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ORIGINAL PAPER

Mycorrhization of Pecan trees (*Carya illinoinensis*) with commercial truffle species: *Tuber aestivum* Vittad. and *Tuber borchii* Vittad

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Abstract Pecan (*Carya illinoinensis*) is an economically important nut tree native to the Mississippi basin and cultivated worldwide. In North America, species of truffles are regularly found fruiting in productive pecan orchards and the truffle genus *Tuber* appears to be abundant in pecan ectomycorrhizal (EM) communities. As an initial step to determine the feasibility of co-cropping European truffle species with pecan, we evaluated whether mycorrhizae of highly esteemed European truffle species (*Tuber aestivum* Vittad. *T. borchii* and *T. macrosporum*) could be formed on pecan seedlings. Seedlings were inoculated with truffle spores and were grown in a greenhouse for 10 months. Levels of EM colonization were estimated visually and quantified by counting EM tips. Ectomycorrhizae were identified both morphologically and molecularly with species-specific amplification and by sequencing of the ITS region of the nuclear ribosomal DNA (nrDNA). Both *T. borchii* and *T. aestivum* spores produced well-formed ectomycorrhizae on pecan seedlings with average root colonization levels of about 62% and 42%, respectively, whereas no ectomycorrhizae of *T. macrosporum* were formed. The anatomy and morphology of these truffle ectomycorrhizae on pecan was characterized. The co-cropping of *T. aestivum* and *T. borchii* may hold promise as an additional stream of revenue to pecan growers, although, further studies are needed to assess whether this symbiosis is maintained after planting in the field and whether truffle production can be supported by this host species.

Keywords *Carya illinoinensis* · Truffle inoculations Co-cropping · *Tuber aestivum* · *Tuber borchii*

Introduction

Truffles are hypogeous fungi, the most famous of which belong to the genus *Tuber* (Pezizales, Ascomycota). Truffles undergo a complex life cycle (Paolocci et al. 2006). After their spores germinate, the growing mycelium must establish a mutualistic ectomycorrhizal (EM) symbiosis with the roots of forest trees (e.g., spp. of *Quercus*, *Corylus*, *Salix*, *Pinus*) or shrubs, such as *Cistus* spp. (Bustan et al. 2006; Fontana and Giovannetti 1978–1979; Hall et al. 2007; Harley and Smith 1983; Norris et al. 1994). Truffles are formed through obligate outcrossing of opposite mating types, although details pertaining to fertilization events are not completely resolved at this time (Riccioni et al. 2008).

Some truffles, especially European ones, are highly valued economically due to their unique organoleptic aroma. Accordingly, they are marketed worldwide. Among these prized species, the winter fruiting species *Tuber magnatum* Vittad. (white truffle) and *T. melanosporum* Vittad. (black truffle) are naturally confined to limited areas of southern Europe. Other species such as *T. aestivum* Vittad. (syn *T. uncinatum* as reported by Wedén et al. 2005) and *T. borchii* Vittad. are widely distributed throughout

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Europe (Bertault et al. 1998; Gryndler et al. 2011). *Tuber borchii* is reported from southern Finland to Sicily and from Ireland west to Hungary. *T. aestivum* is reported latitudinally from Spain and westward across Eastern Europe and China, and longitudinally from Gotland (Sweden) to North Africa (Song et al. 2005). *T. aestivum* is considered the most widespread truffle species in Europe (Gryndler et al. 2011; Hall et al. 2007). In some countries (e.g., Italy, France, New Zealand, United States), *T. aestivum* and *T. borchii* cultivation is feasible and truffles are successfully produced (Chevalier and Frochot 1997; Zambonelli et al. 2002).

An established market for *T. borchii* and *T. aestivum* exists despite the fluctuating price of truffles year by year. In Italy, about the 65% of truffles reaching the processing industry (approximately 80% of the national production of truffles) is attributed to *T. aestivum*, while approximately 10% belongs to *T. borchii* (Pampanini and Martino 2006). The market value of truffles harvested during the season is strongly mediated by weather conditions and annual production levels, but in central Italy for the last year, fresh fruiting bodies of *T. aestivum* were sold for between $110 and $250 per kg, while fresh *T. borchii* sold for $110 up to $180 per kg (commercial truffler; personal information).

Another European truffle species that merits mention is *Tuber macrosporum* Vittad. for several reasons: (1) its excellent organoleptic characteristics and aroma resemble that of the esteemed white truffle (*T. magnatum*); (2) it is widely distributed across Europe; (3) some Italian nurseries have started to produce plants that have been inoculated with *T. macrosporum* spores and several truffle orchards have already been established with this species (Benucci et al. 2011; Vezzola 2005). Although rare in France and Great Britain, *T. macrosporum* appears to be common in Serbia, Hungary, and Romania, and is frequently collected in Italy and Slovenia. There are also reports of *T. macrosporum* in Germany, Czech Republic, Swiss, Ukraine, Croatia, Slovakia, Serbia, and Montenegro (Gógán et al. 2011; Marjanovic et al. 2010). *T. macrosporum* was also recently collected in Turkey (M Bencivenga, personal information).

Truffle cultivation begins by inoculating axenically raised seedlings with truffle spores and, after ectomycorrhizae have formed, out-planting the truffle-infected seedlings into fields managed to meet the ecological requirements of both the truffle fungus and the plant host. This technique (and variations of it) is considered routine today and is used worldwide (Granetti et al. 2005).

At present, nearly all commercial truffle orchards are specialized for the production of truffles alone: host plants are grown and cared for with the sole purpose of promoting and increasing the yield of truffles. Literature on co-cultivation of truffles and economic tree species in which the production of wood or fruits are taken into account are completely lacking. Popular host choices for growing European species of truffles are European species of oak, poplar, willow, and hazelnut with which these truffle species appear to be associated with in nature. In Italy, orchards are often established on marginal lands with low fertility and steep slopes, and many times are inaccessible to farm machineries.

Nevertheless, there are cases where truffles are harvested alongside other commercial crops. For instance, some economically productive hazelnut plantations in Spain also support natural fruitings of *T. melanosporum* and *T. brumale* (Reyna Doménech 2007). In another case, plantations of *Populus* spp. established for wood production in Northern Italy became naturalized in the field by *T. borchii* and *T. magnatum*, and now truffles fruit in these plantations (GMN Benucci, personal observation).

Given that truffle cultivation requires relatively low agricultural inputs (in Europe), Bonet et al. (2006) suggested *T. melanosporum* mycorrhized plants as a valid way to promote reforestation and economic restoration of rural lands and land-use stability.

Recently, Bonito et al. (2011a) showed that truffles are dominant in EM communities of commercial pecan orchards in North American. Ever since the discovery of *Tuber texense* Heimsch with pecan (Heimsch 1958), it has been known that pecan trees support the fruiting of truffles. This truffle species, later synonymized with *Tuber lyonii* Butters by Trappe et al. (1996), is native to North America and is distributed across at least of 13 US states, and Northern Mexico (Bonito et al. 2011a; Hanlin et al. 1989; Taber 1988). Bonito et al. (2011a) hypothesize that pecan orchards could be managed to optimize both truffle and pecan production, given its prevalence in pecan orchards. Despite the fact that limited quantities of *T. lyonii* are sold commercially and are served in restaurants, the market is still struggling because of several factors: (1) its aroma is not as pungent as prized European *Tuber* species; (2) its occurrence and natural production levels fluctuate from year to year, making it difficult to provide a consistent truffle supply to the market; and (3) truffles are harvested by raking, so a mixture of mature and less aromatic immature truffles are collected and marketed, which adversely affects their reputation (Bonito et al. 2011a). Successful mycorrhization of pecan seedlings with European truffle species (e.g., *T. borchii* and *T. aestivum*), which have a flourishing market, could represent the first step for further co-cropping efforts.

Pecan [*Carya illinoinensis* (Wangenh.) K. Koch] belongs to Juglandaceae and is native to the Mississippi basin in North America. *C. illinoinensis* is important agronomically for its nuts (drupe, technically), particularly in the southern

USA. Pecan production in the USA alone is valued at over $433 million (USDA 2008). Large productive orchards have also been established in Brazil, Israel, and Australia (Wakeling et al. 2001).

In Italy, cultivation of *C. illinoinensis* is limited to small plots in southern regions such as Sicily and Puglia and the nuts are marketed locally. Nevertheless, increased plantings of pecan has been proposed to promote timber wood production in Sicily (Raimondo et al. 2009).

In view of co-cultivation systems, *C. illinoinensis* is an attractive option for the production of nuts, truffles, and wood at the end of the cultivation cycle. It is currently not known whether pecan trees planted in Europe associate with European truffle species.

In this study, we aimed to: (1) determine whether ectomycorrhizae of the European truffle species (*T. borchii*, *T. aestivum* and *T. macrosporum)* can be formed on pecan trees using spore slurry inoculations; (2) verify the effectiveness of distinguishing truffle ectomycorrhizae on pecan by morphological and molecular techniques; (3) quantify the level of EM colonization on pecan root systems by comparing two different morphological approaches for the evaluation of the ectomycorrhizal colonization levels; and (4) describe the anatomical and morphological features of European truffle ectomycorrhizae on pecan. We were particularly interested in studying *T. aestivum* and *T. borchii* because they are adapted to a broad range of climatic and soils conditions compared to *T. melanosporum* and *T. magnatum* (Hall et al. 2007; Iotti et al. 2010). Moreover, both *T. aestivum* and *T. borchii* are successfully cultivated (Chevalier and Frochot 1997; Zambonelli et al. 2002) and plants inoculated with these truffles can easily be produced in nursery conditions. This is not yet routine for the esteemed white truffle, *T. magnatum* (Bertini et al. 2005).

Material and methods

Pecan seedling production and truffle spore-slurry inoculation

Pecan seeds were collected from commercial pecan orchards in North Carolina, USA, and were sent to Italy for inoculation studies. Upon arrival, they were soaked in tap water for 10 days at room temperature, changing the water daily to maintain aerobic conditions prior to stratification (Adams and Thielges 1978). For stratification, pecan seeds were placed in zip-lock plastic bags and were stored at 4°C for 30 days. After stratification, seeds were surface sterilized by soaking in a 5% sodium hypochlorite solution for 20 min, rinsed, set in a mix of 50% sterile perlite and 50% sterile vermiculite and placed in a nursery until germination. Ascocarps of *T. borchii*, *T. aestivum* and *T. macrosporum* (collected from central Italy and Hungary, generously provided by truffle collectors) were washed under cold tap water and after accurate morphological identification were stored in plastic bags at −20 ° C. Specimen samples of each truffle were preserved in the Herbarium Universitatis Civitatis Perusii with the following IDs: PERU-182411; PERU-182511; PERU-182611. Spore inocula were prepared by blending frozen truffles for 5 min.

In the summer of 2010, pecan seedlings were selected and cut back to two leaves; then, their taproot was removed and they were transplanted into 9×9×13 cm pots containing a potting mix consisting of sterile natural soil (see Table 1 for soil physicochemical properties), sterile sand, sterile vermiculite and sterile perlite (5:1:2:2). The potting mix was amended with 2g/l NPK (nitrogen, phosphorus and potassium) slow-release fertilizer.

For inoculations, 2g of truffle spore-slurry were dispensed to each seedling. Each of these 2-g doses were diluted in 10 ml tap water and distributed, prior to planting,

Table 1 Physical and chemical characteristics of the natural soil used in the potting mix formulation

Soil texture parameter	Equivalent diameter size (mm)	Units	Quantity
Stone	>2	g/kg	37
Sand	2–0.05	g/kg	325
Silt	0.05–0.002	g/kg	325
Clay	<0.002	g/kg	350
Soil chemical parameter		Units	Quantity
pH_{KCl}	–	pH units	7.4
pH_{H2O}	–	pH units	8.1
Total lime	–	g/kg	180
Soluble $CaCO_3$	–	g/kg $CaCO_3$	88
Organic matter	–	g/kg	21.88
Total nitrogen	–	g/kg	1.42
Assimilable phosphate	–	mg/kg P_2O_5	20.12
Exchangeable potassium	–	meq/100 g	0.76

Soil analyses were carried out according to the government rules on official methodology for soil analysis (Ministero per le Politiche Agricole 1999)

into the transplanting hole immediately below the rooting zone. After mycorrhization procedures were completed, 12 seedlings of each treatment were grouped in plastic pot containers holding 15 pots, each with three negative controls (no inoculum added). These were grown in three blocks side by side in an isolated part of an unheated greenhouse until spring 2011 (Fig. S1).

In total, 36 seedlings were inoculated: 12 with *T. aestivum*, 12 with *T. borchii* and 12 with *T. macrosporum.* They were watered by sprinkling two times a week during the months of June and August, once a week in all other months of their stay in the greenhouse.

Mycorrhizal colonization levels and morphology of ectomycorrhizae

Mycorrhizal colonization levels were tested 6 months after inoculation in two seedlings of each truffle species. Due to the low presence of ectomycorrhizae we decided to let the plant grow for an additional 4 months. In spring 2011, the remaining ten pecan seedlings inoculated with truffle spores were chosen for mycorrhizal evaluation; their stem length, stem diameter, and root system length were also measured. To visualize mycorrhizae, roots were first gently washed in cold tap water and were placed in a glass Petri dish contained distilled water (Avis et al. 2003). EM colonization levels were estimated using two different approaches: (1) visual evaluation (VE) by estimating the percentage of ectomycorrhizae present in the whole root system visually under a stereomicroscope microscope or (2) relative abundance valuation (RAV) following the methodology described by Bencivenga et al. (1995), which nowadays is the standard quality test for truffle-infected plants produced by commercial nurseries in some Italian regions. To calculate the RAV, the root system is divided into two sections: one proximal and one distal. Four portions of the root system are selected for each of these. In every portion, 50 root tips are counted starting from the end of the root, for a total of 400 root tips examined for each seedling. The numbers of mycorrhizal and non-mycorrhizal root tips are then tallied. Non-mycorrhizal tips values were used to determine percentage of EM presence. Mycorrhizae were first observed under a stereomicroscope (Leica Leitz Wild MZ8) and then with a light microscope (Leica Leitz DMRB). Each ectomycorrhiza was considered as a single individual (Bruns 1995; Dickie et al. 2002). EM description and digital photographs were made according to Agerer (1991).

Statistical analyses

Shapiro–Wilk and Anderson–Darling tests (α=0.05) were used to assess whether the data were normally distributed. Significant differences in seedling dimensions (stem length, root system length and stem diameter) and mycorrhizal colonization levels between different inoculation treatments were detected by ANOVA. Tukey's test was used to identify significant differences between means ($p<0.05$).

Molecular analyses of ectomycorrhizae

Genomic DNA was extracted from single ectomycorrhizae at various developing stages, capturing a range of ectomycorrhizae color and mantel thicknesses, and from a pool of ten ectomycorrhizae with or without peritrophic elements (cystidia and emanating hyphae) and coming from different seedling. Extractions were performed with the Extract-N-Amp kit (Sigma). The manufacturer's protocol was followed except that only 20 μl of the extraction and dilution buffer each was used in extracting from individual tips (rather than the 100 μl of each solution as recommended by the manufacturer for extracting DNA from plant leaves).

DNA extracts were then amplified with the ITS1/ITS4 primer pair (White et al. 1990) to amplify the ITS1, 5.8S, and ITS2 regions of the nuclear ribosomal DNA (nrDNA), and with species-specific TBA/TBB primer pair for *T. borchii* (Mello et al. 1999) and species-specific UNCI/UNCII primer pair for *T. aestivum* (Mello et al. 2002). Each 50-μl PCR reaction consisted of 10× PCR buffer (Invitrogen), 200 μM dNTPs, 20 mg of bovine serum albumin, 10 pmol of forward and reverse primer, and 1.75 U of *Taq* DNA polymerase (Invitrogen). PCR thermoprofiles consisted of an initial denaturation of 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, 58°C annealing for 30 s for ITS1/ITS4 (60°C for UNCI/UNCII and 72°C for TBA/TBB with an extension of 1 min) and with a final extension at 72°C for 10 min. Ten microliters of PCR products were run on 1.8% (w/v) agarose gel stained with ethidium bromide. For sequencing the ITS region, amplified products were purified using an Illustra GFXTM PCR DNA kit (GE Healthcare), sequenced in both directions using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and run on an ABI 3130XL DNA Analyzer. Single strand ITS sequences of the same PCR product were merged into a contig with ContigExpress ® Module (Vector NTI Suite, Invitrogen). The similarity of ITS sequences to sequences in GenBank was assessed using the BLASTN algorithm at the NCBI website (National Center for Biotechnology Information; website: http://blast.ncbi.nih.gov/Blast.cgi) (Altschul et al. 1997). Representative sequences from ectomycorrhizal tips have been submitted to GenBank (Benson 2004) with the following accession numbers: JN040498 for *T. borchii* and JN040499 for *T. aestivum*.

Results

Pecan seedlings survivorship, growth and truffle mycorrhization levels

Forty-seven of the 98 stratified seeds germinated (47.9%). Germination happened slowly over a 30-day period. Nonetheless, after a few months the seedlings looked similar in growth and health (Fig. S2).

In April 2011, the remaining ten seedlings from each inoculation treatment were assessed for the mycorrhizal colonization levels. Seedlings had very good aboveground development and looked healthy and well-structured. No statistical differences were detected in the stem length, stem diameter or root system length between *T. borchii*, *T. aestivum* and *T. macrosporum* trials (Table 2).

Seedlings inoculated with *T. borchii* and *T. aestivum* were well colonized with ectomycorrhizae; however, mycorrhizae were absent in roots of seedlings inoculated with *T. macrosporum*.

We did not find any ectomycorrhiza of fungal contaminants in any of the treatment or control seedlings. The level of mycorrhization obtained with VE and RAV approaches gave similar results, and were not statistically different (ANOVA, $p<0.05$). Data obtained by the RAV method showed the average percentage of EM colonization was statistically higher for *T. borchii* (≈0.62) than for *T. aestivum* (≈0.42) (Table 2) (ANOVA, $p<0.05$). Eight of ten seedlings inoculated with *T. borchii* showed >50% EM relative abundance with a maximum value of≈79% and a minimum of≈34%, while only four of ten seedlings inoculated with *T. aestivum* showed >50% of relative abundance with a maximum value of≈83% and a minimum values of≈8%.

Morphological and anatomical traits of *T. aestivum* and *T. borchii* ectomycorrhizae on pecan

Morphological and anatomical traits of *T. aestivum*×*C. illinoinensis* and *T. borchii*×*C. illinoinensis* ectomycorrhizae are reported in Figs. 1 and 2. Measurements for specific anatomical characters are provided in Table 3. The majority of *T. borchii* and *T. aestivum* ectomycorrhizae were well formed on pecan seedlings 10 months after they were inoculated.

T. borchii ectomycorrhizae on pecan are simple or ramified in a monopodial-pinnate pattern (Fig. 1a, b). Single EM tips are straight, fragile, cylindric or club shaped with rounded ends; the color varies considerably as they develop, from light yellow with pale grey shades in the youngest to dark ocher or brownish of the oldest (Fig. 1b, c, d). The mature ectomycorrhizae frequently had a whitish growing tip apex (Fig. 1a, b). Cystidia are the typical of *T. borchii* ectomycorrhizae (Giomaro et al. 2000). They are needle-shaped, simple or connected at the base, smooth, colorless and generally mono-septate (Fig. 1e, g). Cystidia are well distributed over the entire surface of the ectomycorrhiza, preferably in the proximity of the apex that is under development. From our observations the presence of cystidia is not connected to the growth stage of the ectomycorrhiza because both young than old ones could show these emanating elements (Fig. 1b, c). The pseudoparenchymatic mantle type is arranged in few cell layers (4–6) and Hartig net that penetrate the root epidermids until almost second cell layer (Fig. 1f). The outer mantle surface is densely short-spiny, composed by epidermoids cells structured in a uneven regular puzzle-like pattern; cells are variable in shape but less in size (Table 3), varying from ellipsoid and isodiametric to irregular-rectangular and elongated (Fig. 1h).

T. aestivum ectomycorrhizae are almost all simple, sometimes with ramifications present following either a regular monopodial-pinnate or monopodial-pyramidal pattern (Fig. 2a). Simple EM tips are club-shaped or rarely cylindric, with rounded ends. Their color varies with developmental stage, from light amber in the younger ectomycorrhizae to dark brown in the older ones (Fig. 2b, c, d). Cystidia are simple, bristle-like, curley, septate, colorless or light yellow when young, and grow to ocher or brown with darker thick walls when ectomycorrhizae mature (Fig. 2e, f, h). Cystidia are distributed over the surface of the ectomycorrhiza, but are most abundant on the top half or on the apex of the root (Fig. 2e). Presence of cystidia does not appear to be connected to the growth stage of the ectomycorrhiza because the presence and absence of these emanating elements was found in both young than old

Table 2 Values of Pecan seedlings measurement and ectomycorrhizal level (means±standard error, $n=10$)

Soil texture parameter	*T. borchii*	*T. aestivum*	*T. macrosporum*
Stem length (cm)	34.2±1.52	35.0±1.90	34.8±2.03
Root system length (cm)	15.5±0.85	13.8±0.70	13.3±0.90
Stem diameter (mm)	5.02±0.33	6.27±0.55	5.94±0.47
Ectomycorrhizal level			
Visual valuation (VV)	0.61±0.06	0.40±0.08	–
Relative abundance valuation (RAV)	0.62±0.05a	0.42±0.07b	–

Values of relative abundance with different letters are significantly different at $p<0.05$ and $df=1, 18$

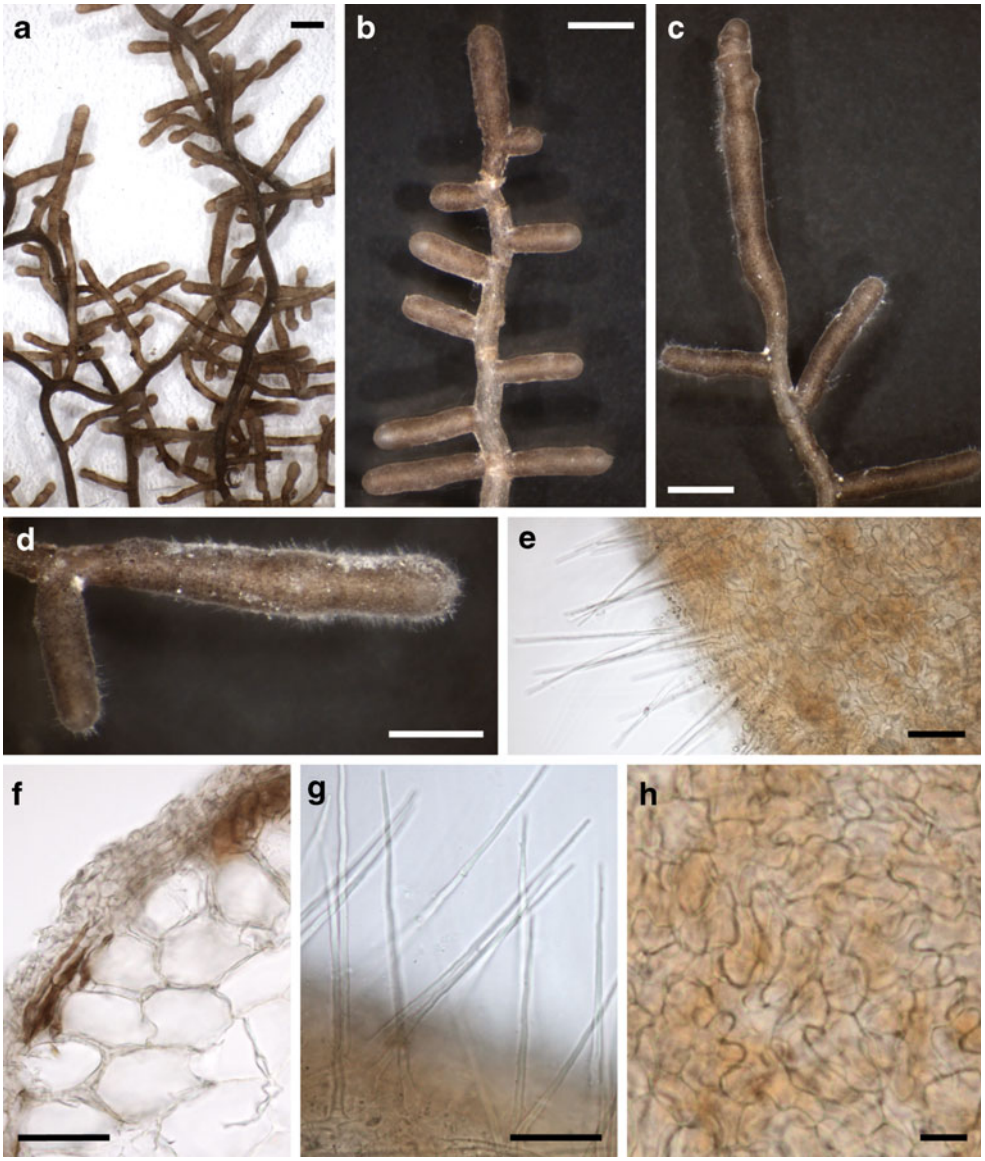

Fig. 1 Anatomical and morphological features of *T. borchii* ectomycorrhizae on pecan trees. **a** Root systems with abundant ectomycorrhizae (bar=700 μm); **b** and **c** simple and ramified ectomycorrhizae (bar=700 μm); **d** ectomycorrhizae with cystidia (bar=500 μm); **e** cystidia and outer mantle pattern (bar=65 μm); **f** section of the mantle and Hartig net (bar=30 μm); **g** needle-like cystidia (bar=30 μm); **h** puzzle-like cells pattern of the outer mantle layer (bar=10 μm)

roots. The outer mantle layer organization of *T. aestivum* ectomycorrhiza on pecan is pseudoparenchymatous; in cross-section the mantle is arranged in few cell layers (4–6) with a periepidermal Hartig net (1–3 cortical cell layers deep) (Fig. 2g). The outer mantle surface is densely long-spiny with cells structured in an angular pattern; cells are variable in shape and less in size (Table 3), varying from rectangular to polygonal-elongated (Fig. 2i).

Molecular verification of *T. aestivum* and *T. borchii* ectomycorrhizae on pecan

Results from molecular species-specific PCR assays and ITS sequence analyses were consistent with our morphological taxonomic assessments. These results were not affected by sample size (single or pooled ectomycorrhizae) or developmental stage. Samples tested with species-specific assays produced expected amplicon sizes of about 430 and 400 bp (Fig. 3) (Mello et al. 1999, 2002). No amplicons were produced in negative controls. Similarity searches using BLASTN algorithm at NCBI confirm that the ITS sequences obtained from ectomycorrhizae were identical to those present in GenBank for the same fungal species with 100% of query coverage, *E* values of 0.0, and identify matches of 99–100%.

Discussion

Ectomycorrhizal formation of European truffle species on pecan

Previous studies found that *Tuber* species are abundant members of the EM community of pecan orchards in USA,

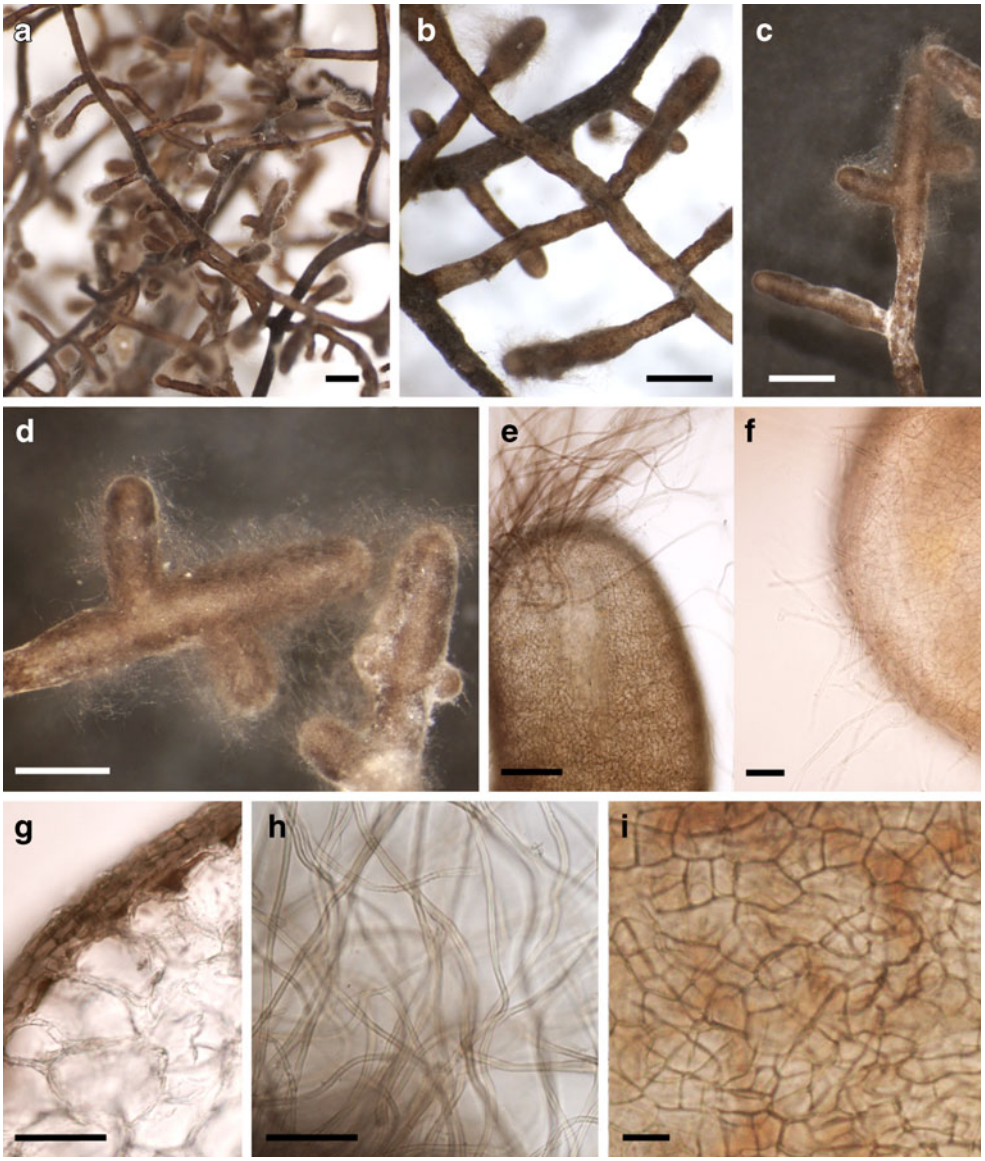

Fig. 2 Anatomical and morphological features of *T. aestivum* ectomycorrhizae on pecan trees. **a** Root systems with ectomycorrhizae (bar=700 μm); **b** and **c** simple and ramified ectomycorrhizae (bar=700 μm); **d** ectomycorrhizae with cystidia (bar=500 μm); **e** apical cystidia and mantle cell pattern (bar=65 μm); **f** apical developing cystidia (bar=20 μm); **g** mantle section of the mantle and Hartig net (bar=30 μm); **h** simple unramified cystidia (bar=30 μm); **i** angular cell pattern of the outer mantle layer (bar=10 μm)

and that pecan seedlings are receptive to the spores of native truffle species (Bonito et al. 2011a). Here we demonstrate that by using standard truffle inoculation practices mycorrhizae of the economically important European truffle species *T. borchii* and *T. aestivum* can also be formed on pecan seedlings. This is the first time European truffle species have been synthesized on this economically important North American host.

In addition to *T. borchii* and *T. aestivum*, we assessed whether mycorrhizae of the economically important truffle species *T. macrosporum* could be formed on pecan. *T. macrosporum* is commonly associated with species in the willow (Salicaceae) oak (Fagaceae) families. Because host-preference below the phylum level appears to be relatively weak in most *Tuber* clades (Bonito et al. 2010), and *T. macrosporum* associates with a range of angiosperms in the Fagacaeae and Betulacae, we predicted that *T. macrosporum* would be able to form ectomycorrhizae on pecan. It was interesting to us that no ectomycorrhizae of this species were produced, as successful mycorrhizal formation of *T. macrosporum* on oaks, hazels and hornbeams through spore-slurry inoculations have been reported by many groups (Giovanetti and Fontana 1981; Granetti 1995; Vezzola 2005; Zambonelli et al. 1993; Benucci GMN, unpublished data), leading us to conclude that *C. illinoinensis* may not be a suitable host for *T. macrosporum*.

Although *T. macrosporum* did not form EM on pecan, *T. borchii* and *T. aestivum* both developed well-formed and abundant EM on this non-native host. It is likely that other economically important *Tuber* species may form stable EM symbiosis with pecan as well. Although it requires a more narrow set of edaphic conditions, the European black truffle *T. melanosporum* may also be a favorable candidate for further study, since this species

Table 3 Anatomical and morphological characteristics of ectomycorrhizae formed by *T. borchii* and *T. aestivum* with *C. illinoinensis* (values are means±standard error, $n=100$)

	T. borchii	*T. aestivum*
Length of the ectomycorrhizae (mm)	1.68±0.07	1.41±0.06
Diameter of the ectomycorrhizae (mm)	0.23±0.01	0.24±0.01
Mantle thickness (μm)	21.76±0.44	22.63±0.64
Length of cystidia (μm)	86.60±2.47	–
Thickness of cystidia (μm)[a]	2.37±0.05	2.31±0.05
Mantle cell, major axis (μm)	16.55±0.46	13.74±0.35
Mantle cell, minor axis (μm)	7.93±0.34	8.86±0.27

[a] For *T. borchii*, thickness was measured at the middle length of each cystidium

associates with a diversity of angiosperm hosts and is being cultivated worldwide (Bonito et al. 2011b).

Ectomycorrhizal morphology and evaluation of ectomycorrhizal colonization levels

The anatomy and morphology of *T. borchii* and *T. aestivum* ectomycorrhizae had attributes characteristic of these respective species (Granetti et al. 2005; Zambonelli et al. 1993). PCR and sequence analyses confirmed morphological identifications, thus ectomycorrhizae of these two species can be reliably distinguished visually. In particular, *T. borchii* possessed straight cystidia and puzzle-like pseudoparenchyma cells (Fig. 1), while *T. aestivum* possessed curly cystidia and angular pseudoparenchyma cells (Fig. 2).

We compared two methods (VE and RAV) for evaluating the proportion of ectomycorrhizae on seedlings. We found that differences between these two methods were small and not significantly different from each other. This result is important in cases of routine seedling tests on mycorrhizal colonization levels, such as is done when truffle-infected plants produced by commercial nurseries are certified on percentage of ectomycorrhizae for marketing and outplanting. *T. borchii* formed the most ectomycorrhizae on pecan, averaging≈62%, but *T. aestivum* also formed abundant ones averaging≈42%, based on RAV data.

Co-cultivation of nuts and prized truffles

The modern scale and economy of agriculture has led many farmers to seek alternatives to traditional crops. There is growing interest in producing wealth in an environmentally sound and sustainable manner. Truffle orchards can be established on marginal lands with low fertility and steep slopes, so not to compete with most food production systems. Truffles are now being cultivated worldwide, through the growth of plant hosts that have been inoculated with truffle spores (Wang and Hall 2004). However, these orchards are often established as monocultures and with the sole aim of truffle production. Cultivating truffles with other compatible crops could improve the sustainability and economic productivity of truffle farming. We suggest that co-cultivation of pecans and prized truffle species represents a novel strategy for boosting rural and disadvantaged economies. Pecans are cultivated across Southern USA and in other countries including Australia, Italy, Brazil, and South America (Wakeling et al. 2001; Herrera 2000). Pecan trees do well with the high levels of calcium carbonate required for truffle production (Sparks 1976), and the two crops could provide independent revenue streams. In such a dual cropping system, the risks may also be reduced because multiple products would be produced. As a first step in determining the feasibility of co-cropping truffles, we have shown that using standing inoculation methods two economically important European truffle species (i.e., *T. borchii* and *T. aestivum*) formed healthy well-colonized EM with *C. illinoinensis* in a nursery setting. Additional studies are needed to address whether *Tuber* EM are maintained when seedlings are planted out in an orchard setting, and whether these truffle species will fruit with pecan.

Another important consideration is whether the management of commercial truffle and pecan orchards is compatible. With certain pecan varieties and in particular regions, trees may be at risk of foliar, fruit or root disease and may require the use of biocides (Jones and Ritchie 1999; Powell et al. 1968). In some cases fertilizers are used to boost production or reduce disease symptoms. These applications are likely to adversely affect the truffle symbionts and other belowground microbes (Bunemann et al. 2006; Zambonelli and Iotti 2001). It is unknown whether these applications accumulate inside truffles. In the light of this, reports and strategies for organic or low-input management of pecan

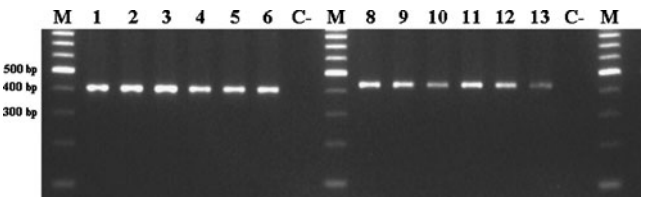

Fig. 3 Amplification products obtained with UNCI/UNCII and TBA/TBB specific primers for *T. aestivum* and *T. borchii*, respectively, and electrophoresed on a 1.8% (w/v) agarose gel. *M*, GeneRuler DNA ladder (Fermentas); *C−*, negative control (no genomic DNA added to PCR reaction); *lines 1–2*, amplification fragments obtained starting from pools of ten *T. aestivum* ectomycorrhizae; *lines 3–6*, amplification fragments obtained starting from single *T. aestivum* ectomycorrhizae at different developing stage; *lines 8–9*, amplification fragments obtained starting from pools of ten *T. borchii* ectomycorrhizae; *lines 10–13*, amplification fragments obtained starting from single *T. borchii* ectomycorrhizae at different developing stage

orchards (Diver and Ames 2000) are of interest for future truffle–pecan compatibility studies.

In conclusion, our results show that the economic tree host *C. illinoinensis* can establish ectomycorrhizal symbiosis with *T. borchii* and *T aestivum*. Where ecological conditions are suitable for the development of the truffle EM symbiosis, pecan may represent a promising alternative host for truffle cultivation.

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References

Adams JC, Thielges BA (1978) Seed treatment for optimum pecan germination. Tree Planters' Notes 35:13–14

Agerer R (1991) Characterization of ectomycorrhiza. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology: techniques for the study of mycorrhiza. Academic Press, San Diego, pp 25–73

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Avis PG, McLaughlin DJ, Dentinger BC, Reich PB (2003) Long-term increase in nitrogen supply alters above- and below-ground ectomycorrhizal communities and increases the dominance of *Russula* spp. in a temperate oak savanna. New Phytol 160:239–253

Bencivenga M, Donnini D, Tanfulli M, Guiducci M (1995) Tecnica di campionamento delle radici e degli apici radicali per la valutazione delle piante micorrizate. Micol Ital 2:35–47

Benson DA (2004) GenBank. Nucleic Acids Res 33:D34–D38

Benucci GMN, Raggi L, Massimo GD, Baciarelli-Falini L, Bencivenga M, Falcinelli M, Albertini E (2011) Species-specific primers for the identification of the ectomycorrhizal fungus *Tuber macrosporum* Vittad. Mol Ecol Resour 11:378–381

Bertault G, Raymond M, Berthomieu A, Callot G, Fernandez D (1998) Trifling variation in truffles. Nature 394:734. doi:10.1038/29428

Bertini L, Rossi I, Zambonelli A, Amicucci A, Sacchi A, Cecchini M, Gregori G, Stocchi V (2005) Molecular identification of *Tuber magnatum* ectomycorrhizae in the field. Microbiol Res 161:59–64

Bonet JA, Fischer CR, Colinas C (2006) Cultivation of black truffle to promote reforestation and land-use stability. Agron Sustain Dev 26:69–76

Bonito GM, Gryganskyi AP, Trappe JM, Vilgalys R (2010) A global meta-analysis of *Tuber* ITS rDNA sequences: species diversity, host associations and long-distance dispersal. Mol Ecol 19:4994–5008

Bonito G, Brenneman T, Vilgalys R (2011a) Ectomycorrhizal fungal diversity in orchards of cultivated pecan (*Carya illinoinensis*; Juglandaceae). Mycorrhiza. doi:10.1007/s00572-011-0368-0

Bonito G, Trappe JM, Donovan S, Vilgalys R (2011b) The Asian black truffle *Tuber indicum* can form ectomycorrhizas with North American host plants and complete its life cycle in non-native soils. Fungal Ecol 4:83–93

Bruns TD (1995) Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. Plant Soil 170:63–73

Bunemann EK, Schwenke GD, Van Zwieten L (2006) Impact of agricultural inputs on soil organisms — a review. Aust J Soil Res 44:379–406

Bustan A, Ventura Y, Kagan-Zur V, Roth-Bejerano N (2006) Optimized conditions for mycorrhiza formation between the pink rockrose (*Cistus incanus*) and the black Périgord truffle (*Tuber melanosporum*). Isr J Plant Sci 54:87–96

Chevalier G, Frochot H (1997) La truffe de Bourgogne (*Tuber uncinatum* Chatin). Edition Pétrarque, Levallois-Perret, p 257

Dickie IA, Koide RT, Steiner KC (2002) Influences of established trees on mycorrhizas, nutrition, and growth of *Quercus rubra* seedlings. Ecol Monogr 72:505–521

Diver S, Ames G (2000) Sustainable Pecan Production. ATTRA (Appropriate Technology Transfer for Rural Areas), HTML Production, ©NCAT, IP077 (https://attra.ncat.org/attra-pub/pecan.html)

Fontana A, Giovannetti G (1978–79) Simbiosi micorrizica fra *Cistus incanus* L. spp. Incanus e *Tuber melanosporum* Vitt. Allionia 23:5–11

Giomaro G, Zambonelli A, Sisti D, Cecchini M, Evangelista V, Stocchi V (2000) Anatomical and morphological characterization of mycorrhizas of five strains of *Tuber borchii* Vittad. Mycorrhiza 10:107–114

Giovannetti G, Fontana A (1981) Simbiosi micorrizica di *Tuber macrosporum* Vitt. con alcune Fagales. Allionia 24:13–17

Gógán CA, Bratek Z, Illyés Z, Dimény J (2011) Studies on *Tuber macrosporum* Vittad. natural truffle habitats in the Carpatho-Pannon region. In: Atti 3°Congresso internazionale di Spoleto sul Tartufo. Tipolitografia Federici, Terni, pp 182–188

Granetti B (1995) Caratteristiche morfologiche, biometriche e strutturali delle micorrize di *Tuber* di interesse economico. Micol Ital 2:101–117

Granetti B, De Angelis A, Materozzi G (2005) Umbria terra di tartufi. Umbriagraf, Terni, p 303

Gryndler M, Hršelová H, Soukupová L, Streiblová E, Valda S, Borovička J, Gryndlerová H, Gažo J, Miko M (2011) Detection of summer truffle (*Tuber aestivum* Vittad.) in ectomycorrhizae and in soil using specific primers. FEMS Microbiol Lett 318:84–89. doi:10.1111/j.1574-6968.2011.02243.x

Hall IR, Brown GT, Zambonelli A (2007) Taming the truffle. The history, lore and science of the ultimate mushroom. Timber Press, Oregon, p 304

Hanlin RT, Wu M, Brenneman TB (1989) The occurrence of *Tuber texense* in Georgia. Mycotaxon 34:387–394

Harley JL, Smith SE (1983) Mycorrhizal Symbiosis. Academic Press, London, p 483

Heimsch C (1958) The first recorded truffle from Texas. Mycologia 50:657–660

Herrera E (2000) Economic Importance of the Pecan Industry. New Mexico State University and USDA electronic distribution: http://aces.nmsu.edu/pubs/_z/z-501.html

Iotti M, Lancellotti E, Hall I, Zambonelli A (2010) The ectomycorrhizal community in natural *Tuber borchii* grounds. FEMS Microbiol Ecol 72:250–260

Jones RK, Ritchie DF (1999) Some Common Pecan Diseases and Their Control in North Carolina, In: Ornamental Disease Information — Note 3, North Carolina Cooperative Extension Service

Ministero per le Politiche Agricole (1999) Metodi ufficiali di analisi chimica del suolo. D.M. del 13/09/99, Gazzetta Ufficiale n. 248 del 21/10/99

Marjanovic Z, Grebenc T, Markovic M, Glisic A, Milenkovic M (2010) Ecological specificities and molecular diversity of truffles

(genus *Tuber*) originating from mid-west of the Balkan Peninsula. Sydowia 62:67–87

Mello A, Garnero L, Bonfante P (1999) Specific PCR-primers as a reliable tool for the detection of white truffles in mycorrhizal roots. New Phytol 141:511–516

Mello A, Cantisani A, Vizzini A, Bonfante P (2002) Genetic variability of *Tuber uncinatum* and its relatedness to other black truffles. Environ Microbiol 4:584–594

Norris JR, Read DJ, Varma AK (1994) Techniques for mycorrhizal research: Methods in microbiology. Academic Press, San Diego, p 928

Pampanini R, Martino G (2006) L'importanza del tartufo nell'economia della montagna. Micol Ital 3:3–17

Paolocci F, Rubini A, Riccioni C, Arcioni S (2006) Reevaluation of the life cycle of *Tuber magnatum*. Appl Environ Microbiol 72:2390–2393

Powell WM, Hendrix FF, Marx DH (1968) Chemical control of feeder root necrosis of pecans caused by *Pythium* species and nematodes. Plant Dis Rep 52:577–578

Raimondo FM, Ilardi V, Mazzola P (2009) Nuove prospettive per l'arboricoltura da legno in Sicilia: la coltivazione del noce Pecan. In: Atti del Terzo Congresso Nazionale di Selvicoltura, Taormina (ME), 16–19 ottobre 2008. Accademia Italiana di Scienze Forestali, Firenze, pp 1535–1538

Reyna Doménech S (2007) Truficultura. Fundamentos y técnicas. Ediciones Mindi-Prensa, Madrid, p 686

Riccioni C, Belfiori B, Rubini A, Passeri V, Arcioni S, Paolocci F (2008) *Tuber melanosporum* outcrosses: analysis of the genetic diversity within and among its natural populations under this new scenario. New Phytol 180:466–478

Song MS, Cao JZ, Yao YJ (2005) Occurrence of *Tuber aestivum* in China. Mycotaxon 91:75–80

Sparks D (1976) Soil pH and the pecan. 67th Annual Report of the Northern Nut Growers Association, pp 93–99

Taber RA (1988) Observations on the ecology of the Texas truffle, *Tuber texense*. In: Atti del Secondo Congresso Internazionale sul Tartufo, 24–27 Novembre 1988. Grafiche P. & P. (ed), pp 331–336

Trappe JM, Jumpponen AM, Cazares E (1996) NATS truffle and truffle-like fungi 5: *Tuber lyonii* (=*T. texense*), with a key to the spiny-spored *Tuber* species groups. Mycotaxon 60:365–372

USDA (2008) National Agricultural Statistics Service. Noncitrus fruits and nuts: Fr Nt 1–3 (09). United States Department of Agriculture. http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1

Vezzola V (2005) Primi risultati produttivi con piante micorrizate da *T. macrosporum* Vittad. In: Atti Seminario sullo stato attuale della Tartuficoltura Italiana, 21–22 febbraio 2004. Grafiche Millefiorini, Spoleto-Norcia, IT, pp 51–55

Wakeling LT, Mason RL, D'Arcy BR, Caffin NA (2001) Composition of pecan cultivars Wichita and Western Schley [*Carya illinoinensis* (Wangenh.) K. Koh] grown in Australia. J Agricult Food Chem 49:1277–1281

Wang Y, Hall IR (2004) Edible ectomycorrhizal mushrooms: challenges and achievements. Can J Bot (Rev Can Bot) 82:1063–1073

Wedén C, Danell E, Tibell L (2005) Species recognition in the truffle genus *Tuber* — the synonyms *Tuber aestivum* and *Tuber uncinatum*. Environ Microbiol 7:1535–1546

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: A guide to methods and applications. Academic Press, San Diego, pp 315–322

Zambonelli A, Iotti M (2001) Effects of fungicides on *Tuber borchii* and *Hebeloma sinapizans* ectomycorrhizas. Mycol Res 105:611–614

Zambonelli A, Salomoni S, Pisi A (1993) Caratterizzazione anatomomorfologica delle micorrize di *Tuber* spp. su *Quercus pubescens* Willd. Micol Ital 3:73–90

Zambonelli A, Iotti M, Giomaro G, Hall I, Stocchi V (2002) *T. borchii* cultivation: an interesting perspective. Edible mycorrhizal mushrooms and their cultivation. In: Hall I, Yun W, Danell E, Zambonelli A (eds) Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, 3–6 July 2001. Christchurch, New Zealand (CD-Rom)