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ORIGINAL ARTICLE

Multi-cropping edible truffles and sweet chestnuts: production of high-quality *Castanea sativa* seedlings inoculated with *Tuber aestivum*, its ecotype *T. uncinatum*, *T. brumale*, and *T. macrosporum*

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Abstract The plantation and management of sweet chestnut (*Castanea sativa* Mill.) orchards is a common and traditional land use system in many areas of Europe that offers the advantage of simultaneous production of nuts and timber. During the last decades, sweet chestnut has declined dramatically in many regions because of the profound social changes in rural areas coupled with pathogen attacks. Truffles, the hypogeous ascocarps of the ectomycorrhizal genus *Tuber*, are currently cultivated using host trees inoculated with these fungi for improving production in truffle orchards. The production of good forestry quality chestnut seedlings inoculated with European truffles in nurseries is essential for multi-cropping plantation establishment, but so far, it has not been implemented in agroforestry practices. Moreover, it is necessary to assess the physiological condition of the seedlings due to the high calcium amendment needed for the growth of *Tuber* spp. mycelium that can become toxic for the host plants. In this study, seedlings of *C. sativa* were inoculated with *Tuber aestivum* and its ecotypes *T. uncinatum*, *T. brumale*, and *T. macrosporum* and were grown in a greenhouse using culture conditions favorable for the production of high-quality plants for forestry purposes. At the end of the assay, levels of root colonization and morphological and physiological parameters of the seedlings were measured. The colonization of *C. sativa* with *T. aestivum*, its ecotype *T. uncinatum*, and *T. brumale* was successful, and the seedlings showed normal growth. Inoculation protocols with *T. macrosporum* need to be improved. *Tuber* species formed well-developed ectomycorrhizae on *C. sativa* in nursery conditions.

Keywords *Castanea sativa* · *Tuber aestivum* · *Tuber uncinatum* · *Tuber brumale* · *Tuber macrosporum* · Forestry quality seedlings · Multi-cropping

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Introduction

The ectomycorrhizal (ECM) genus *Tuber* P. Micheli ex. F.H. Wigg. (Pezizales, Ascomycota) includes species that associate with ecologically important tree and shrub species in Mediterranean ecosystems (Benucci et al. 2012b). Truffles, the belowground fruiting bodies of *Tuber* spp., are edible and harvested for commercial purposes due to their exceptional organoleptic characteristics (Díaz et al. 2003; Pacioni et al. 2014). In Spain, mainly two *Tuber* species are collected in the wild (*T. melanosporum* Vittad. and *T. aestivum* Vittad.) due to the ease of their marketability; however, *T. brumale* Vittad., another edible species of economic interest, can also be found. Truffles grow in a wide range of habitats associated with a broad range of host species like oak, willow, poplar, hazel, and some shrubs like rockroses (Napoli et al. 2010). They all require calcareous soils with pH 7–8 (Mello et al. 2006). The market price of truffles (€200–€3000 per kg) varies depending on the species and their geographic origin, and it is negatively correlated with the abundance of seasonal production. The Piedmont truffle (*Tuber magnatum* Pico) is the most prized, followed by *T. melanosporum* (Benucci et al. 2012c).

Tuber aestivum, the summer truffle, is the most widespread truffle species in Europe (Hall et al. 2007; Gryndler et al. 2011; Benucci et al. 2012a). It occupies a broad range of ecological environments, and it is more appreciated in foreign than in Spanish markets. *Tuber uncinatum* Chatin is now considered the same species as *T. aestivum* based on molecular studies, although they were originally considered two different species because of their differences in ecology and fructification period (Paolocci et al. 2004; Wedén et al. 2005). In this study, despite considering them as two ecotypes, we maintain the two species names and test their performance separately. *Tuber brumale* is present in natural truffle grounds in Spain, although it is not collected for commercial use. It grows in areas with slightly low soil moisture and thrives in some truffle plantations in the north of the Iberian Peninsula (Reyna 2007). *Tuber macrosporum* Vittad. is widely distributed in Europe (Benucci et al. 2012c) forming mycorrhizas with many different tree species (Granetti et al. 2005; Vezzola 2005; Bencivenga and Falini 2012; Hilszczańska et al. 2013). It grows in fresh, wet, and rich in clay calcareous soils with variable levels of calcium carbonate, often in lowlands or near rivers (Vezzola 2005; Marjanović et al. 2010; Benucci et al. 2014). Despite its excellent organoleptic characteristics resembling those of the Piedmont truffle, it has been ignored because it is usually sold intermixed with other truffle species of inferior quality (Iotti et al. 2002), and its market is still incipient or non-existing in many countries like Spain.

Currently, due to the decline in production of *T. melanosporum* in natural truffle grounds over the last decades (Hall et al. 2003), the use of plants inoculated with this truffle species for complementing its production in the wild has been a breakthrough in silvicultural and agronomic practices in Europe. Inoculated plants are grown in nurseries under controlled environmental characteristics for 1 or 2 years, the time necessary for the formation and development of ectomycorrhizae in their roots; afterwards, they are transferred to the field (Reyna 2007; Morcillo et al. 2015). In recent years, some initiatives have encouraged the combination of timber and/or fruit production with the collection of edible mushrooms as a secondary forest product in multi-crops (Martins et al. 2011; Bonet et al. 2014). Truffles are usually cultivated as a monocrop, but there is a high potential for sustainable multi-cropping of truffles with economically important host plants like sweet chestnut, oak, and hazelnut for multiple crop yields like food, fuel, and fiber (Porter et al. 2009; Benucci et al. 2012c; Lancellotti et al. 2014). In fact, the use of black truffle mycorrhizal plants has been proposed to promote reforestation in rural areas while improving the economic profitability of forestry activities (Bonet et al. 2006).

The success of a reforestation program depends mainly on the environmental conditions of the site where it is taking place and on the ability of the nursery plants to establish and grow in those conditions (Grossnickle 2000). The latter is determined by the quality of the forestry plant and varies depending on the needs and aims of the specific reforestation program (Navarro and Pemán 1997). Therefore, forestry quality refers to the ability of a plant to survive and grow, meeting the expectations of a specific growing season (Duryea 1985), and it is reflected by its morphological and physiological traits that allow for adaptation to the reforestation site (Cortina et al. 2006). In the specific case of the production of forestry quality plant inoculated with *Tuber* spp. for truffle production purposes in plantations, the quality of the plant is also reflected in the development of a root system highly colonized by the desired *Tuber* species (Andrés-Alpuente et al. 2014; Murat 2015).

In Mediterranean ecosystems, for instance, where summer drought is the most important limiting abiotic factor (Castro et al. 2004; Villar-Salvador et al. 2013), reforestation success is closely linked to the quality of the plant used, which largely depends on how it has been grown in the nursery. For instance, nitrogen fertilization plays a very important role in plant quality and outplanting performance (Oliet et al. 2009; Villar-Salvador et al. 2012). Moderate and high levels of nitrogen (> 100 mg N/plant) positively influence the morphological and physiological quality of deciduous plants (Jacobs et al. 2005; Salifu and Jacobs 2006; Patterson and Dimov 2013). However, available data related to the influence of the application of fertilizers to *Tuber* inoculated plants in the nursery are scarce and the results contradictory, usually ignoring the recommendations to obtain high forestry quality plants (Dupré et al. 1982; García-Barreda et al. 2016). Moreover, once formed, truffle ascocarps are still dependent on the carbon supply from their plant hosts (Le Tacon et al. 2013, 2015) which is directly linked to the plant condition; this makes the production of high-quality plants essential.

Belonging to Fagaceae, the genus *Castanea* includes many species; among them, *C. sativa* (European or sweet chestnut), *C. crenata* (Japanese chestnut), *C. mollissima* (Chinese chestnut), and *C. dentata* (American chestnut) are the most common and economically important. *Castanea sativa* is the only native species in Europe, and it is mainly distributed across the Mediterranean region, spreading from the Caucasus to Portugal and South England (Martín et al. 2012) and covering a wide range of environmental conditions (Ciordia et al. 2012).

In Spain, *C. sativa* grows in areas with significant humidity in summer, spreading from north to south, including the Canary Islands (Ruíz de la Torre 2006; Blanco et al. 1997). It prefers well-drained and deep soils, with no late frosts, a drought period that does not exceed 2 months, and summer rainfall of ca. 50 mm. It grows from sea level up to 1500 m asl (Ruíz de la Torre 2006; Cuenca Valera and Majada Guijo 2012), in both acidic and basic soils and preferentially with lime not exceeding 4% (Flórez et al. 2001). *Castanea sativa* plantations are exploited both for fruit and timber (Cuenca Valera and Majada Guijo 2012). In the nursery, sweet chestnut is commonly planted bare root, although the recent increase in its

production has led to the use of large forest containers at higher seedling densities (Cuenca Valera and Majada Guijo 2012). The use of sweet chestnut in reforestation is limited, and it is usually planted together with *Quercus ilex* L., *Quercus faginea* Lam., *Quercus pyrenaica* L., *Quercus robur* L., and even *Quercus suber* L. (Ruíz de la Torre 2006; Cuenca Valera and Majada Guijo 2012). *Castanea sativa* is adapted to a wide range of climatic and soil conditions, overlapping in many cases with those suitable for truffle growth. Although it has been always speculated that the European chestnut is able to naturally associate with *Tuber* spp. (Benucci et al. 2012c), the production of truffles in chestnut woodlands has not been proved until very recently in Gerona, Spain (Morcillo et al. 2015).

Castanea sativa mycorrhizal seedlings inoculated with *T. borchii* and *T. aestivum*/*uncinatum* have been previously obtained following traditional plant production methods based on the use of sterilized and/or solarized soils and without fertilization treatments (Zambonelli and Branzanti 1988, 1990). Nursery cultivation procedures can strongly determine the functional characteristics of the seedlings and their field performance (Villar-Salvador et al. 2004). In fact, fertilization treatments are common in nursery production of plants for forestry purposes, since they enhance the quality of the plants and strongly influence their functional traits (e.g., biomass, nutrient content, photosynthetic capability).

Based on a successful production of high-quality mycorrhizal *C. sativa* seedlings with species of *Tuber*, a multi-cropping system could be established to promote rural or marginalized economies by providing farmers with a valuable source of income (Benucci et al. 2012b). Therefore, the aims of this study were to (1) produce *C. sativa* seedlings of high forestry quality while inoculated with edible truffle species and using high nitrogen fertilization regimes and (2) test the morphological and physiological quality of the inoculated seedlings in substrates with high calcium content, optimal for the growth of *Tuber* spp., and compare it to the quality of non-inoculated seedlings in substrates commonly used for chestnut seedling growth.

Material and methods

Seedling production in the nursery

Seeds from *C. sativa* of provenance ES02 (Galicia, north of Spain) were surface sterilized in 5% sodium hypochlorite solution for 20 min and rinsed in distilled water several times. They were then placed in trays filled with peat moss (Kekkilä F0, Finland), moistened with distilled water, and maintained in a germination chamber (Incubig model, Selecta, Spain) at 20 °C until germination. When the root emerged and reached 3 cm in length, seedlings were transferred to five forest trays with 32,400 mL cells (190/400, Plasnor, Spain). The trays were filled with a mixture of peat moss (Kekkilä F6) and perlite (0–10 mm) 4:1 (*v*/*v*). Substrate was amended with 90% dolomitic calcium to three different pHs for the optimal growth of each truffle species: pH = 7.2 for *T. macrosporum* (Benucci et al. 2016), pH = 7.8 for *T. aestivum* and *T. uncinatum* (Stobbe et al. 2013), and pH = 8.1 for *T. brumale* (Reyna 2007). The substrate of the control plants was adjusted to pH = 6 recommended for *C. sativa* (Pintos et al. 2000).

Inoculum production and seedling inoculation

Truffles used for inoculum had different provenances: *T. macrosporum* from Hungary and *T. aestivum*, *T. uncinatum*, and *T. brumale* from Spain. In this study, we considered *T. aestivum* and *T. uncinatum* separately, to test if we could find differences in colonization levels and plant performance that reflected the different ecotypes. The identity of all ascocarps was confirmed macroscopically and microscopically. In April 2012, after an adaptation period of 2 months, plants were inoculated using an aqueous suspension of spores incorporated into the substrate. The inoculations were made by manual injection with 2 g of fresh truffle per seedling. One forest tray containing 32 plants was inoculated per truffle species (128 inoculated seedlings in total), and 32 non-inoculated seedlings on a forest tray were used as control plants.

Plant growth conditions

Inoculated plants were grown in a greenhouse at a minimum temperature of 4 °C and maximum of 32 °C. No shading was applied during the summer. NPK fertilizer (19-5-17; Hakaphos, BASF, Germany) was applied weekly from June to November 2012 resulting in 200 mg of N per plant at the end of the experiment. The trays were rotated in the greenhouse during the experiment each 15 days to avoid possible effects of position.

Root ECM colonization levels and physiological and morphological parameters of the seedlings

Plants were maintained for 2 years in the greenhouse to ensure the presence and full development of *Tuber* ectomycorrhizae while allowing for signs of potential negative effects on the inoculated seedlings of the high calcium concentration of the substrates used to reach the optimum pH for the different truffle species growth. The assessment of seedling quality was based on the morphological and physiological parameters of the plants (Villar-Salvador 2003) and the *Tuber* mycorrhizal colonization levels of the seedlings. The physiological plant measurements were recorded in a subset of 10 randomly selected individuals per treatment. Potential nutritional deficiencies in the seedlings were estimated measuring chlorophyll

fluorescence in a fluorimeter (FSM, Hansatech): the maximum photosystem II quantum yield that is the variable fluorescence and maximum fluorescence ratio (Fv/Fm) at dawn and at midday in plants previously adapted to the dark and the quantum yield of photosystem II (Φ_{PSII}), which estimates the efficiency of photosystem II lighting (Maxwell and Johnson 2000), were recorded. In addition, water potential measurements in a pressure chamber at dawn and at midday were performed following Scholander et al. (1964) and Tyree and Hammel (1972) to differentiate and identify potential variations in water use by plants inoculated with the different truffle species.

At the end of the experiment, in winter 2014, full root systems were thoroughly rinsed with tap water and the level of root ECM colonization was estimated as in Chevalier and Grente (1979), rating seedling mycorrhizal quality on a 1 to 5 scale. For statistical purposes, this scale was converted to percentages according to Trouvelot et al. (1986) (Andrés-Alpuente et al. 2014). Ectomycorrhizae were identified at species level by their morphological and anatomical characteristics as described in Agerer (1987–2012) using a stereomicroscope (Nikon SMZ100) and an inverted microscope (Nikon Ti-U). Photographs were taken utilizing a Nikon DS-5mC and a DS-2mV digital camera. Plant morphology was assessed by measuring the height of the seedlings and the diameter at the root collar; the plants were then dried in an oven during 48 h at 60 °C, and the dry weight of the roots and shoots was measured.

Molecular analyses of ectomycorrhizae

The molecular identification of *Tuber* ectomycorrhizae was performed at the Institute of Agrifood Research and Technology (IRTA, Catalonia, Spain). The DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) was used for genomic DNA extraction from a composite sample of 10 ECM tips per truffle species. The following species-specific primers were used for PCR depending on the target species to be detected: Tu1sekvF/Tu1sekvF (Gryndler et al. 2011) for *T. aestivum* and *T. uncinatum*, ITSB/ITS4LNG (Paolocci et al. 1999) for *T. brumale*, and TmacrF/TmacrR (Benucci et al. 2011) for *T. macrosporum*. The PCR reactions were performed using the Biotools mix (Biotools B6M, Madrid, Spain) in a GeneAmp 9700 (Applied Biosystems). Two positive controls for each truffle species obtained from identified ascocarps and a negative control with HPLC water were included in each run.

Statistical analysis

Shapiro and Wilk (1965) and Levene (1960) tests were used to verify normality and homoscedasticity, respectively, of the variables measured ($\alpha = 0.05$). Variables were transformed when needed, and when normality and homoscedasticity were not met, a Kruskal-Wallis test ($p < 0.05$) was performed. To test for differences in the morphological and physiological variables measured among the truffle species, an analysis of variance (one-way ANOVA) was performed. Differences between means were addressed using the Tukey test ($p < 0.05$). Statistical analyses were performed using R v. 2.15.0 (R Foundation for Statistical Computing 2012).

Results

Morphology of ectomycorrhizae and root colonization levels

The inoculation of the seedlings was successful; *Tuber* ectomycorrhizae were observed in all treatments species, and they were morphologically and anatomically similar to those described previously as *T. aestivum* and *T. uncinatum* (Benucci et al. 2012b; Müller et al. 1996), *T. brumale* (Fischer et al. 2004), and *T. macrosporum* (Benucci et al. 2012d) (Fig. 1).

Briefly, mycorrhizal systems of all the inoculated *Tuber* species (Fig. 1a, b, e, f, i, j) were simple or ramified with monopodial-pinnate or monopodial-pyramidal pattern, and with short-distance exploration type (Agerer 2001, 2006). Cystidia of *T. aestivum* and *T. uncinatum* (Fig. 1a, b) are long, simple, bristle like, curly, septate, and from yellowish in young mycorrhizae to ochre-brown in older ones (Fig. 1c), and the outer mantle is pseudoparenchymatous with angular cells (Fig. 1d). In *T. brumale* (Fig. 1e, f), cystidia in the outer mantle are short, needle like, yellow-amber, and usually monoseptated, with warts on the surface, and with a basal septum and broad base (Fig. 1g); the outer mantle is pseudoparenchymatous with epidermoid cells sometimes rounded and thickened cell walls (Fig. 1h). *Tuber macrosporum* (Fig. 1i, j) cystidia are long, simple, bristle like, sinuous, septate, and yellow to ochre in oldest mycorrhizae (Fig. 1k), and the outer mantle is pseudoparenchymatous with epidermoid cells that form a regular puzzle-like pattern (Fig. 1l).

Seedlings inoculated with *T. brumale*, *T. aestivum*, and *T. uncinatum* showed high levels of ECM colonization as in Donnini et al. (2014), with over 30% of the root tips colonized, and no other ECM fungi found in their roots. Levels were significantly lower ($F = 9.60$; $p < 0.001$) only in seedlings inoculated with *T. macrosporum*. Across seedlings, the percentage of root tip colonization by *T. macrosporum* ranged from 5 to 70% with an average of 19% (± 24%). Seedlings with *T. aestivum* and *T. uncinatum* ectomycorrhizae showed similar colonization levels, from 30 to 70% with an average of 46% (± 17 and ± 14%, respectively). *T. brumale* seedlings showed the highest colonization levels, ranging from 30 to 80% with an average of 62% (± 18%). Molecular analyses

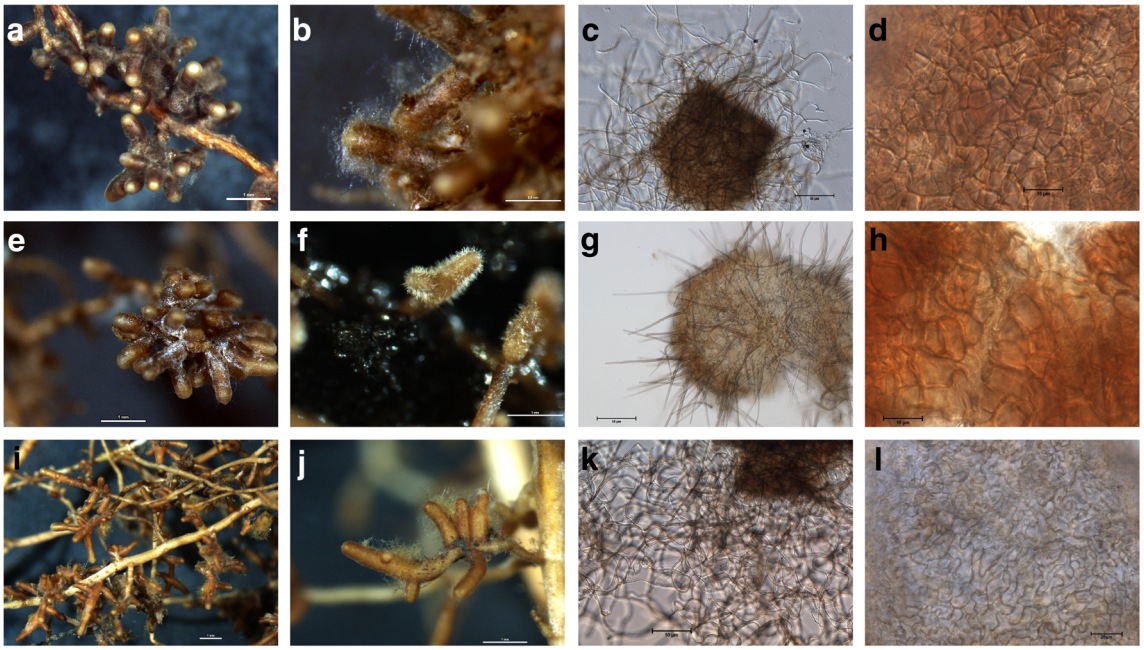

Fig. 1 Anatomical and morphological features of *Tuber* ectomycorrhizae with *Castanea sativa*. **a–d** *Tuber aestivum*. **a**, **b** Ectomycorrhizae. **c** Cystidia. **d** Outer mantle. **e–h** *T. brumale*. **e**, **f** Ectomycorrhizae. **g** Cystidia. **h** Outer mantle. **i–l** *T. macrosporum*. **i–j** ectomycorrhizae. **k** Cystidia. **l** Outer mantle. **m–p** *T. uncinatum*. **m**, **n** Ectomycorrhizae. **o** Cystidia. **p** Outer mantle

confirmed the identification of the three *Tuber* species in roots of *C. sativa*. All ectomycorrhizae gave the expected amplicon size corresponding to those obtained from ascocarps of each *Tuber* species. No amplifications were observed in the negative controls.

Morphological and physiological parameters of *C. sativa* seedlings

In general, the inoculated seedlings growing at higher pH did not show lower morphological or physiological quality than the non-inoculated seedlings growing at lower pH (Tables 1 and 2). However, some significant differences were found: *T. brumale* seedlings showed smaller diameter and greater height and root biomass than the control plants, whereas *T. macrosporum* and *T. uncinatum* seedlings had less shoot biomass. *T. aestivum* seedlings did not differ significantly from the non-mycorrhizal plants in any of the plant parameters measured.

Shoot biomass showed variation depending on the treatment. The seedlings with *T. uncinatum* and *T. macrosporum* mycorrhizae were significantly smaller in biomass than the control plants or plants with *T. brumale*, while plants with *T. aestivum* had an intermediate biomass. Root colonization by *Tuber* positively influenced the growth of the root system. Control seedlings and seedlings with a poor level of mycorrhization with *T. macrosporum* developed root systems with lower biomass, while *T. aestivum* and *T. uncinatum* seedlings formed root systems of intermediate biomass, tending to be lower in the latter. *T. brumale* plants formed the largest root system.

All plants showed high Fv/Fm ratios at dawn, from 0.84 to 0.86, and they did not differ across treatments ($H_{(4.50)} = 5.86$;

Table 1 Morphological parameters measured in *Castanea sativa* seedlings 18 months after inoculation with different *Tuber* species (mean ± SD, $n = 10$). Values within rows showing different letters are significantly different (Tukey test, $p < 0.05$)

Variable	Control		*T. macrosporum*		*T. aestivum*		*T. uncinatum*		*T. brumale*		Statistic	
											p	F
Height (cm)	36.1 ± 5.6	ab	33.1 ± 10.2	a	43.1 ± 8.9	ab	34.1 ± 6.6	a	45.1 ± 8.9	b	< 0.001	4.4
Diameter (mm)	10.3 ± 1.6	bc	9.6 ± 1.1	ab	10.7 ± 0.9	bc	11.5 ± 2.2	c	8.1 ± 0.8	a	< 0.001	8.2
Dry weight root (g)	5.8 ± 2.9	a	5.9 ± 1.6	a	8.5 ± 3.2	ab	7.0 ± 2.9	ab	8.7 ± 2.0	b	< 0.05	2.9
Dry weight shoots (g)	8.7 ± 3.3	b	4.5 ± 1.7	a	7.1 ± 2.3	ab	4.8 ± 1.6	a	9.3 ± 2.0	b	< 0.001	9.7

Table 2 Physiological parameters measured in *Castanea sativa* seedlings 18 months after inoculation with different *Tuber* species (mean ± SD, $n = 10$). Fv/Fm_{dawn}: maximum photosystem II quantum yield at dawn; Fv/Fm_{noon}: maximum photosystem II quantum yield at noon; Φ_{PSII}: quantum yield of photosystem II; $WaterPot_{dawn}$: water potential at dawn; $WaterPot_{noon}$: water potential at noon. Statistical differences are presented in ANOVA test or Kruskal-Wallis test. Values within rows showing different letters are significantly different

Variable	Control	*T. macrosporum*	*T. aestivum*	*T. uncinatum*	*T. brumale*	Statistic	
						p	Test
Fv/Fm_{dawn}	0.84 ± 0.03 a	0.80 ± 0.06 a	0.84 ± 0.02 a	0.85 ± 0.01 a	0.86 ± 0.04 a	> 0.05	$H = 5.86$
Fv/Fm_{noon}	0.61 ± 0.25 b	0.67 ± 0.11 b	0.80 ± 0.07 ab	0.72 ± 0.16 ab	0.84 ± 0.04 a	0.023	$H = 16.60$
Φ_{PSII}	0.17 ± 0.10 b	0.16 ± 0.11 b	0.35 ± 0.09 a	0.23 ± 0.08 b	0.30 ± 0.14 ab	< 0.001	$F = 6.43$
$WaterPot_{dawn}$	− 0.67 ± 0.10 a	− 0.70 ± 0.27 a	− 0.63 ± 0.21 a	− 0.93 ± 0.45 a	− 0.64 ± 0.13 a	> 0.05	$H = 4.38$
$WaterPot_{noon}$	− 2.36 ± 0.63 a	− 2.35 ± 0.53 a	− 2.36 ± 0.40 a	− 2.11 ± 0.45 a	− 1.55 ± 0.15 b	< 0.001	$F = 5.83$

$p > 0.2$). However, we found significant differences at noon ($H_{(4.50)} = 16.60$; $p < 0.005$); *T. brumale* seedlings showed higher Fv/Fm ratios than control and *T. macrosporum* seedlings, while *T. aestivum* and *T. uncinatum* showed intermediate values. The estimated quantum yield of photosystem II (Φ_{PSII}) measurements differed among seedlings from different treatments ($F = 6.43$; $p < 0.001$). *T. aestivum* and *T. brumale* seedlings showed significantly higher values than the rest of the treatments. *T. uncinatum* and *T. macrosporum* seedlings did not differ from controls. At dawn, seedling water potential did not differ among treatments ($H_{(4.50)} = 4.39$; $p > 0.35$), ranging from 0.64 to 0.93. At noon, all treatments showed lower water potential than at dawn. Only *T. brumale* seedlings showed significantly higher water potential values than the other treatments. No significant differences were observed among the other treatments and the control seedlings, all showing averaged values below − 2.0 MPa.

Discussion

In the 1960s, with the aim to overcome the declining of wild black truffle production in Europe, scientists and landowners began to develop methods for the cultivation of *T. melanosporum* in orchards (Pruett et al. 2009). Currently, truffle cultivation is feasible too for other species such as *T. aestivum* and *T. borchii*, obtaining even greater success and using a broader range of hosts and habitats (Benucci et al. 2012c; Bencivenga and Falini 2012). The ecological width of *Tuber* spp. has allowed the development of this symbiosis with new hosts in order to multi-crop truffles with other forest products like nuts or wood (Benucci et al. 2012b; Lancellotti et al. 2014).

C. sativa is the only native species of the genus in Europe with wide distribution throughout Southern Europe, showing its ability to adapt to a variety of environmental conditions (Lauteri et al. 1998; Martín et al. 2010). It is one of the species of major economic importance in the Mediterranean basin, managed for timber as well as for fruit production and other forest secondary products (Conedera et al. 2016). The active management of chestnut woodlands and orchards has resulted in the extension of its distribution at the limits of its ecological range (Conedera et al. 2016).

Due to its adaptability to different environments and the benefits obtained from its multiple uses, it might be a good candidate as host for edible truffle species in multi-cropping plantations in Europe. When experimenting with new hosts in truffle inoculation trials, special attention should be paid to the plant health and nutrition to survive with the high pH and calcium concentration required by the truffle partner (Benucci et al. 2012c). This is of special importance in the case of the sweet chestnut, which is a calcifuge species (Flórez et al. 2001) and could be affected by those conditions during the nursery stage or once the plants are transferred to calcareous soils.

In this study, we obtained mycorrhizal plants of *C. sativa* inoculated with different *Tuber* species aiming for the high quality that these plants would need to establish and grow in substrates rich in calcium and with a high pH, optimum conditions for truffle growth. Common nursery culture techniques do not include fertilizers since they can have a negative effect on the mycorrhizal levels (e.g., Dupré et al. 1982 in *T. melanosporum* mycorrhizae). However, more recent studies show that there are no changes in the mycorrhizal colonization of *Tuber* spp. under moderate applications of fertilizer (Benucci et al. 2012b, d; García-Barreda et al. 2016). Our study shows that using common techniques of controlled inoculation and relatively high levels of nitrogen fertilization, the production of quality seedlings of *C. sativa* with ectomycorrhizae of edible truffles like *T. aestivum/T. uncinatum* and *T. brumale* is possible. To our knowledge, this is the first time that sweet chestnut is recorded as a host for *T. brumale* and *T. macrosporum*.

So far, *T. macrosporum*, a truffle of increasing economic value (Benucci et al. 2016), has only been found in association with broadleaf hosts (Benucci et al. 2012b) and the synthesis of ectomycorrhizae using spore inoculum only described with oaks, hazel, and hornbeam (Giovannetti and Fontana 1981;

Zambonelli et al. 1993; Granetti et al. 2005; Vezzola 2005; Benucci et al. 2012d). The low percentages of ECM colonization in *T. macrosporum* seedlings in this study are similar to those described by Benucci et al. (2012c) in hazel and oak. Further inoculation trials are needed to standardize and optimize the production of high-quality mycorrhizal plants with this truffle in a routine nursery setting.

As opposed to *T. macrosporum*, *T. aestivum/T. uncinatum* and *T. brumale* formed abundant ectomycorrhizae in chestnut roots. *T. aestivum/T. uncinatum* have been recorded as symbiotic partners of many host species like *Quercus pubescens*, *Q. ilex*, *Q. robur*, *Q. faginea*, and *Corylus avellana* (Zambonelli and Govi 1983; Chevalier and Frochot 1990; Pruett et al. 2008; Donnini et al. 2014). Seedlings inoculated with the ecotypes *T. aestivum/T. uncinatum* did not show differences in ECM colonization levels and plant performance parameters; only in the case of yield of photosystem II, *T. aestivum* seedlings showed higher values, similar to those for *T. brumale* seedlings. *T. brumale* also forms ectomycorrhizae with several hosts including *Pinus pinea*, *Pinus nigra*, *Pinus halepensis*, *Q. pubescens*, *Q. ilex*, *Q. robur*, *Tilia platyphyllos*, and *C. avellana* (Palenzona et al. 1969, 1972; Chevalier 1973; Chevalier and Couteudier 1975; Zambonelli et al. 1993, 1995; Zeppa et al. 2005). For both *Tuber* species, the inoculation was successful and high root colonization levels were obtained.

ECM fungi have an influence in the morphology and development of their host roots and in their photochemical capacity, which can affect plant performance in plantations particularly in Mediterranean areas where drought and nutrient limitation are common (Villar-Salvador et al. 2012). ECM fungi enhance nutrient and water acquisition by the plants, having an influence in plant growth (Smith and Read 2008). Previous studies have shown that inoculation with *Tuber* species stimulates the development of the root system and total plant biomass (Bencivenga and Venanzi 1990; Nardini et al. 2000; Domínguez-Núñez et al. 2006). Larger aerial parts allow increased production of photosynthates leading to higher growth. However, water consumption increases due to increased respiration, which is compensated by a more developed root system and greater uptake of water by the mycelium (Nardini et al. 2000). In addition, nutrient demand from bigger plants would be higher, but ectomycorrhizae compensate with more efficient nutrient uptake from the rhizosphere (Smith and Read 2008).

Some differences were observed in the morphological parameters of the seedlings. The growth of the aerial part varied among the different *Tuber* mycorrhizal plants, and the plants inoculated with *T. brumale*, *T. aestivum*, and *T. uncinatum* showed a higher root biomass than the non-inoculated seedlings and the lower colonized *T. macrosporum* seedlings. These results agree with other studies where the inoculation of seedlings with *Tuber* spp. enhanced the development of the root system (Bencivega and Venanzi 1990; Nardini et al. 2000; Domínguez-Núñez et al. 2006).

T. brumale is able to use compounds from substrates rich in calcium for massively pumping this element to the roots (Ricard 2003; Valverde-Asenjo et al. 2009; García-Montero et al. 2012). This can poison the host plant leading to serious nutritional damage. The plant would then respond by stimulating and encouraging the greater growth of roots. Further nutritional studies on the host plants would be needed to confirm this, but together with their higher ECM colonization levels could explain the larger root systems of *T. brumale* seedlings. All seedlings, including controls, were fertilized during the experiments which might have masked the effect of *Tuber* inoculation; moreover, there might be a threshold of ECM colonization levels at which their effect is more noticeable in the plant's growth.

C. sativa is sensitive to high concentrations of calcium (Flórez et al. 2001) which might have resulted in the failure of *Tuber* survival once in the field in previous studies where the plants were planted in high pH soils (Belloli et al. 2001). In this study, despite the high concentration of calcium in the substrate, most of the inoculated plants did not show evident symptoms of nutritional deficiencies as reflected by photosystem II activity (Table 2). Under stress conditions, the ratio Fv/Fm can fall below 0.70 (Maxwell and Johnson 2000; Misra et al. 2001); this was only observed in control and *T. macrosporum* seedlings, reflecting the low ECM colonization levels of the latter that probably led to lower water and nutrient uptake (Bending and Read 1995; Griffiths and Caldwell 1992; Harley and Smith 1983). In Mediterranean environments, nutritional deficiency can lead to difficulties in growth, water management, and survival, which can be prevented by good fertilizer management in nursery (Jacobs et al. 2005; Oliet et al. 2009; Villar-Salvador et al. 2012). The fertilizer applied to the seedlings during the experiment has probably palliated potential nutrient deficiencies, more evident in un-colonized and poorly colonized plants as reflected by photosystem II activity. Water potential measurements at noon indicate a positive effect of *T. brumale* ectomycorrhizae in the water status of seedlings. This has been previously shown for *T. melanosporum* in situations of water stress, especially in summer, when this fungus enhances plant water status (Domínguez-Núñez et al. 2006, 2008, 2009).

More fertilization experiments in nurseries are needed to produce high-quality plants for forestry while well inoculated with *Tuber* spp., to avoid their failure to adapt to field conditions and to improve plant establishment in plantations and consequent truffle production. Improving the nutrient status of the plants while maintaining or enhancing the mycorrhizal colonization levels might lead to earlier and/or better truffle yields in plantations (Bonet et al. 2006).

In summary, the inoculation of *C. sativa* with *T. aestivum/uncinatum* and *T. brumale* at their optimum growth conditions

is feasible and does not decrease the quality of the plants, improving in general their performance. Therefore, multicropping *C. sativa* with edible truffles offers great potential, especially with *T. brumale*, which significantly improves plant performance in the nursery. Sweet chestnut seedlings mycorrhizal with *T. macrosporum* can also be produced; however, inoculation and culture protocols need to be improved to increase mycorrhizal colonization levels. Further studies are needed to better understand and manage these symbioses in chestnut. Long-term monitoring of plant performance in the field becomes essential to assess the stability of this plant-fungal relationship in plantations.

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