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ORIGINAL PAPER

Can NPK fertilizers enhance seedling growth and mycorrhizal status of *Tuber melanosporum*-inoculated *Quercus ilex* seedlings?

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Abstract Although successful cultivation of the black truffle (*Tuber melanosporum*) has inspired the establishment of widespread truffle orchards in agricultural lands throughout the world, there are many unknowns involved in proper management of orchards during the 6–10 years prior to truffle production, and there are conflicting results reported for fertilizer treatments. Here, we systematically evaluate the combined effects of nitrogen, phosphorous, and potassium with different doses of each element, applied to either foliage or roots, on plant growth parameters and the mycorrhizal status of outplanted 3-year-old seedlings in five experimental *Quercus ilex–T. melanosporum* orchards. Fertilization did not significantly improve seedling aboveground growth, but the plants treated with the fertilizer 12-7-7 applied to the roots (HNr) displayed longer field-developed roots. Only the fertilizer with the highest dose of K (10-6-28) applied to the foliage (HKf) increased the probability of fine root tip colonization by *T. melanosporum* in field-developed roots. However, the plants treated with the same fertilizer applied to the soil (HKr) presented the highest probability for colonization by other competing mycorrhizal soil fungi. Potassium seems to have an important role in mycorrhizal development in these soils. Apart from *T. melanosporum*, we found 14 ectomycorrhizal morphotypes, from which seven were identified to species level, three to genus, two to family, and two remained unidentified by their morphological characteristics and DNA analyses.

Keywords Black truffle · Truffle orchard · Ectomycorrhiza · Morphotyping · DNA analysis · Nitrogen · Phosphorous · Potassium

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Introduction

Truffles are the fruiting bodies of hypogeous ectomycorrhizal (ECM) fungi belonging to the genus *Tuber* F. H. Wigg. (Ascomycota). The black truffle (*Tuber melanosporum* Vittad. or *Tuber nigrum* Bull.) has high commercial value due to its excellent organoleptic properties. The decline in wild black truffle production during the last century has led to the development of truffle orchards, using *T. melanosporum*-inoculated seedlings (mainly oak and hazelnut), but truffle production in orchards is difficult to predict. In the establishment of these orchards, the early years are particularly critical for survival and growth of both plant and fungal symbionts.

Fertilization is one of the standard treatments to favor early plant growth, but for truffle-inoculated plants, both positive and negative effects of fertilization have been reported from greenhouse and field experiments. Dupré et al. (1982) found that the optimum application of inorganic nitrogen and phosphorous (NP) fertilizations to promote plant growth for *Quercus pubescens* Wild. did not correspond with the doses favorable for *T. melanosporum* colonization. In productive truffle beds, application of high doses of the commercial fertilizer specific for truffle culture, Fructitruf®, decreased the ascoma production (Sourzat 2000) while the same fertilizer applied in a proper dose increased the size of the truffles but only during 3 years (Sourzat 2002) after which production decreased. However, additions of fertilizers, particularly N and P, have been shown to reduce mycorrhizal colonization (Newton and Pigott 1991; Treseder 2004), most probably due to reducing the host investment in the fungal symbiont to sequester soil nutrients.

Foliar fertilization is a common practice to correct nutritional deficiencies caused by a limited or unavailable supply of soil nutrients. The fertilizer application pathway (foliar vs. soil) could have different effects on plant and fungal development because with foliar fertilization, nutrients are directly available to the plant without retention and transformation by soil organisms.

We hypothesize that fertilization in truffle orchards could be beneficial because trees with greater growth parameters, including diameter and height, display earlier burns—the bare area surrounding the host tree which is caused by the phytotoxic effect of the *T. melanosporum* mycelium (Fasolo-Bonfante and Fontana 1971)—and yield greater truffle production than smaller trees (Shaw et al. 1996; Suz et al. 2008). Increased tree growth is likely to support increased ECM growth (Wallander 2006) because greater host leaf area will result in greater carbon assimilation and allocation to ECM roots. Also, we hypothesize that possible negative effects of fertilization on *T. melanosporum* development could be avoided by the use of foliar fertilizers instead of soil fertilizers.

The objective of this study was to find a fertilization treatment adequate for both partners of the symbiosis, improving the growth of the trees and supporting the presence and development of *T. melanosporum* during the establishment and preproduction phase of the truffle orchard. In a field study, we evaluated the effects of four commercially available NP and potassium (NPK) fertilizers, applied to foliage or soil, on the development of *T. melanosporum* mycorrhizae in inoculated *Quercus ilex* seedlings. We also studied the effect of the fertilizers on the composition of the mycorrhizal fungal community in the roots of the seedlings.

Materials and methods

Experimental design and study sites

The experiment was a randomized block design with fertilization as the main factor. The treatments were replicated in five blocks in different provinces in Spain: site 1 (Lleida), sites 2 and 3 (Castellón), site 4 (Huesca), and site 5 (Álava). Site and soil characteristics of each study orchard are described in Table 1. In each block, there were nine experimental units. The treatments consisted of

Table 1 Site and soil characteristics for study sites

Parameter	Site 1	Site 2	Site 3	Site 4	Site 5
Locality (province)—Spain	Corbins (Lleida)	Morella (Castellón)	Todolella (Castellón)	Santorens (Huesca)	Vitoria (Álava)
Altitude (m)	210	950	800	1,049	600
pH (1:2.5 H_2O)	8.0	8.3	8.2	8.1	8.2
Organic material (%), Walkley–Black	4.4	3.5	2.0	4.0	1.9
Calcium carbonate (%), Bernard	26	27	44	46	44
Nitrogen (%), Kjeldahl	0.31	0.23	0.13	0.23	0.15
Phosphorus (ppm), Olsen	34	5	54	4	23
Potassium (ppm), Am. Ac. Ex.	244	241	235	263	183
USDA classification	Loam	Clay-loam	Loam	Loam	Clay-loam
Total sand (%), $0.05<D<2$ mm	30.7	33.2	45.1	40.6	19.9
Coarse silt (%), $0.02<D<0.05$ mm	14.0	9.7	14.4	13.4	16.1
Fine silt (%), $0.002<D<0.02$ mm	31.7	27.8	20.9	21.6	33.3
Clay (%), $D<0.002$ mm	23.6	29.3	19.6	24.4	30.7

Am. Ac. Ex. ammonium acetate extraction

eight fertilization treatments applied to *T. melanosporum*-inoculated seedlings, including a nonfertilized control. An additional treatment consisted of noninoculated *Q. ilex* seedlings with no fertilizer treatment to compare the effects of *T. melanosporum* inoculation on plant growth and to monitor the mycorrhizal species composition in oak roots 3 years after outplanting at five different orchards. Each of the fertilization treatments was randomly assigned to 6 m^2 experimental units containing ten inoculated seedlings. Each experimental unit had a 6-m border and was separated from the contiguous one with a single row of noninoculated seedlings to control for border effects (Online Resource 1).

Plants and inoculation

A total of 400 inoculated *Q. ilex* 1-year seedlings and 173 noninoculated seedlings (including border seedlings) were planted in autumn of 1998. Both inoculated and noninoculated seedlings were examined for their mycorrhizal status before plantation according to the method of Fischer and Colinas (1996). Noninoculated seedlings were planted as bioassays of ECM species composition in the different soils, as some mycorrhizal fungi do not colonize seedlings previously colonized with *T. melanosporum* (Mamoun and Olivier 1993; Domínguez Núñez et al. 2006) or seedlings that have been fertilized (Reyna 2000).

Fertilization treatments

Three commercial NPK fertilizers with higher proportion of nitrogen (HN), phosphorus (HP), or potassium (HK) were selected to test for the effect of a higher dose of each element in the combination. Additionally, a slow release fertilizer with a high P content (HPSR) was chosen to test for the rate of release and applied only as a soil treatment. Fertilization treatments are described in detail in Table 2. For foliar application, seedling leaves were sprayed manually with a mist solution at the dose recommended by manufacturers. Leaves were saturated with care to avoid fertilizer application to the surrounding vegetation. The same dose of each fertilizer was applied to the soil, close to the base of the stem. Control seedlings were treated with water. Fertilizers were applied yearly in spring for 3 years, except the HPSR fertilizer treatment, which was applied only once when seedlings were planted following manufacturer's instructions.

Data collection

After 3 years, in 2001, we removed one plant per experimental unit with its complete root system and transported the seedlings with soil to the laboratory where they were stored at 4°C until processed. Plants were cleaned of soil carefully without damaging the root tips. Height, root collar basal diameter, and taproot length of each seedling were measured. Roots were separated into two categories: older roots developed in the nursery (plug) and newer roots developed in the field. All roots were separated into fine roots (<0.5 mm diameter) and coarse roots (>0.5 mm diameter). Root length was estimated using the grid intersect method (Newman 1966; Marsh and Marsh 1971). Foliage, stem, and roots were separated, dried, and weighed.

Morphological and anatomical identification of *T. melanosporum* mycorrhizae were carried out following the descriptions of Rauscher et al. (1995). Colonization by *T. melanosporum* ectomycorrhizae and by other ECM fungi was estimated following the method of Fischer and Colinas (1996). The proportion of *T. melanosporum* on roots [PT= number of *T. melanosporum* mycorrhizae/(number of nonmycorrhizal tips + number of mycorrhizae from other fungi)] and the proportion of other competing ectomycorrhizae [PC=number of mycorrhizae of other fungi/number of *T. melanosporum* mycorrhizae] were calculated.

Table 2 Commercial name, application route, dose, release rate, and composition of the commercial fertilizers applied

Commercial name	Application route (code)	Dose plant/year	Release rate	NPK	% Ammonia	% Nitrate	% Urea	Oligo-element content
Medramás	Roots (HPSR)	80 g[a]	Slow	8-16-8	8 (NH_2)	0.0	0.0	10% CaO, 5% MgO, 6% SO_2, 1% Fe, 0.01% B
Quimur K	Leaves (HKf) Roots (HKr)	0.12 g	Fast	10-6-28	1.8	8.2	0.0	2.0 p/p MgO, 2.4% B
Proferfol 1	Leaves (HPf) Roots (HPr)	0.14 ml	Fast	7-21-7	5.0	0.0	2.0	0.05% B, 0.05% Cu, 0.1% Fe, 0.1% Mg, 0.05% Zn
Proferfol 2	Leaves (HNf) Roots (HNr)	0.14 ml	Fast	12-7-7	3.0	1.0	8.0	Same as for Proferfol 1

NPK formulation is expressed as % N, % $P_2 0_5$, and % $K_2 0$

[a] The HPSR fertilizer was applied only once in the soil when seedlings where planted (two tablets of 40 g/seedling)

Table 3 Ectomycorrhizal fungi collected on roots of the noninoculated and *T. melanosporum*-inoculated *Q. ilex* seedlings indicating in which fertilization treatment and sites they were encountered

Morphotype name	Treatment	Site	Relative abundance	ECM tip morphology[a]	GenBank accession number	Closest GenBank match (identities)	E value	Maximum identity (%)
Tuber melanosporum	All excepting NI	All	PT=6.6	Rauscher et al. (1995)	Site 1: GQ254869[b]	Site 1: U89359 *T. melanosporum* (587/587)	0.0	100
					Site 3: GQ254870[b] GQ254871[b]	Site 3: U89359 *T. melanosporum* (594/595; 595/595)	0.0	99–100
AD type (Giraud 1988)	HKr	3	PC=0.15	(a) Not branched; (b) brown; (c) wolly hairy; (d) loosely woven, PTC; (e) PSP, polygonal cells; (f) absent; (g) common, colored, branched in 90° angles; (h) absent	GQ254872	EU822506–EU822507: vouchered mycorrhizae "*Quercirhiza quadratum*" (178/182)	6E−83 2E−82	97 98
						FN377852 Uncultured fungus (159/161)	5E−74	98
						DQ402506 Uncultured ECM fungus "AD type" (142/164)	2E−38	86
Tuber brumale (with cystidia)	NI	1, 5	PF=0.48	Fischer et al. (2004)	Not sequenced			
Tuber brumale (without cystidia)	NI	1, 5	PF=0.48	Similar to previous but without cystidia	Site 1: GQ254852[b]	Site 1: AF106891 *Tuber oligospermum* (615/628)	0.0	97
					Site 5: GQ254851	Site 5: AF106891 *Tuber oligospermum* (607/620)	0.0	97
Cenococcum like	NI	All	PF=0.47	(a) Not branched/monopodial; (b) black; (c) grainy–woolly and shiny; (d) PTC hyphae star-like arranged; (e) PTC; (f) absent; (g) dark brown, thick walls, no clamps; (h) absent	GQ254866	EF434154 Uncultured fungus (673/692)	0.0	97
						DQ179119 *Cenococcum geophilum* (659/681)	0.0	96
Sphaerosporella like	HNr NI	1	PC=0.014 PF=0.003	(a) Not branched; (b) ochre yellowish brown to dark brown; (c) fibrous; (d) PTC, polygonal elongated cells (e) transitional between PTC and PSP; (f) absent; (g) with occasional ornamentations without clamps; (h) absent	Not sequenced			
Suillus like	NI	1	PF=0.003	(a) Monopodial sometimes bifurcate; (b) white-pinkish; (c) cottonish; (d, e) PTC; (f) absent; (g) abundant, red-brownish lipid drops; (h) absent	Not sequenced			
Tuber like	NI	2, 3, 4	PF=0.12	(a) Not branched; (b) light to dark brown; (c) smooth; (d) PSP epidermoid cells, very thick walls; (e) PSP; (f) absent; (g) absent; (h) absent	Site 2: GQ254857, GQ254858[b]	Site 2: DQ402504 Uncultured mycorrhiza (*Tuberaceae*; 525/537, 525/537[b])	0.0	97
						EF362474 *Tuber rufum* (443/475, 443/476[b])	0.0	93

					Site 3: GQ254860	Site 3: DQ402504 Uncultured mycorrhiza (*Tuberaceae*; 202/209)	7E−93	96
								97
					Site 4: GQ254859	Site 4: DQ402504 Uncultured mycorrhiza (*Tuberaceae*; 525/537)	0.0	93
						EF362474 *Tuber rufum* (443/475)	0.0	93
ECM1	HPf	2	PC=0.04	(a) Not branched; (b) gray-brown; (c) hairy; (d) PSP, dark brown polygonal cells; (e) PSP; (f) abundant, long, thin, and rigid, shark in the very tip, branched in straight angle; (g) absent; (h) absent	GQ254864	EF635843 Uncultured *Herpotrichiellaceae* (557/558)	0.0	99
ECM2	NI	2, 3, 4	PF=0.22	(a) Not branched or irregular branching; (b) white-yellow to brownish; (c) stringy; (d) PTC; (e) transitional PSP; (f) absent; (g) abundant retaining substrate particles.; (h) absent	Site 2: GQ254856	Site 2: FJ196904 Uncultured ectomycorrhiza (*Clavulinaceae*; 630/687)	0.0	91
					Site 4: GQ254853[b], GQ254854[b], GQ254855[b]	Site 4: FJ196904 Uncultured ectomycorrhiza (*Clavulinaceae*; 631/688, 631/687, 632/687)	0.0	91
ECM3	HNr	2	PF=0.09	(a) Monopodial tortuous forming big glomerula, (b) silver-golden (external hyphae) and brown, (c) tringy, brilliant hydrophobic, (d) PTC ring-like arrangement, (e) PTC, (f) absent, (g) absent, (h) present	GQ254867	AJ555517 *Melanogaster broomeianus* (333/333)	3E−173	100
					GQ292556	FJ861415 *Nectria radicicola* (188/188)	6E−193	100
ECM4	HNr	3	PC=0.04	(a) Not branched, club-shaped; (b) light brown; (c) matte plush; (d) PTC; (e) PSP; (f) absent; (g) common, curved, or tortuous, with clamps; (h) absent	Not sequenced			
ECM5	HNf	3	PC=5.9	(a) Bent tips, irregular branching, forming glomerula; (b) light to dark brown; (c) stringy; (d, e) PSP; (f) absent; (g) common, long, reddish, with clamps, pigments in membranes and vacuoles; (h) common, reddish-brown, common branching, type F (Agerer 1987-2001)	GQ254861[b]	AJ629406 *Astraeus hygrometricus* (641/646)	0.0	99
					GQ254862[b]	AJ629406 *Astraeus hygrometricus* (638/646)	0.0	98
					GQ254863[b]	AJ629406 *Astraeus hygrometricus* (640/650)	0.0	98
ECM6	HKr	5	PC=3.6	(a) Monopodial pyramidal, tortuous unramified ends; (b) white transparent to grey-brownish; (c) Felty; (d, e) PTC; (f) absent; (g) common, bent hyaline hyphae with clamps; (h) absent	GQ254865	DQ068971 *Tomentella ellisii* (461/473)	0.0	97
						EU700261 Uncultured ECM (*Tomentella*; 461/474)	0.0	97

Table 3 (continued)

Morphotype name	Treatment	Site	Relative abundance	ECM tip morphology[a]	GenBank accession number	Closest GenBank match (identities)	E value	Maximum identity (%)
ECM7	Control INOC	3	PC=0.035	(a) Not branched, tortuous; (b) brown to black; (c) grainy and hairy, reflective; (d) PTC; (e) PSP, polygonal cells; (f) absent; (g) common, tortuous, brownish; (h) absent	GQ254868	EF411080 Uncultured ECM (*Tomentella*; 615/636)	0.0	96

Relative abundance of each morphotype is given in proportions (PT, PC, and PF). ECM tip morphology followed methodology of Agerer (1987–2001) and Rauscher et al. (1995), and molecular identification was based on sequencing of ITS nrDNA followed by BLAST search in the NCBI GenBank DNA database

PTC plectenchymatous, *PSP* pseudoparenchymatous, *PT* proportion of *T. melanosporum* (number of *T. melanosporum* mycorrhizae/number of total root tips), *PC* proportion of the competing fungus (number of tips colonized by the morphotype/number of tips colonized by *T. melanosporum*), *PF* proportion of fungus in noninoculated plants (number of tips colonized by the morphotype/number of total root tips), *HPSR* 8-16-8, *HK* 10-16-28, *HP* 7-21-7, *HN* 12-7-7, *f* foliar application, *r* root application, *NI* noninoculated and nonfertilized plants, *Control INOC T. melanosporum*-inoculated and nonfertilized plants

[a] ECM tip morphology: (a) macroscopic description, (b) color, (c) texture, (d) outer mantle, (e) inner mantle, (f) cystidia, (g) emanating hyphae, and (h) rhizomorphs

[b] Sequences resulted from cloning.

Other ectomycorrhizae were described according to Agerer (1987–2001); in Table 3, some of the features used for morphotyping are indicated. Mycorrhizae were freeze-dried and stored at −20°C for molecular analyses.

DNA isolation and PCR amplification

Genomic DNA from mycorrhizae was isolated using the E.Z.N.A. Fungal DNA miniprep kit (Omega Bio-Tek, Doraville, GA, USA) without mercaptoethanol. Three to five tips were used for identification of each morphotype. Polymerase chain reaction (PCR) and nested PCR, when needed, were performed using puRe Taq Ready-to-Go PCR Beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Initial PCR was carried out using the pair of primers ITS1F and ITS4, and nested PCR was carried out with the primers ITS5 and ITS2 (Gardes and Bruns 1993; White et al. 1990) following cycling conditions proposed by Martín and Winka (2000). Amplicons were visualized in 2% agarose gels stained with SYBR® safe (Invitrogen, Eugene, OR, USA) under visible light.

Specific PCR probes were performed to confirm the identity of *Tuber brumale* mycorrhizae, using the primer pair ITSB and ITS4LNG (Rubini et al. 1998; Paolocci et al. 1999), following the cycling conditions recommended by the authors. Amplicons were visualized as described above. The PCR products were subjected to restriction digestion (restriction fragment length polymorphism, RFLP) by three enzymes (*Alu* I, *Eco* RI, and *Hinf* I) according to the manufacturer's recommendations. The restriction fragments were separated by electrophoresis in 2% agarose gels. Patterns were compared to those obtained from an ascoma of *T. brumale* (Fischer et al. 2004).

Sequencing and cloning

Gel bands from agarose gels were excised and cleaned using QIAquick Gel PCR purification kit (Qiagen, Valencia, CA, USA). Two sequencing reactions were done corresponding to both primers used in the amplification. Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA) was used to identify the consensus sequence from the two strands of the ITS nrDNA of each isolate.

When polymorphisms in ITS sequences were obtained, cloning was performed using the pGEM®-T Easy Vector II cloning kit (Promega, Fitchburg, WI, USA) and purified with QIAPrep Spin Miniprep (Qiagen, Hilden, Germany). Three clones with inserted products were selected for sequencing. To confirm that the inserted product was correct, prior to sequencing, 2 µl of the purified plasmid DNA was digested with *Eco* RI following the instructions of the manufacturers. Both strands were sequenced separately using vector specific primers T7 and SP6.

Sequencher was used to identify the consensus sequence from the two DNA strands of each isolate.

Nucleotide Basic Local Alignment Search Tool (BLAST) searches (megablast) were used to compare the sequences obtained in this study against other DNA sequences in the National Center for Biotechnology Information (NCBI; Altschul et al. 1997). New sequences were deposited in GenBank (NCBI) under the accession numbers indicated in Table 3.

Statistical analysis

Analysis of variance (ANOVA) with fertilization as the main factor and locality as block effect was performed (Data Desk® 6.2, Ithaca, NY, USA) to determine significant differences between treatments, followed by least significant difference grouping at $p<0.05$ (Ramsey and Schafer 1996). Each fertilizer treatment was evaluated by comparing the fertilized *T. melanosporum*-inoculated plants with the nonfertilized *T. melanosporum*-inoculated plants. The assumptions of the ANOVA analysis were achieved by adjusting the best Box–Cox transformation of the dependent variable in each case. When variables were transformed, their means were back-transformed to the original scale and reported as medians (Ramsey and Schafer 1996). The effect of treatments for variables reported as proportions was analyzed by logistic regression using the GENMOD procedure of SAS/STAT® software version 8.02 (Schabenberger and Pierce 2002). Confidence intervals (CI) at 95% are reported for mean values.

Results

Noninoculated vs. nonfertilized *T. melanosporum*-inoculated plants

After 3 years in the field, there were no differences in growth parameters (plant height, root collar basal diameter, taproot length, lengths of field- and nursery-developed roots, fine and coarse roots, and dry weights of foliage, stem and roots) between the noninoculated and *Tuber*-inoculated plants.

In the noninoculated plants, no *T. melanosporum* ectomycorrhizae were found, but seven ECM morphotypes from other fungi were observed (Table 3): *Cenococcum* like, *Tuber* like, *T. brumale* with needle-like cystidia, *T. brumale* without cystidia, *Sphaerosporella* like, *Suillus* like, and morphotype ECM2 (Fig. 1). From *Sphaerosporella*-like and *Suillus*-like morphotypes, only morphological identification was performed due to insufficient number of mycorrhizae. *Sphaerosporella* like probably belongs to *Sphaerosporella brunnea* (Alb. & Schwein.) Svrček & Kubička, but no ascocarps were observed to confirm the identity. The identity of *T. brumale* with cystidia was confirmed by its PCR-RFLP patterns. Four morphotypes were identified by their ITS sequences (Table 3). According to the BLAST search, the sequence obtained from the *T. brumale* morphotype without cystidia belongs to *Tuber oligospermum* (Tul. & C. Tul.) Trappe, and the morphotype *Cenococcum* like to *Cenococcum geophilum* Fr. The morphotypes *Tuber* like and ECM2 displayed the highest BLAST similarity with a sequence close to *Tuber rufum* (Baciarelli-Falini et al. 2006) and an uncultured ectomycorrhiza from the family *Clavulinaceae*, respectively. The number of nonmycorrhizal tips/seedling was higher in the noninoculated plants ($p=0.036$).

T. melanosporum-inoculated plants: fertilized vs. nonfertilized plants

There were no significant differences in growth between fertilized and nonfertilized *T. melanosporum*-inoculated plants, although the ratio of length of field roots vs. nursery roots was significantly greater ($p=0.009$) in the HNr fertilizer treatment compared with plants from nonfertilized control treatment (Table 4).

Across all treatments, average seedling data were (mean ± SE): taproot length 67±2.6 cm, height 33.9±2.28 cm, root collar basal diameter 1.09±0.05 cm, and total root length 8.81±0.89 m (coarse root length 7.41±0.85 m and fine root length 1.4±0.13 m). The mean number of root tips per seedling was 2,927±343, and the mean number of *T. melanosporum* mycorrhizae per seedling was 2,012±329. Over these 3 years in the field, the average proportion of *T. melanosporum* mycorrhizae (PT=6.6; CI 2.3–10.9) increased with respect to the proportion observed in the nursery-grown seedlings prior to plantation (PT=1.02, CI 0.79–1.24).

The probability of a tip from the field grown roots to be colonized by *T. melanosporum* was higher for plants treated with HKf than for nonfertilized inoculated plants ($p=0.0019$; Table 4). In addition, for inoculated seedlings treated with the same fertilizer applied to the roots (HKr), the probability of a tip to be colonized by an ECM fungus other than *T. melanosporum* was higher than for the nonfertilized inoculated plants ($p<0.0001$; Table 4).

In the fertilized *T. melanosporum*-inoculated plants, seven morphotypes apart from *T. melanosporum* were observed: AD type, *Sphaerosporella* like (probably *S. brunnea*), ECM1, ECM3, ECM4, ECM5, and ECM6. The mycorrhiza AD type, named for the 90° angles observed in the branching external hyphae, “angle droit” in French (Giraud 1988), was identified by its morphological and anatomical traits (Online Resource 2). Furthermore, BLAST search confirmed the identification of this mycobiont, showing the maximum identity (97–98%) with two

Fig. 1 Ectomycorrhizal morphotypes. The specific name after the morphotype code is the closest GenBank megablast match with sequences from ectomycorrhizal fungal specimens. **a** AD type: *Quercirhiza quadratum* (Águeda et al. 2008), **b** *T. brumale*, **c** *C. geophilum*, **d** ECM1: unknown, **e** ECM2: *Clavulinaceae*, **f** ECM3: *M. broomeianus*, **g** ECM4: unknown, **h** *Tuber* like: *Tuberaceae*, **i** ECM5: *A. hygrometricus*, **j** ECM6: *T. ellisii*, **k** *T. melanosporum*, **l** ECM7: *Tomentella* sp., **m** *Sphaerosporella* like, **n** *Suillus* like. *Bar*=300 µm

Table 4 Effects of the fertilizer treatments on the (1) mean ratio of length of field- vs. nursery-developed roots, (2) median of the proportion of *T. melanosporum* mycorrhizae with respect to total number of root tips in the field-developed roots, and (3) median of the proportion of number of mycorrhizae of fungi other than *T. melanosporum* with respect to the total number of root tips per seedling

NPK fertilizer treatment	Root length ratio FR/PR	*T. melanosporum* mycorrhizae Tmm/TT (%)	Non-*T. melanosporum* mycorrhizae Ofm/TT (%)
HPSR	0.91a	53.67a	0.029a
HKf	1.01a	82.70b	0.006a
HKr	1.43a	15.86a	7.852b
HPf	1.11a	48.09a	0.333a
HPr	1.40a	60.89a	0.013a
HNf	1.35a	39.05a	3.364a
HNr	1.90b	49.14a	0.416a
Control INOC	0.99a	49.14a	0.165a

Values followed by different letters differ significantly ($p<0.05$) to those from the control, accordingly to ANOVA in the two first variables and to logistic regression in the third one

HPSR 8-16-8, *HK* 10-6-28, *HP* 7-21-7, *HN* 12-7-7, *f* foliar application, *r* root application, *Control INOC* nonfertilized *T. melanosporum*-inoculated plants, *FR* roots developed in the field, *PR* roots developed in the nursery plug, *Tmm T. melanosporum* mycorrhizae, *TT* total number of root tips, *Ofm* mycorrhizae of fungi other than *T. melanosporum*

sequences identified as *Quercirhiza quadratum* (Águeda et al. 2008) and also classified as belonging to AD type. After molecular analyses (Table 3), the morphotype ECM3 was identified as *Melanogaster broomeianus* Berk. (100% similarity), and on the same tips, DNA from *Nectria radicicola* was identified. A picture of the mantle of ECM3 is shown in Online Resource 3. The morphotype ECM4 could not be identified by its DNA due to insufficient number of mycorrhizae. The morphotype ECM5 (Online Resource 3) showed the highest similarity with a sequence from *Astraeus hygrometricus* (Pers.) Morgan. The morphotype ECM6 was identified as *Tomentella ellisii* (Sacc.) Jülich & Stalpers. The mycobiont of the morphotype ECM1 could not be identified since the sequence obtained from these mycorrhizae belonged to the *Herpotrichiellaceae* family, nonectomycorrhizal fungi. In nonfertilized *T. melanosporum*-inoculated plants, the unique morphotype found, named ECM7, was identified by its nrITS DNA sequence as *Tomentella* sp.

Discussion

This study has challenged us to evaluate the dynamics of the mode of application, the dosages applied, and the ECM fungal community interactions in truffle orchards. Our results did not provide a straight-forward conclusion to the fertilizer question. It highlights the unknown role of potassium in truffle soils and its uptake by *T. melanosporum*. By systematically testing commercially available fertilizers utilizing several combinations in order to test elevated doses of a specific nutrient, we have observed that this is more than a nutrient-driven system, and we have identified several of the ECM fungal species which do proliferate in these truffle orchards, established on abandoned agricultural lands with naturally occurring high pH soils. Clearly, there is more work to do.

The sharper plant growth improvement in response to fertilization was in seedlings treated with HNr with higher ratios of length of field roots vs. nursery roots than nonfertilized plants. In the long term, this faster growth of the roots could lead to colonization of new tips by other fungi if *T. melanosporum* is not able to grow at the same rate as roots (Olivier 2000). HNr-fertilized plants had three different ECM fungi in their roots—*S. brunnea*, ECM3 (*M. broomeianus*), and the morphotype ECM4—although none was present in proportions higher than *T. melanosporum*. The same fertilizer applied to the leaves (HNf) did not produce any significant effect in plant or fungal growth in our experiment, but the unique morphotype found in this treatment, ECM5 (*A. hygrometricus*), predominated over *T. melanosporum* (Table 3). Bonet et al. (2006) observed no fertilization effect on survival, growth, or mycorrhizal status of *Q. ilex* seedlings in the first 18 months in the field, after a single foliar application of HNf at two doses. Olivera (2005) applied the same two doses of HNf as Bonet et al. (2006) to *Q. ilex* seedlings during 4 years in the field. They did not find differential growth in fertilized plants, but the higher dose of fertilizer decreased the number of *T. melanosporum* mycorrhizae. In this study, we applied only the lower recommended doses to minimize potential negative effects of high doses on mycorrhizal development. Possibly, the precaution of using low doses of the fertilizers resulted in the lack of any outstanding change in vegetative growth of the seedlings. In addition, across all treatments, after 3 years in the field, the proportion of *T. melanosporum* mycorrhizae (PT) increased compared to PT in nursery seedlings before planting. Thus, fertilization at these levels did not inhibit the development of *T. melanosporum* mycorrhizae.

Foliar fertilization is useful because nutrients are immediately available to the plant and not subject to soil processes. A foliar application of a fertilizer may result in plant synthesis of carbohydrates directly available to the ECM fungi in the roots, while soil application of the fertilizer involves utilization of the nutrients by other microorganisms. This could be the case of the effect of the fertilizer HK; when applied to the leaves, it favors *T.*

melanosporum already colonizing the roots in the plants, but when applied to the roots, HK favored colonization by non-*T. melanosporum* fungi. Little is known about K absorption by *T. melanosporum* mycorrhizae, although Domínguez Núñez et al. (2006) suggested that this fungus might limit K uptake. Further research in this topic is needed to address the role of K in this plant–fungus symbiosis.

In roots of plants where HKr was applied, we found mycorrhizae of *T. ellisii* and of the AD morphotype type. *Tomentella* species are common in truffle environments, but they can be overlooked in sporocarp inventories due to their inconspicuous fruiting bodies (De Miguel et al. 2001). The mycorrhiza AD type has been found several times in naturally occurring truffle woodlands and orchards (Bencivenga et al. 1995; De Román and De Miguel 2005; Baciarelli-Falini et al. 2006; Águeda et al. 2008). This mycorrhiza displayed the highest similarity with ITS sequences from vouchered mycorrhizae belonging to the AD-type group (Águeda et al. 2008) and with sequences from other ectomycorrhizae belonging to *Pyronemataceae* family collected in truffle environments (Baciarelli-Falini et al. 2006) and also classified as AD type. In our study, these mycorrhizae colonized 15% of the root tips in the seedling where it was found.

Improved plant performance in response to colonization by various *Tuber* species seems to depend on host–*Tuber* spp. combination (Bencivenga and Venanzi 1990). Domínguez Núñez et al. (2006) found improved seedling growth and survival of summer drought for *Q. ilex* and *Quercus faginea* Lam. seedlings inoculated with *T. melanosporum*, 20 months after outplanting. The same authors reported improved growth and increased P uptake for *T. melanosporum* inoculated, *Quercus petraea* Liebl. and *Pinus halepensis* Mill. seedlings (Domínguez Núñez et al. 2008); theirs were nursery seedlings whose growth response was measured 6 months after inoculation. In our study, the lack of differences in growth parameters between noninoculated and *Tuber*-inoculated plants may be due to the effectiveness of field-borne or other nursery mycorrhizal fungi. However, after 3 years in the field, the number of nonmycorrhizal tips per seedling was still higher in the noninoculated seedlings.

In the noninoculated seedlings, seven different ECM morphotypes were found, of which only *S. brunnea* was also present in the roots of *Tuber*-inoculated seedlings. This fungus may have come from the nursery since it is a typical ECM greenhouse species (Bencivenga et al. 1995). In noninoculated plants, we observed mycorrhizae of *C. geophilum*, *T. brumale*, *T. oligospermum*, two morphotypes belonging to the family *Tuberaceae* and *Clavulinaceae*, respectively, and the morphotype *Suillus* like. *C. geophilum* was also present in roots of all nonfertilized inoculated seedlings. This ascomycete is a very common ECM species found in several types of environments, including truffle orchards (De Román and De Miguel 2005; Domínguez Núñez et al. 2006; García-Montero et al. 2007; Bonet et al. 2006).

T. brumale has been recorded several times in other studies in *Q. ilex*–*T. melanosporum* orchards (De Miguel and Sáez 2005; Domínguez Núñez et al. 2006). It can be problematic in *T. melanosporum* truffle orchards because it is a very competitive species whose truffles are of lower quality than those of *T. melanosporum*. *T. oligospermum* was found together with *T. brumale* and misidentified with this species in the morphotyping process, due to a lack of cystidia. The morphotype belonging to *Tuberaceae* showed a high similarity to a sequence close to *T. rufum* (Baciarelli-Falini et al. 2006). The absence of cystidia and the thickness of the mantle walls agree with this identification.

Of the 40 inoculated seedlings examined, *T. melanosporum* was the predominant fungus in all but two seedlings. The proportion of *Tomentella* sp. in one plant treated with HKr was 3.6 times higher than the proportion of *T. melanosporum*, while mycorrhizae of *A. hygrometricus*, in other seedling treated with HNf, were found in a number six near times higher than *T. melanosporum*. Fruiting bodies of *A. hygrometricus* are frequent in truffle forests, and they are also able to produce a burn effect around the stem of the tree, similar to that produced by *T. melanosporum* (Giraud 1988).

The capacity for *T. melanosporum* to predominate and proliferate in *Q. ilex* seedlings has been observed in other studies. Martínez de Aragón (2005) studied the role of soil born inoculum as competitor for Holm oak seedlings inoculated with *T. melanosporum* shortly after a catastrophic forest fire. Four and a half years after plantation, he found that truffle-inoculated Holm oaks maintained 36% of their root tips colonized with *T. melanosporum*. Domínguez Núñez et al. (2006) observed a decrease in colonization from spontaneous soil fungi in outplanted *Q. ilex* seedlings inoculated with *T. melanosporum* as compared to non-inoculated plants. It is possible that *T. melanosporum* and *Q. ilex* form a perennial symbiosis that may exclude competing ectomycorrhizal fungi, especially in Mediterranean conditions where investment in drought tolerance is crucial.

The morphotypes of ECM fungi observed in roots of noninoculated plants were different from those from inoculated plants. Inoculum of ECM fungi other than *T. melanosporum* may have originated from the nursery or reside in orchard soils, and the presence of black truffle mycorrhizae may influence the ECM assemblage able to colonize the roots of the *Tuber* plants. None of the noninoculated oak seedlings picked up *T. melanosporum* from any of the five truffle orchard sites suggesting the lack of native *T. melanosporum* inoculum in these sites.

We have seen that black truffle mycorrhization levels have increased over the 3 years in the field, maintaining its

predominance in the roots of these oaks. We have also observed that the doses of fertilizers used did not reduce or inhibit this colonization, and the foliar application of a high K fertilized enhanced the development of *T. melanosporum* mycorrhizae in roots developed after planting in the field. Our study provides an inventory of the ECM fungal community in these truffle orchards with an insight to what fungal species might compete with *T. melanosporum* for root colonization sites in young truffle orchards and might influence black truffle colonization and future truffle production.

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