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ORIGINAL ARTICLE

Success and failures in the inoculation of five introduced trees in Chile with *Tuber magnatum* Pico: First advances for the domestication of the white truffle in South America

Éxitos y fracasos en la inoculación de cinco especies arbóreas introducidas en Chile con *Tuber magnatum* Pico: Primeros avances en la domesticación de la trufa blanca en Sudamérica

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ABSTRACT

Truffles are highly demanded edible fungi. They belong to the genus *Tuber* and have very high economic value, with *Tuber magnatum* being one of the most expensive and gastronomically used. This study evaluates the potential of five introduce tree species as mycorrhizal hosts for *T. magnatum* in Chile. Seeds from *Quercus cerris*, *Q. robur* and *Corylus avellana* were harvested from adult trees. Cuttings from *Populus nigra* and *Salix caprea* were harvested in the field and asexually propagated under greenhouse conditions. After two months, they were transplanted to 260 cc pots containing sterilized composted pine bark. A spore suspension (10^6 spores/plant) of *T. magnatum* was injected directly into plant roots. Three months after inoculation we observed spore germination and the presence of mycelium around the roots in some seedlings. After seven months, we observed mycorrhizae from *T. magnatum* only in *Q. cerris*, *Q. robur* and *C. avellana*, characterized by their epidermoid mantle and awl-shaped, bristle-like cystidia. The identification the mycorrhizal structures was confirmed by sequencing of the nuclear ITS-rDNA region. This study provide the first advances for the domestication of this highly valuable truffle in Chile and South America and the successful mycorrhized plants could be used in further field assays. Some *Quercus*, *Populus* and *Salix* species have been used as ornamental plants and are naturalized in Chile for over 100 years. Additionally, hazelnut (*Corylus avellana*) is currently grown in the country under intensive silviculture. These tree species could act as a non-intentional host for truffles, dispersed from production sites.

Keywords: *Corylus avellana*, ITS-rDNA, mycorrhizal synthesis, *Quercus cerris*, *Q. robur*, white truffles.

RESUMEN

Las trufas son hongos comestibles muy demandados. Pertenecen al género *Tuber*, siendo de alto valor económico, con *Tuber magnatum* uno de los más caros y usados gastronómicamente. Aquí se evaluó el potencial de cinco especies arbóreas introducidas como huésped de *T. magnatum* en Chile. Semillas de

Quercus cerris, *Q. robur* y *Corylus avellana* fueron recolectadas de árboles adultos. Segmentos de *Populus nigra* y *Salix caprea* se recolectaron en terreno y propagaron asexualmente en invernadero. Luego de dos meses, se trasplantaron a potes de 260 cc con compost de corteza de pino esterilizado. Luego, se inyectó una suspensión esporal (10^6 esporas/planta) de *T. magnatum* directamente en las raíces. Tres meses después, se observó germinación de las esporas y micelio alrededor de las raíces en algunas plántulas. Luego de siete meses, se observaron micorrizas de *T. magnatum* solo en *Q. cerris*, *Q. robur* y *C. avellana*, caracterizadas por su manto epidermoide y cistidios en forma de aguja. La identificación de las estructuras micorrícicas se confirmó con secuenciación de la región nuclear ITS-rDNA. Este estudio provee los primeros avances para domesticar esta trufa altamente valiosa en Chile y Sudamérica, y las plantas exitosamente micorrizadas podrían usarse en futuros ensayos de campo. Algunas especies de *Quercus*, *Populus* y *Salix* se usan en Chile como plantas ornamentales, naturalizadas desde hace más de 100 años. Adicionalmente, el avellano europeo (*Corylus avellana*) se cultiva en el país bajo silvicultura intensiva. Estas especies arbóreas podrían actuar como huéspedes no intencionales de trufas, dispersadas desde lugares de producción.

Palabras clave: *Corylus avellana*, ITS-rDNA, *Quercus cerris*, *Q. robur*, síntesis micorrícica, trufa blanca.

INTRODUCTION

Truffles are edible fungi with unique aromas (Mello *et al.* 2001; Paolocci *et al.* 2006). The genus *Tuber* includes 180 to 230 species distributed worldwide (Bonito & Smith 2016) and around 30 of them in Europe (Reyna 2000; De Miguel & Reyna 2007; Gregori 2007). Not all truffle species have culinary value and only approximately a dozen species have commercial interest (Bonito *et al.* 2010). Among them, *Tuber magnatum* Pico (Italian white truffle or Piedmont truffle), *Tuber melanosporum* Vittad. (Périgord black truffle), *Tuber aestivum* Vittad. (summer or Burgundy truffle) and *Tuber borchii* Vittad. (bianchetto truffle) hold the highest gastronomic value, with *T. magnatum* and *T. melanosporum* reaching the highest values in international markets (Hall *et al.* 2007; Zambonelli *et al.* 2010; Salerni *et al.* 2014; Reyna & García-Barreda 2014; Zambonelli *et al.* 2015). In France, Italy, Spain and Australia truffles are currently a multi-million industry (Reyna & García-Barreda 2014). Along their natural distribution, truffles are not only harvested in the wild but also in plantations using truffle inoculated seedlings (Reyna & García-Barreda 2014). Truffles not only harbor and economical value but also important social and ecological value (Benucci *et al.* 2012a) living in mycorrhizal symbiosis with the roots of suitable host plants (Paolocci *et al.* 2006; Zambonelli *et al.* 2012a; Bonito *et al.* 2013). *Tuber* spp. were thought to only form ectomycorrhizae (Zambonelli *et al.* 2015), but recently they have been found forming arbutoid mycorrhizae (Lancelloti *et al.* 2014), endomycorrhizas in orchids (Selosse *et al.* 2004) and they might also act as a root endophytes in non-ectomycorrhizal plants (Schneider-Maunoury *et al.* 2018).

Truffles naturally grow in areas with a particular weather, soil and vegetation, like those of the Mediterranean Europe (Reyna & De Miguel 2007, Sáez & De Miguel 2008). The loss and deterioration of their natural habitats (Benucci *et al.* 2012a) due to the reduction of traditional practices, over-harvesting, deforestation, successive wildfires and prolonged droughts have substantially decreased their production in the last decades (Moreno-Arroyo *et al.* 2005; Honrubia *et al.* 2006; Reyna 2007). As a consequence of this decline, establishing plantations of inoculated tree seedlings, compatible with *Tuber* spp., has allowed their culture (Meotto & Bassi 1994; Chevalier & Frochot 1997; Reyna & Colina 2007; Bencivenga *et al.* 2009; Benucci *et al.* 2012b; Zambonelli *et al.* 2015).

Tuber melanosporum was first cultivated in France in the 19th century (Olivier *et al.* 1996; Reyna & García-Barreda 2014) and it is currently cultivated worldwide, mainly in regions with Mediterranean-like climate (Reyna & García-Barreda 2014). More recently, other *Tuber* species have also been successfully cultivated, such as *Tuber aestivum* and its ecotype *T. uncinatum, Tuber borchii* and the *Tuber indicum* complex (Chevalier & Frochot 1997; Zambonelli *et al.* 2000; Hu *et al.* 2005). Much effort but without success has been devoted to *T. magnatum* (Gregori 2007; Bencivenga *et al.* 2009; Zambonelli *et al.* 2015). The failure of *T. magnatum* cultivation is partly due to the poor scientific knowledge gathered for this truffle during the past few decades (see Zambonelli *et al.* 2012b) and to the mischaracterization of *T. magnatum* in inoculated seedlings (Riccioni *et al.* 2016) which led to the presence of other truffle species in established plantations. In the present study we evaluate the potential of five non-native host trees species present in Chile as potential

hosts for *T. magnatum* with the aim of obtaining mycorrhizal plants in controlled conditions to be used in white truffle plantations. The tested tree species are commonly found in Chile as ornamental plants and for hazelnut production (*Corylus avellana*) and, if found suitable host for *Tuber*, could eventually form wild truffle populations.

MATERIALS AND METHODS

Plant material and inoculation of seedling/cuttings

Seeds of *Quercus cerris* L., *Quercus robur* L. and *Corylus avellana* L. were harvested from healthy individuals in Los Ángeles (Chile) and later stratified at 4°C for two months in moist conditions. Seeds were then superficially sterilized with 10% sodium hypochlorite solution for 5 min and sown in a Styrofoam seedbed filled with a vermiculite-based substrate. Cuttings of *Populus nigra* L. and *Salix caprea* L. were obtained from the field form healthy trees growing in the field. Cuttings were then asexually propagated in a greenhouse in plastic pots filled with river sand (particle size of 2-3 mm). The sand was previously autoclaved at 121 °C for 1 h during three consecutive days. Two months after seed germination and rooting of the cuttings, plant material was placed in 260 cc pots filled with previously steam-sterilized composted bark of *Pinus radiata* D.Don. Substrate pH (5.9 in natural condition) was set to 7.7 adding a commercial plant supplement (Magnecal 15 Inacal Company, Copiapó, Chile) containing $CaCO_3$ equival. > 95%, Mg 15-18%, CaO 31-36%, SO_3 < 0.50%, K_2O < 0.20%, Na_2O < 0.30%, and moisture 0.5%. Plants were then inoculated with a *Tuber magnatum* spore suspension (10^6 spores/plant) corresponding approximately to ~2 g of fresh previously identified ascomata, injected directly into the root system (Honrubia *et al.* 1995; Brundrett *et al.* 1996; Palazón & Barriuso 2007; Pereira *et al.* 2010). A *T. magnatum* ascoma (Fig. 1a, b) collected in a natural truffle-ground in Piamonte, Italy (Guzmán Gastronomía SL-Spain) and identified according to Pegler *et al.* (1993) was used as inoculum. The gleba from the ascoma was cut and dried at room temperature, grinded and stored until inoculum production (Honrubia *et al.* 1995; Palazón & Barriuso 2007; Pereira *et al.* 2010). A total of 25 seedlings/cuttings were inoculated for each plant species (125 plants in total). After inoculation, plants were grown for seven months in a growth chamber (photoperiod of 16/8-hours light/dark, RH 65 % ± 5, and T° = 24 ± 1 °C) and watered at field capacity once or twice per week, according to their developmental state. After the culture period, the mycorrhizal status of the plants was evaluated following previous protocols (Donnini 2005; Palazón & Barriuso 2007). We carefully extracted segments of long roots with adhering short roots from three different depths of the root system of each plant (proximally, intermediately, and distally in relation to the root collar). Roots were then cut in segments of 2 to 3 cm long. Fifteen inoculated and 15 control seedlings of each plant species were evaluated. Six hundred root tips, randomly selected from these segments, were screened under a dissecting microscope for mycorrhizal formation. The results were expressed in percent of mycorrhizal root tips.

Identification of ectomycorrhizae by morphological features

Fresh mycorrhizal root tips were observed under a dissecting microscope (Olympus SZ2-ILST) and a compound microscope (Olympus CX32) and documented by micro-photography using a Moticam 2000 2.0 Mpixel digital camera. Morphological and anatomical features such as color and surface structures of the mycorrhizae, as well as shape of cystidia and mantle layers, were determined according to Agerer (1986, 1991) and compared with reference descriptions of *T. magnatum* ectomycorrhizae (Zambonelli *et al.* 1993; Meotto & Bassi 1994; Rubini *et al.* 2001; Mello *et al.* 2001). Line drawings of diagnostic details were made with a Leitz Dialux compound microscope, equipped with a camera lucida drawing device at ×100 magnification of water-mounted, fresh specimens. Selected reference specimens corresponding to individual ectomycorrhizae were preserved in 2% CTAB at 4 °C for subsequent molecular analysis.

Molecular identification of ectomycorrhizae

Three to five mycorrhizae previously assigned to *T. magnatum* based on morphological characteristics were randomly selected from *C. avellana*, *Q. cerris and Q. robur* roots systems. The same number of root tips were randomly selected from *P. nigra* and *S. caprea* roots for molecular analyses. Genomic DNA was isolated using the E.Z.N.A. Fungal DNA miniprep kit (Omega Bio-Tek, Doraville, GA, USA) without mercapto-ethanol. Polymerase chain reaction (PCR) was performed using PuReTaq Ready-to-Go PCR beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The internal transcribed spacer region (ITS) of the nuclear ribosomal DNA was amplified using the fungal specific primer ITS1F (Gardes and Bruns 1993) and the eukaryotic primer ITS4 (White *et al.* 1990), following cycling conditions proposed by Martín & Winka (2000). A positive control consisting of DNA from a *T. magnatum* ascoma and a negative control without DNA were included. Amplicons were visualized in 2% agarose gels stained with SYBR® safe (Invitrogen, Eugene, OR, USA) under visible light. PCR products were then cleaned using the E.Z.N.A.® Cycle-Pure kit (Omega Bio-Tek) and sequenced bi-directionally. Sequences were edited with Sequencher v. 4.2 (Gene Codes Corp., Ann Arbor, MI, USA). The identification

of the sequences was performed through BLAST searches in GenBank (Altschul *et al.* 1990). The obtained ITS sequences were included in GenBank under the accession numbers MG992594, MG992595 and MG992596.

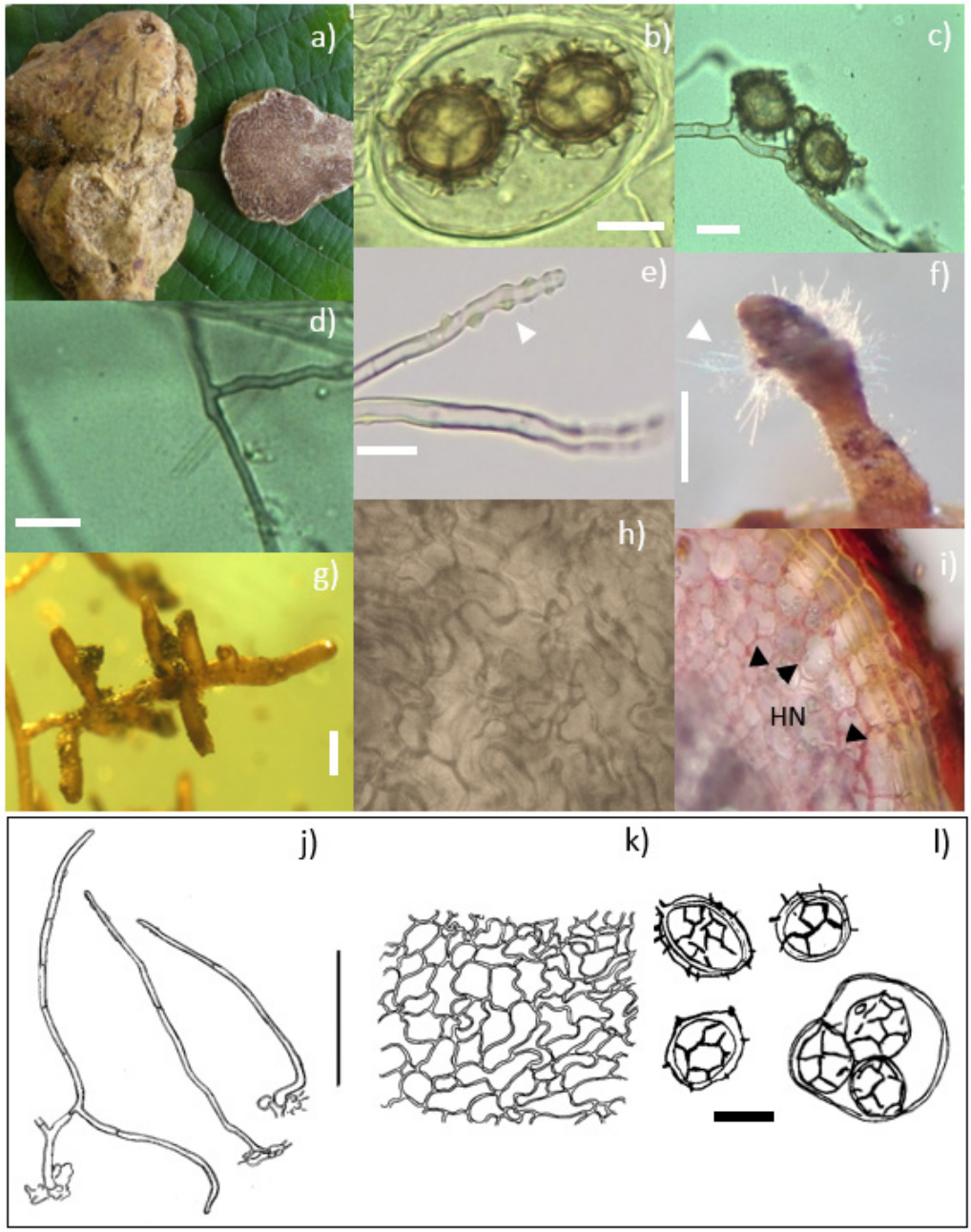

Figure 1. a) *Tuber magnatum* ascocarps, b) Asci and spores of *T. magnatum* (bar= 20 µm), c) Germinating spores of *T. magnatum* (bar= 20 µm), d) Segment of a cystidium with orthogonal ramification (bar = 20 µm), e) Colourless emanating hyphae with crystals (bar= 10 µm), f) Monopodial pinnate ectomycorrhizal (bar= 0.5 mm), g) Old ectomycorrhizae with no cystidia (bar= 0.5 mm), h) Outer layer of fungal mantle in plain view with epidermoid-pseudoparenchymatous cell pattern, i) Root cross section and Hartig net (HN, arrowhead), j-k) Diagnostic details of ectomycorrhizae formed by *T. magnatum* and *Q. cerris* (bar = 50 µm), j) Large cystidium, k) Outer layer of fungal mantle), and l) Spores (bar = 20 µm). / a) Ascocarpos de *Tuber magnatum*, b) Ascas y esporas de *T. magnatum* (barra= 20 µm), c) Esporas de *T. magnatum* germinando (barra = 20 µm), d) Segmentos de un cistidio con ramificación ortogonal (barra = 20 µm), e) Hifas incoloras emanando con cristales (barra = 10 µm), f) Ectomicorriza pinnada monopodial (barra = 0,5 mm), g) Ectomicorriza vieja sin cistidios (barra = 0,5 mm), h) Capa externa del manto fúngico con patrón celular epidermoide-seudoparenquimatoso, i) Sección transversal de la raíz y red de Hartig (HN, flecha), j-k) Detalles diagnósticos de la ectomicorriza formada por *T. magnatum* y *Q. cerris* (barra = 50 µm), j) Cistidios grandes, k) Capa externa del manto fúngico), y l) Esporas (barra = 20 µm).

Statistical analysis

Mycorrhizal colonization levels were compared among hosts by one-way ANOVA, and significant differences between them were determined using a Tukey test ($p<0.05$) for multiple comparisons (Steel & Torrie, 1989). Analyses were performed using the software STATISTICA 6.0 (Statsoft, USA).

RESULTS

Three months after inoculation with a *T. magnatum* spore solution, we detected the presence of mycelium of this belowground ascomycetes only in the root systems of specimens from *Quercus* and *Corylus*; we found germinated spores and growing mycelia with a simple septum and a branching pattern at approximately a right angle (90°) adjacent to the septum (Fig. 1c, d) with presence of crystals (Fig. 1e). Five months after inoculation, mycorrhizal structures typical of *Tuber* species were observed in secondary fine roots in *Q. cerris*. Ectomycorrhizae of *T. magnatum* were unbranched (simple, straight mycorrhiza, Fig. 1f) with infrequent pinnate monopodial structures (Fig. 1g), with or without cystidia. Young structures were light-brown/amber in color, turning red-brown when mature. Smooth surface, sometimes with hyphae, branching in approximately 90° with rounded tips of 1-2.5 mm long and 0.1-0.3 mm wide. Pseudoparenchymatous and epidermoid mantle formed by long cells with rounded ends (Fig. 1h, k). Hartig net extended to the third layer of cortical cells in the root (Fig. 1i). Seven months after inoculation, we observed ectomycorrhizae of *T. magnatum* in *Q. cerris*, *Q. robur* and *C. avellana*, but we did not find signs of root colonization by *T. magnatum* in *P. nigra* and *S. caprea*. We found the highest colonization levels in *Q. cerris* with a 32% of the root tips showing *T. magnatum* ectomycorrhizae, followed by *Q. robur* (21%) and *C. avellana* (17%) (Fig. 2). The ITS sequences obtained from the ectomycorrhizae in both oak and hazel confirmed their identity as Italian white truffle, showing maximum similarity with *T. magnatum* sequences from GenBank. We found ectomycorrhizas of *Geospora* sp. in the root system of *P. nigra* and *S. crapea*, but we did not observe symbiotic association with *T. magnatum*.

Substrate organic material was 31.5%. Once substrate pH was corrected (5.9 to 7.7), we measured a decrease in N and in the C/N ratio. We also observed an increase in P, K, Ca, Mg and in electric conductivity from 0.13 to 0.56 dSm^{-1} (Table 1).

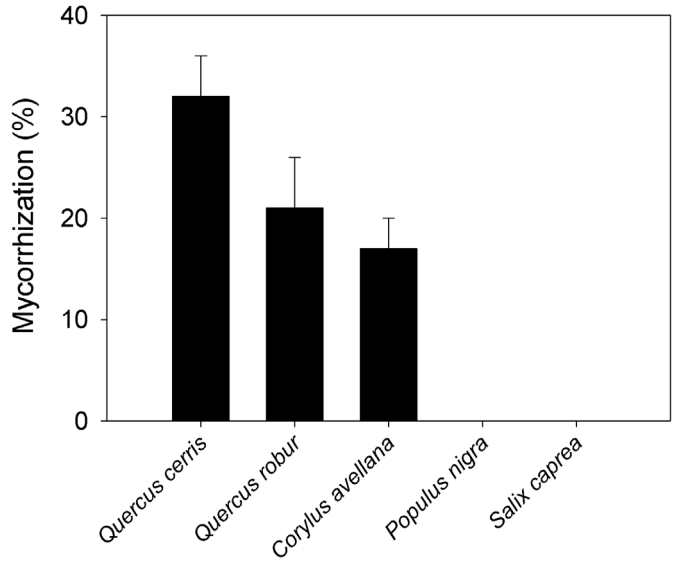

Figure 2. Percentage of mycorrhizal roots in *Q. cerris*, *Q. robur*, *C. avellana*, *P. nigra* and *S. caprea* nurse plants. / Porcentaje de raíces micorrizadas en plantas nodrizas de *Q. cerris*, *Q. robur*, *C. avellana*, *P. nigra* y *S. caprea*.

Table 1. Chemical properties of pine bark substrate used for inoculation of woody plants with *T. magnatum* before and after liming. OM=organic matter; EC=electric conductivity. / Propiedades químicas del sustrato de corteza de pino usado para la inoculación de plantas leñosas con *T. magnatum* antes y después de aplicar cal. OM = materia orgánica; EC = conductividad eléctrica.

Liming	pH	OM (%)	Total Nitrogen (%)	P_2O_5 (%)	K_2O (%)	CaO (%)	MgO (%)	C/N	EC dS m^{-1}
Before	5.9	31.5	0.60	0.17	0.19	1.42	0.84	38.3	0.13
After	7.7	31.5	0.44	0.20	0.24	7.02	1.43	28.5	0.56

DISCUSSION

The most important technological advance in truffle cultivation has been the large scale production of quality inoculated plant seedlings (Chevalier & Pargney 2014). Modern truffle cultivation is based on planting high quality colonized plants grown under controlled conditions in greenhouses into locations with suitable soils and climates (Zambonelli *et al.* 2010, 2015). The spore inoculation technique used in this study is the most commonly used method for producing *Tuber*-colonized plants because of its simplicity and the impossibility of obtaining enough mycelium

due to its slow growth in *Tuber* (Mello *et al.* 2006; Zambonelli *et al.* 2010; Hall & Zambonelli 2012). Mycelium as inoculum is only used for a reduced group of truffle species (Zambonelli *et al.* 2010; Lancellotti *et al.* 2014; Iotti *et al.* 2016) which could allow to select the fungal strains adapted to specific climatic, edaphic conditions and hosts (Zambonelli *et al.* 2010, 2015). *Tuber magnatum* produces the world's most expensive truffle (Murat *et al.* 2005; Mello *et al.* 2006; Zambonelli *et al.* 2012b; Iotti *et al.* 2014; Riccioni *et al.* 2016). This species is distinct from other *Tuber* species for the unique aroma of its ascomata, its limited distributional range, and the difficulties of its cultivation (Mello *et al.* 2001; Riccioni *et al.* 2016). Much effort has been devoted to the artificial culture of *T. magnatum*, but without success (Gregori 2007; Bencivenga *et al.* 2009; Benucci *et al.* 2012a).

To our knowledge, this is the first successful artificial mycorrhization with *T. magnatum* in South America, and Chile in particular. Five months after inoculation *Quercus* and *Corylus* seedlings showed *T. magnatum* ectomycorrhizae (between 17-32%) without detectable presence of other mycorrhizal fungi colonizing the same roots systems. These three species have been previously described as hosts of *T. magnatum* (Mischiati & Fontana 1993; Meotto & Bassi 1994; Bencivenga *et al.* 2009). Unlike other edible truffle species, the mycorrhization with *T. magnatum* is a very difficult and delicate process, usually hard to reproduce in different sites/ conditions (Rubini *et al.* 2001; Zambonelli *et al.* 2012a; Benucci *et al.* 2012a; Riccioni *et al.* 2016). In fact, Mello *et al.* (2001) suggests that *T. magnatum* has a lower ability to form ectomycorrhizas compared to other *Tuber* species, possibly related to low spore germination (Gregori 2002; Hall & Zambonelli 2012; Murat 2015). However, three months after inoculation, we detected germinating spores of *T. magnatum* in the root system of the plants (see Fig. 1c). Riccioni *et al.* (2016) state that difficulties in obtaining genuine *T. magnatum* ectomycorrhizas on nursery-inoculated host plants might result from the scarce competitive ability of this fungus and/or from the ability for sustaining these structures on their hosts over several months at field conditions. Few studies obtained ectomycorrhizae of *T. magnatum* in controlled conditions (Mischiati & Fontana 1993; Glamoclija 2000; Mello *et al.* 2001; Rubini *et al.* 2001). We obtained higher mycorrhizal levels (17 and 32%) that Mischiati & Fontana (1993) for *Tilia cordata* (12,5%), *Q. robur* (11%) and *Corylus avellana* (16,5%) using mycelium as inoculum. These are relatively low compared to previous reports on other *Tuber* species. For example, *T. borchi* can reach colonizations levels of 62%, *T. aestivum* of 42%, and *T. melanosporum* of 74% in controlled conditions (Benucci *et al.* 2011; Benucci *et al.* 2012b; Pereira *et al.* 2013). However, due to the technical difficulties in *T. magnatum* cultivation, the obtaining of mycorrhizal plant can be considered a success, despite the relatively low percentage.

The optimum level of mycorrhizal colonization in the roots mostly depends on the climatic and soil characteristics of the plantation site, which affect competitiveness, extent and rate of colonization of the inoculated species (Iotti *et al.* 2012a). Zambonelli *et al.* (2005) showed that a 30% initial rate of root colonization with *T. aestivum* reached values of 50–70% in mycorrhizal seedlings after 5 years when planted in a suitable soil. *Tuber magnatum* is a particularly intriguing ectomycorrhizal fungus: it produces ectomycorrhizae in controlled conditions (Iotti *et al.* 2012b), but they are rarely found in the field in productive truffle-grounds (Murat *et al.* 2005; Bertini *et al.* 2006; Mello *et al.* 2006; Zambonelli *et al.* 2012b; Iotti *et al.* 2014). *Tuber magnatum* mycorrhizae area also absent in natural productive areas (Leonardi *et al.* 2013; Salerni *et al.* 2014) and when present, mycorrhization percentage in usually below 5% (Murat *et al.* 2005; Bertini *et al.* 2006). In fact, the mycorrhizas of this truffle seem to disappear when the plants are transplanted in the field (Hall *et al.* 2007). In contrast, other species of *Tuber* such as *T. rufum*, *T. brumale*, *T. maculatum* and *T. borchii* can achieve a mycorrhization of 9,6 to 48,9 % in the field (Murat *et al.* 2005; Bertini *et al.* 2006; Iotti & Zambonelli 2006).

The Italian white truffle can forms ectomycorrizae in roots of poplars, willows, oaks, aspen, alder and hazelnut (Meotto *et al.* 1992; Donnini *et al.* 2000; Murat *et al.* 2005; Bencivenga *et al.* 2009; Luigi *et al.* 2010). However, seven months after inoculation, seedlings of the Salicaceae species *Populus nigra* and *Salix caprea* did not show colonization of *T. magnatum*. In our study, cuttings of *P. nigra* and *S. caprea* developed highly lignified root systems, which were probably not suitable for the mycorrhizal association with *T. magnatum*. Additionally, in these two genera, ectomycorrhizas of *Geopora* were found. Fungal species such as *Sphaerosporella brunnea* and *Pulvinula constellatio* can be frequent contaminant in the production of mycorrhizal plants inoculated with *T. magnatum* as well as other truffle species such as *T. maculatum* and *T. borchii* (Amicucci *et al.* 2001; Bertini *et al.* 2006; Riccioni *et al.* 2016). Inoculum quality is one of the most important requirements when *Tuber* infected plants are produced (Zambonelli *et al.* 2010). When large quantities of ascomata are used to prepare inoculum, operators may accidentally incorporate less valuable species of white truffles which can then become established on the host plants (Zambonelli *et al.* 2010; Murat *et al.* 2016). *Tuber magnatum* mycorrhizas can be barely distinguished morphologically from those of other related white truffles (Mello *et al.* 2001), therefore DNA-base identification is needed (Riccioni *et al.* 2016). The introduction of molecular methods for the identifications has allowed to

detect less valuable *Tuber* species such as *T. maculatum* and *T. borchii*, in *T. magnatum*-inoculated plants (Iotti *et al.* 2012a; Zambonelli *et al.* 2015).

The type of soil used to grow inoculated plants affects the rate of root colonization by target fungal species and potential contaminants (Iotti *et al.* 2012a). Various substrate types have been tested during the development of truffle culture, ranging from natural soil from truffle woodlands to different types and mixtures of natural and artificial components such as soil, peat, perlite, vermiculite, sand, dolomite, and varieties of compost (Palazon & Barriuso 2007; Iotti *et al.* 2012a). However, specific details about the composition of the potting mixes, inoculum amounts, amendments, and greenhouse conditions vary among nurserymen, and most remain a trade secret (Hall *et al.* 2003). The results of our assay confirm that compost of *P. radiata* bark, a cheap and available product in forestry industry, is an adequate substrate after an adjustment of its natural pH of 5.9 by application of lime such as Magnecal 15 which consists mostly of $CaCO_3$ and other mineral components that add important micronutrients during the nursery phase of the inoculated seedlings. *P. radiata* plantations occupy a large area in Central-South Chile and this could be a potentially interesting use for its bark. The incomplete composting of the pine bark may have a negative impact on seed germination and seedling growth and thus hamper the establishment of mycorrhizal symbiosis but this does not seem to be the case in our study where the substrate pH was adjusted to 7.7, suitable for synthesis of *T. magnatum* mycorrhizae. In general, truffle species prefer low organic matter mixtures supplemented with calcium carbonate components (Pruett *et al.* 2009) to raise pH to ~8.0 (Iotti *et al.* 2012a). Organic matter content in the substrate used here was 31.5%, which can be considered relatively high. In Europe, soils from areas where *T. magnatum* naturally occur have been used for the cultivation of mycorrhizal plants (Iotti *et al.* 2012a), with the associated economic and environmental costs that this practice implies. These natural calcareous soils in Italy have a pH close to 8 and organic matter content range from close to 2.5 to 3% (Bencivenga & Baciarelli 2012). Conductivity reflects the concentration of soil solution salts in the substrate; a high conductivity may be due to the substrate material but also to excessive fertilization, which can negatively affect plant performance and mycorrhizal formation. Low levels of this parameter are recommended for truffle culture; in fact, natural truffle grounds have not been found in saline or chalky soils (García-Montero *et al.* 2007).

The artificial culture of truffles, especially *T. melanosporum*, is common in different parts of the world (Hall *et al.* 2007; Chevalier & Pargney 2014; Reyna & García-Barreda 2014; Zambonelli *et al.* 2015; Murat *et al.* 2016; Zambonelli *et al.* 2017). In Chile, there are close to 400 ha of *T. melanosporum* currently in production. The new challenge for the country is the introduction, and future production of other *Tuber* species, especially those with high demand and economic value in international markets. Here, we contribute to the understanding of the controlled mycorrhization of tree species with *T. magnatum*, using a composted *P. radiata* bark as a substrate, after pH adjustments though liming. Additionally, we described the macroscopic and microscopic characteristics of the mycorrhizal association between *T. magnatum* and *Q. cerris*. Future studies should test longer viverization periods to increase mycorrhization percentage. Additionally, field assays are required to evaluate the behavior of the mycorrhizal fungi in natural soils with artificially corrected pH. This information could be used in the future domestication and production of white truffle, and other edible truffles, in Chile. Additionally, *Quercus*, *Populus* and *Salix* species are commonly used as ornamental plants and urban trees and are common in rural settings. These species have been present in Chile for over 100 years and are naturalized. *C. avellana* plantations are also present in Central-South Chile. It should be taken into account that *Tuber* spores (or other types of inoculum) could be dispersed from production sites into compatible host trees in urban or rural areas. The ecological consequences of this potential formation of wild populations of truffles should be further studied. However, due to the host-specificity and the pH requirements of *Tuber magnatum* we hypothesize that an ecological impact on native plants and/or fungi is unlikely. This truffle, as stated above, require basic soils which are not usually found in Chile.

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