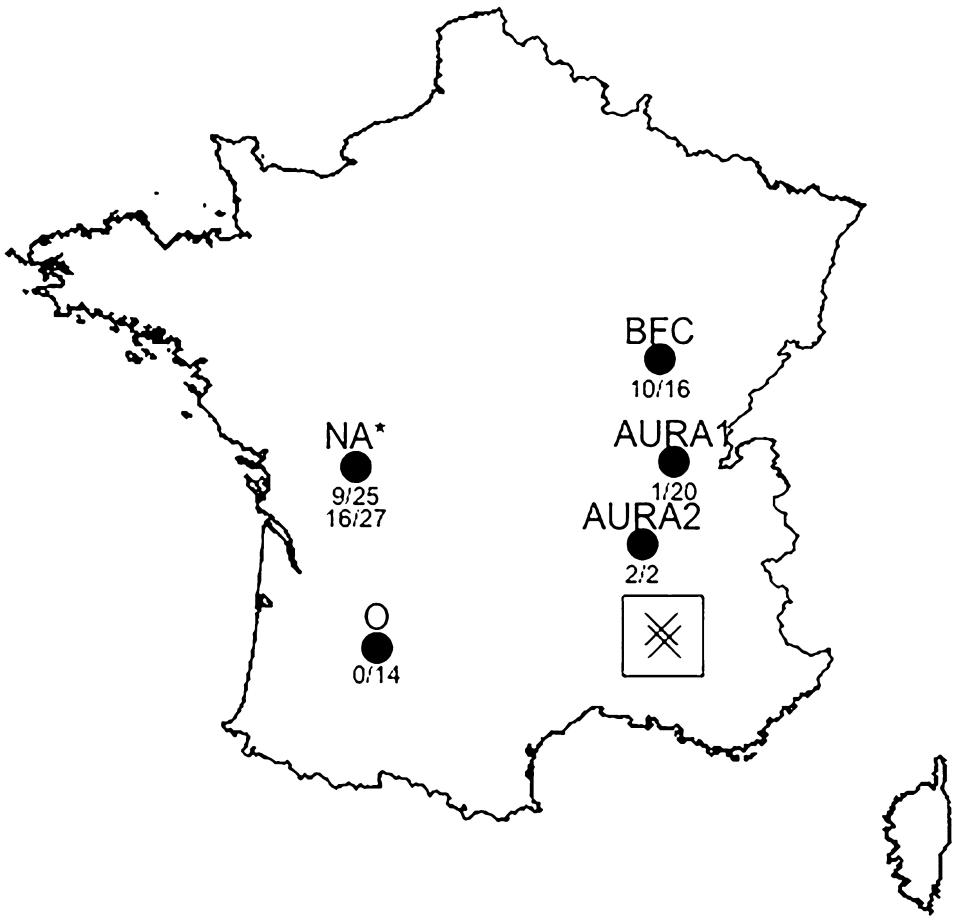

Fig. 1 Map of France with the localization of the sampled orchards (black circle) as well as the position of two known natural *T. magnatum* productive sites (crosses). The area where *T. magnatum* is naturally present is indicated by a square. The number of soil samples with a positive amplification for *T. magnatum* is indicated below the black circle according to the total number of samples for the 2018 sampling. For NA, the result of the 2020 soil sampling is also indicated below. An asterisk identifies the orchard (NA) where *T. magnatum* ascocarps were harvested in 2019 and 2020. Abbreviation: AURA1 Auvergne Rhône-Alpes 1, AURA2 Auvergne Rhône-Alpes 2, BFC Bourgogne Franche-Comté, NA Nouvelle-Aquitaine, O Occitanie

magnatum harvests in Europe is a business of about 0.9 billion € year^{-1} (Lovrić et al. 2020).

To date, the harvest of *T. magnatum* has occurred exclusively in spontaneous forests; its cultivation has been difficult in contrast to other truffle species such as the Perigord black truffle (*Tuber melanosporum* Vittad). *T. melanosporum* is now mainly harvested in dedicated orchards realized with mycorrhized plants that prevent production decline in France. It has even been introduced onto other continents (Olivier et al. 2012; Le Tacon et al. 2014; Chen et al. 2016). Attempts to cultivate *T. magnatum* started in the 1970s in Italy with more than 500,000 mycorrhized plants sold (Riccioni et al. 2016). However, only a few dozen *T. magnatum* orchards have been productive 15–20 years after planting; these were all in areas where *T. magnatum* is naturally occurring. Therefore, it is possible that indigenous *T. magnatum* strains were eventually responsible for the ascocarp production (Riccioni et al. 2016).

The problem for the cultivation of this species can be explained first by its very demanding ecological requirements that have been described only recently (Marjanović et al. 2015; Bragato and Marjanović 2016). Additionally, the morphological description of *T. magnatum* ectomycorrhizae has long been incorrect until molecular DNA analyses allowed the correct identification (Mello et al. 2001; Rubini et al. 2001). Therefore, most plants sold in Italy were not effectively mycorrhized by *T. magnatum*, and those that were truly mycorrhized with *T. magnatum* have been discarded (Riccioni et al. 2016). After a 9-year INRAE/Robin research program, *T. magnatum* mycorrhized plants have been commercialized in France since 2008 (Murat 2015). This has led to dedicated orchards, but several questions remain open: Does *T. magnatum* persist in the soil after planting? Which are the suitable cultivation techniques? Is it possible to harvest ascocarps in such orchards? The aims of this study were to assess the persistence of *T. magnatum* in the soil of plantations and to characterize the first *T. magnatum* orchard that produced ascocarps outside the known geographic natural range of this species.

Materials and methods

Site selection and soil sampling

In order to test the persistence of *T. magnatum* after planting mycorrhized plants, five orchards were selected in different regions of France (Fig. 1). They correspond to

the five owners that responded positively to our solicitation. Due to confidentiality constraints, the exact geographic coordinates cannot be indicated. In all orchards, commercial seedlings mycorrhized with *T. magnatum* (Robin nursery, St. Laurent du Cros, France) were planted from 2011 to 2015. Planted seedlings were 1-year *Q. pubescens* produced in 430 cm^3 pots. Due to the low frequency of *T. magnatum* ectomycorrhiza in the field (Murat et al. 2005; Leonardi et al. 2013), *T. magnatum* mycelium was traced in the soil. Soil cylinder cores (5-cm diameter × 10-cm depth) were sampled in the four cardinal directions (North, South, East, West) of the trees at about 0.7 m from the trunk and at a depth of 20 to 30 cm. The four soil cores were then pooled in a single plastic bag. A total of 77 trees were sampled from March to June 2018. In June 2020, an additional 27 trees were sampled in the NA orchard after ascocarps were harvested in 2019 (see below). Ten soil samples were also harvested outside the NA orchard under *Salix* spp. trees. Soil samples were cleaned from woody debris and roots (> 2 mm), homogenized, and kept at − 20 °C. Total DNA from soil samples was extracted using the PowerSoil DNA extraction Kit (Qiagen, Courtaboeuf, France) following the manufacturer's protocol and stored at − 20 °C for molecular analysis.

Amplification of *T. magnatum* mycelium in the soil

The quantity of *T. magnatum* mycelium in a complex matrix such as soil is extremely variable; it is expected to be low in some soils. A nested qPCR protocol was used for maximum sensitivity. The nested qPCR protocol is composed of three steps: a first enzymatic reaction step performed on the PCR mixture without the template DNA. The first 8-µL mixture is composed of 2X REDExtract-N-Amp™ PCR ReadyMix™ (5 µL), 10-µM TmgITS1 for (0.25 µL; Iotti et al. 2012), 10-µM primer Back3 (0.25 µL; Rubini et al. 2001), water (2.2 µL), and MboI restriction enzyme (0.1 µL). This was placed at 37 °C for 30 min for activation and at 95 °C for 2 min for inactivation. This first step avoided contamination during the nested PCR (Carroll et al. 1999; Zampieri et al. 2010). After decontamination, PCR amplification was performed by adding the template DNA (2 µL) to the previous reaction to reach a final volume of 10 µL on a C1000 Touch™ Thermal Cycler (Bio-Rad, Schiltigheim, France) with denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. The last step is a real-time PCR amplification step performed in 10-µL mixture composed of Takyon ™ ROX Probe MasterMix dTTP Blue ™ (5 µL), 10-µM TmgITS1 for (0.4 µL; Iotti et al. 2012), 10-µM TmgITS1 rev (0.4 µL; Iotti et al. 2012), 5-µM TaqMan TmgITS1 probe (0.4 µL; Iotti et al. 2012), water (2.8 µL), and template DNA (PCR product of the previous step (1 µL)), carried out on a StepOnePlus™ Real-Time PCR System machine provided with the StepOne software v. 2.3 (Life Technologies, Applied Biosystem) on 96-well plates with carryover prevention at 50 °C for 2 min, Takyon™ activation at 95 °C for 3 min, 30 cycles of denaturation at 95°C for 5 s, and annealing at 60 °C for 15 s. The specificity of the nested qPCR was tested against the DNA of different *Tuber* spp. (i.e., *T. aestivum*, *T. borchii*, *T. brumale*, *T. magnatum*, *T. melanosporum*, *T. mesentericum*, and *T. rufum*).

Climate, description, management, soil hydric potential, and soil features of the NA orchard

The NA orchard is located in Nouvelle Aquitaine region (France) at about 80 m above sea level. It has a southeast slope of 3% and a Cfb (temperate oceanic) climate according the Köppen-Geiger climate classification (Beck et al. 2018). The NA orchard was planted in March 18, 2015, with 52 *Q. pubescens* at 4-m rows with 6 m between rows; the field had been used for pasture and cultivation of alfalfa for decades (Fig. S1 and S2). The orchard was surrounded by agricultural fields (i.e., maize and wheat).

A black plastic cover (A12V 80 µm; Robin nursery, St Laurent du Cros, France) of 1.5 m of width in the rows was installed to control the weeds and maintain soil water; it was removed in November 2017 (Fig. S2). The extremities of the cover were buried to hold it in place in the direction of the row. The owners manage this orchard similarly to their *T. melanosporum* orchards. From 2015 to 2018, monthly soil tilling with a disc cultivator and vibro-cultivator was performed between rows. In February 2018, the shoot tips were lightly pruned while the soil was tilled with a vibro-cultivator; a power arrow was used in March/April. Since ascospores are involved in the truffle sexual reproduction, a commonly used cultural practice is to inoculate soil with crush truffle that is rich in ascospores (Olivier et al. 2012; Le Tacon et al. 2016; Murat et al. 2016). Soil inoculation consisting of crushed truffle was performed on April 8, 2020, for plants 395, 396, 424, 425, and 429 with the first three ascocarps harvested in 2019. All ascocarps (minus pieces used for morphological and DNA analyses (about 100 g)) were crushed and incorporated in 10 litres of water with 10 tablespoons of honey. Honey is often used by truffle farmers in the inoculum preparation (Murat et al. 2016), but to date, there is no experimental demonstration it has a real impact on truffle production. For each plant, two inoculations were realized at about 30 cm from the trunk in the east and west directions. Each inoculation consisted of 1 L of mixture incorporated in the soil by planting a shovel and incorporating the liquid underneath. No chemical inputs were used on this site.

The rainfall was recorded by owners with a manual rain gauge. Watering was realized with a sprinkler installed close to each plant (Fig. S2). In 2019, three watering sessions of 15 mm each were realized on July 4 and 22 as well as September 4. Nine watering sessions of 25 mm each were realized in 2020: April 15; May 31; June 26; July 11, 17, and 28; August 6 and 25; and September 9. In June 2020, two TEROS21 probes (Meter-Group, Munich, Germany) connected to an EM50 data logger (Meter-Group, Munich, Germany) were installed at 1 m from tree 429 at 12 cm of depth toward the northeast and southwest direction. The pF (i.e. soil hydric potential) was recorded to follow the soil water availability. Trees 397, 429, and 424 produced one ascocarp in 2019; soil samples collected under these trees were sent to the INRAE soil laboratory analysis (Arras, France) for chemical and physical analyses.

Molecular analyses of the ascocarps harvested in NA orchard

A piece of each ascocarp was used for spore morphology identification and DNA analysis. After a first check of the spores with a microscope, DNA from one ascocarp harvested in 2019 and the two ascocarps harvested in 2020 was extracted using DNA Plant Minikit (Qiagen, Courtaboeuf, France) following the manufacturer's protocol and stored at − 20 °C for molecular analysis. For the 2019 ascocarp, the internal transcribed spacer (ITS) was amplified using fungal universal primers ITS1F and ITS2 (Gardes and Bruns 1993), and sequenced by Eurofins (Ebersberg, Germany). The sequence was deposited in Genebank with the following accession number: MT937196. This sequence was aligned using ClustalW in MEGA version 10.1.8 (Kumar et al. 2018) with those of other Tuber spp: *T. aestivum*, *T. mesentericum*, *T. panniferum*, *T. excavatum*, *T. magnatum*, *T. borchii*, *T. rufum*, *T. brumale*, and *T. indicum*. A neighbour-joining tree was realized with MEGA. A bootstrap test with 1000 repetitions was realized. The DNA of the two 2020 ascocarps were analysed using a direct and specific *T. magnatum* qPCR protocol (Iotti et al. 2012).

Result and discussion

To trace the mycelium of *T. magnatum* in the soil, a nested qPCR protocol amplifying only *T. magnatum* was developed (Fig. S3). Nested PCR was chosen because it increases the sensitivity versus direct PCR (Zampieri et al. 2010). Using this protocol, we observed the *T. magnatum* mycelium presence in the soil samples collected under 22 out of the 77 trees in 2018; 3 to 7 years after planting (Fig. 1). Only the soil of the O orchard had no *T. magnatum* mycelium (Fig. 1). The lack of amplification could be due to an absence of *T. magnatum* in the soil, low mycelium levels, non-uniform distribution of the truffle in the soil, or a sampling period that is not optimal due to the temporal dynamics of the mycelium in the soil (Salerni et al. 2014). However, we did demonstrate that *T. magnatum* persisted in the soil several years after planting.

The key result of this work was the harvest of *T. magnatum* ascocarps in the NA orchard (Fig. 2; Fig. S4 and S5). The owners harvested one ascocarp (40 g) on October 10, 2019, under tree 429 and two (37 g and 31 g) on November 9, 2019, under tree 424 and 397, respectively. In the first check of the 2020 season, the owners harvested one ascocarp (45 g) on September 13, 2020, under tree 401 (Fig. S4) and another (56 g) on September 28, 2020, under tree 403 in the presence of two INRAE agents (Fig. 2; Fig. S6). The five ascocarps were harvested under five different trees; 5.5 years after planting, 10% of the plants produced one ascocarp (Fig. S1). The spore morphology for all ascocarps, the ITS sequencing (accession number: MT937196) for one ascocarp, and the qPCR amplification of two others confirmed that they belong to *T. magnatum* (Fig. S6 and S7). These truffles were the first confirmed *T. magnatum* ascocarps in a man-made orchard outside the natural geographic range of the species ever harvested.

Few *T. magnatum*-producing natural sites are known in France but all are located in the southeast of the country in Dfb and Csa climates (Beck et al. 2018). The NA orchard is located in western France in Cfb climate (Fig. 1). Mycelium of *T. magnatum* was not detected in soils harvested under local trees (*Salix* spp) close to the stream (Fig. S8) implying that *T. magnatum* was not naturally present in the vicinity of the orchard. We conclude that harvested ascocarps originated from strains thriving on planted mycorrhized plants instead of natural inoculation by indigenous strains. The mycelium of *T. magnatum* in the soil of this orchard was more widely distributed in 2020 (16 soil samples out of 27) compared with the sampling of 2018 (9 samples out of 25) (Fig. S1). In 2020, positive amplifications were detected in soil samples collected under the three productive trees in 2019. Unfortunately, the soil of the two trees that produced ascocarps in autumn 2020 was not sampled in June 2020 (Fig. S1).

The NA orchard is located in a marl with chalky limestone from the superior Cretaceous. The soil is classified as clay loam (Fig. S9) with a pH in water of 8.1 (Table S1). All values are in the range of those observed in productive *T. magnatum* forest in Italy (Bragato and Marjanović 2016). The annual rainfall in 2019 was about 800 mm (Fig. S11). From January to August 2020, 329 mm of rainfall was recorded (Fig. S10). *T. magnatum* is commonly harvested in soil with high water content. In the NA orchard, water was always available since the pF never reached 4.2 (i.e., wilting point) (Fig. S11). In 2020,

Fig. 2 Picture of the ascocarp harvested in September 28, 2020, in the NA orchard. Ascocarp was found 60 cm from the trunk close to a sprinkler

nine watering sessions were performed (see method section) in order to maintain some soil water availability. July 2020 was exceptionally dry (4 mm; Fig. S10 and S11). For *T. melanosporum*, maintaining a pF less than 4 allowed for an increased production (Le Tacon et al. 1982; Bach and Murat 2020). For *T. magnatum*, the best growth of its mycelium in soil occurred at water saturation confirming that *T. magnatum* mycelium has a high demand for water (Marjanović et al. 2015; Iotti et al. 2018).

The soil temperature of NA orchards in the summer is about 20 °C (Fig. S11), which corresponds to the optimal temperature for soil mycelium according to Iotti et al. (2018). The soil hydric potential and temperature in the NA orchard are thus optimal for *T. magnatum* soil mycelium growth. For *T. melanosporum* and *T. magnatum* a correlation between soil mycelium quantity and ascocarp production have been proposed (Suz et al. 2008; Parladé et al. 2013; Iotti et al. 2012). Water and temperature conditions in the NA orchard favoured the formation of the ascocarps. These conditions were obtained via the management techniques applied by the owner. The use of a black plastic cover avoided weed growth and maintained soil humidity probably favouring growth of *T. magnatum* soil mycelium. Frequent soil tilling led to good aeration of the soil; watering in tandem with the black plastic prevented the soil from becoming excessively dry. Soil tilling, watering, tree pruning (that just starting in the NA orchard), and spore inoculation (started in 2019 in the NA orchard) are common practices in *T. melanosporum* (Olivier et al. 2012) and *T. aestivum* orchards (Todesco et al. 2019). This suggests that similar management techniques could be suitable for different truffle species with different natural habitats and ecological requirements.

The production occurred very quickly (4.5 years after planting), which is similar to *T. melanosporum*, and *T. aestivum* orchards (Olivier et al. 2012; Todesco et al. 2019). The harvesting of ascocarps in two consecutive seasons under different trees suggests that production will be perennial. These results demonstrate the feasibility of *T. magnatum* cultivation worldwide by planting mycorrhized plants while maintaining suitable soil water and temperature condition. Truffle cultivation is in accordance with agroecology policies because it does not require the use of chemical treatment. This culture can be a biodiversity hotspot in agricultural regions as showed by Therville et al. (2013). The cultivation of *T. magnatum* is thus a real opportunity for farmers, and it can help them respond to the high demand for this high-priced food.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00572-020-01013-2.

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Compliance with ethical standards

Conflict of interest INRAE and Robin nursery have a know-how license based on a common INRAE/Robin process for the production and commercialization of plants mycorrhized with *T. magnatum*.

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