Microbiol. Biotechnol. Lett. (2022), 50(1), 40–50
http://dx.doi.org/10.48022/mbl.2109.09012
pISSN 1598-642X eISSN 2234-7305

MBL
Microbiology and Biotechnology Letters

Mycorrhizal Synthesis of Périgord Black Truffle (*Tuber melanosporum*) with Mexican Oak Species

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Received: September 26, 2021 / Revised: January 6, 2022 / Accepted: January 17, 2022

The Périgord black truffle (*Tuber melanosporum*) is an edible fungus and among the most expensive foods worldwide. It is the basis of a multimillion-dollar bio-business. Truffle farming does not exist in Mexico, and no formal studies have been conducted on its culture. This report describes the mycorrhizal synthesis (i.e., artificial union of fungus with host) of *T. melanosporum* with oak species native to Mexico (*Quercus polymorpha*, *Q. fusiformis*, and *Q. canbyi*). The mycorrhizal association was successful in *Q. polymorpha* and *Q. fusiformis*, as confirmed morphologically and using *T. melanosporum* molecular primers (ITSML/ITS4LNG). The effect of the ectomycorrhizal fungus on host growth (stem diameter) was statistically significant. Illustrations of the study are presented.

Keywords: **Truffle farming, black diamond, Mexico, black forest gold**

Introduction

The Périgord black truffle (also known as the black diamonds of forest or gastronomy), is an edible hypogeous fungus belonging to the genus *Tuber* (family Tuberaceae, order Pezizales, class Discomycetes) and is the most widely cultivated truffle specie in the world [1, 2]. It is native to Europe grown mainly in France, Italy, Spain, and part of the former Yugoslavia [1]. Its cultivation in New Zealand in limed acid soils has also been quoted and reported successful results [2–4]. In the USA, this bio-industry emerged in the west (California) and east (Tennessee), and is the first productive truffle farm in North America [2, 5, 6]. The cultivation of *Tuber melanosporum* Vittad. has also been reported in Africa, Asia, South America (Chile, Argentina) with varied results [2, 6, 7]. The Périgord Black truffle is of great economic importance due to its high price (700–1500 €/kg) depending on season and weather conditions [2, 6, 8] and for its high demand in national and international haute cuisine which has established a multimillion-dollar forest industry (bio-enterprises) in Europe [3, 7–10]. The black truffle also known as the Périgord truffle is likewise, of great nutritional importance since it contains proteins, minerals as well as an excellent aroma and flavor [9, 11–14]. In Mexico, the trufficulture does not exist, however, in the 19th century, a "truffle" *sensu lato* was consumed by the Tarahumara communities in summer on the mountains of the Sierra Madre Occidental in Northwest of Mexico. Every year in July this "truffle", now known as *Melanogaster umbriniglebus* Trappe & Guzman, was collected with help of dogs that knew how to find them on their own [15]. A pioneer preliminary study on the cultivation of native truffles in

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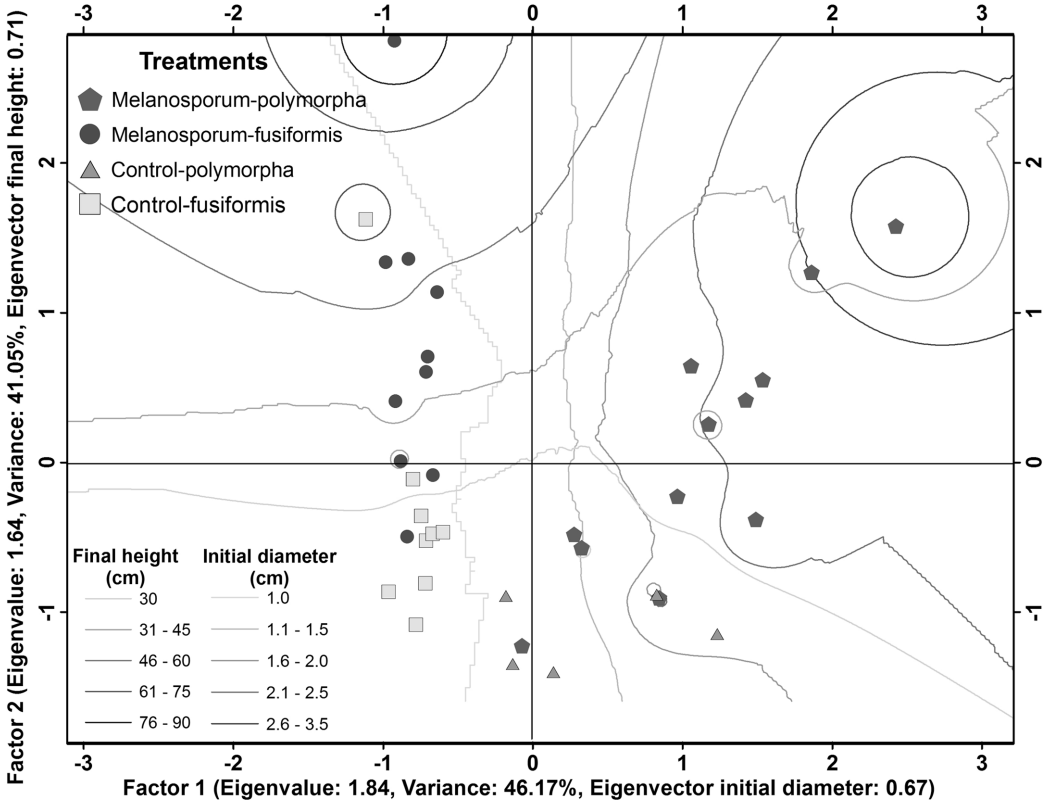

Fig. 1. Scatter plot from the factor analysis. The order of the observations is represented with different symbologies according to the treatment and the species of oak used. The interpolated values of the main morphological variables that explain the variations between treatments are also presented. The *Q. fusiformis* plants inoculated with *T. melanosporum* are ordered in relation to higher values of final height. While *Q. polymorpha* inoculated plants were ordered in relation to both high final height values and higher stem diameter.

México was carried in 2009 [16], the researchers reported the isolation and synthesis of *Tuber* sp. (*Tuber miquhuanensis* Guevara, Bonito & Cazares) with *Quercus canbyi* Trel. native species from northeastern Mexico. The almost nonexistent scientific papers on truffle cultivation in México contrast remarkably with those performed in Europe.

Due to the great importance of the Périgord black truffle, the absence of the truffle culture in Mexico, and the discovery of the Mexican black truffle *T. regimontanum* Guevara, Bonito & Rodr. (Fig. 2b) aroused interest in its cultivation and formulated the question could the Périgord black truffle be cultivated in Mexican oak species? To answer this question, a synthesis bioassay was performed using the Périgord black truffle with Mexican oak symbionts. The mycorrhizal synthesis was morphologically confirmed and molecularly corroborated.

Materials and Methods

Germination of oak seeds

Seeds of *Quercus polymorpha* Cham. & Schltdl., *Q. fusiformis* Mill. (Live oak) and *Q. canbyi* Trel. were collected from several trees adapted to the edaphic (vertisol soil), altitude (325 mosl) and climatic (dry and hot, range temperature of 2℃ in January, 38℃ in August) conditions of Ciudad Victoria, Tamaulipas, México. The oak species were morphologically (taxonomically) determined using specialized literature and herborized specimens were deposited at José Castillo Tovar Herbarium (ITCV). The seeds were submerged in tap water in a container and all floating acorns were discarded. The selected seeds were then disinfected with chlorine 2% for 15 s, ethyl alcohol 70% 10 s, and oxygenated water 3% 30 s, each time rinse with sterile water.

http://dx.doi.org/10.48022/mbl.2109.09012

Fig. 2. (a) Fresh fruiting bodies of the French Périgord *Tuber melanosporum*. Fruiting bodies and cross section of the American black truffle *T. regimontanum* (b). Germination of oak acorns (c). Oak seedlings after a month of germination (d). Inoculum solution of *T. melanosporum* (e). Inoculation of one month old oak seedlings with truffles ascospore suspension (f).

The 10 healthy selected seeds of each species were immediately germinated in a black clay soil (vertisol)/sand mixture (2:1, pH of 7.0–8.0) previously sterilized at 120℃ for two h at 15 lbs. Fifty nursery bags (180 × 60 mm), previously sterilized with 2% chlorine and then rinsed with sterile water, were poured with a soil volume of 400 ml in each bag to germinate the seeds, and placed in a greenhouse under natural daily temperature (2℃ in January, 38℃ in August)/light conditions and watered (200 ml) every third day. Seedlings emerged one month after planting (Fig. 2c, d).

Preparation of spore inoculum

Ascomata of *T. melanosporum* were obtained from

Tennessee Truffle, LLC USA. Five grams of dry ascoma were dissolved in 500 ml of sterile water during 12 h and homogenized with a blender for 3–5 s for the preparation of the spore suspension. Five ml of inoculum (1 million ascospores estimated with a Neubauer counting chamber), were deposited at the base of the one-month-old seedlings of *Q. polymorpha*, *Q. fusiformis,* and *Q. canbyi* with 10 replicates each including their controls according to the specialized literature [17–19]. A duplicate of the ascoma of *T. melanosporum* (Fig. 2a) used in this research was herborized and deposited in the Herbarium José Castillo Tovar (ITCV).

Colonization and ectomycorrhizal characterization

To determine the degree of mycorrhization, seedlings of 6 and 12 months after inoculation were analysed. Ten rootlets between 3–5 cm long were cut at random from fifteen mycorrhizal seedlings of *Q. polymorpha*, *Q. fusiformis*, *Q. canbyi* respectively, and from the not inoculated control, and the degree of root mycorrhization was measured by counting the total number of colonized and non-colonized tips and expressed as a percentage of mycorrhized tips with *T. melanosporum* in the function of the total number of examined tips.

The root system of the inoculated and control plantlets was washed and observed under the stereoscopic microscope Carl Zeiss for the recognition of ectomycorrhizae and photographed. The characterization of the mycorrhiza was carried out following specialized literature [20, 21] to recognize the type of mantle (puzzle), cystidia, the shape of the hyphae, dimensions, and color under a compound microscope [19].

Statistical analysis

The effect of the black truffle on the development of the height and the diameter of the oak stem was contrasted simultaneously between the plants inoculated with *Tuber melanosporum* and the control groups (without inoculation), in both oak species (*Q. polymorpha* and *Q. fusiformis*). For this, the two-way PERMANOVA test was used with the Morisita Index as the measure of distance considering the differences between the sample sizes of the treatments [22, 23]. The variables contribution and redundancy was determined by an ordination test known as Factor Analysis, used the Principal Component Analysis as extraction method and the varimax rotation method [24]. The two-way PERMANOVA test was carry out in the software PAST 4.06 [23], while the Factor Analysis in IBM SPSS 26 (IBM®, USA, 2019) and the graph were designed with in ArcGIS 10.3 (ESRI®, USA, 2014).

Molecular analysis

After one year of inoculation, colonized root tips by *T. melanosporum* with *Q. polymorpha*, and *Q. fusiformis* were analyzed following previously described methods [25–27]. The roots were removed and carefully washed with tap water. Fresh mycorrhizae formed by *T. melanosporum* were separated under a dissecting microscope and used for DNA extraction. Selected colonized root tips were vortexed in a microcentrifuge tube for 10 s and then centrifuged for 2 min at 14000 rev min^{-1} to separate and remove all residual soil particles from symbiotic tissue. Under a dissecting microscope with a Petri dish containing sterile distilled water, the mantle was first cleaned from surrounded mycelium to prevent PCR contamination by other undesirable fungi. Then the mantle was peeled with a needle and a small portion (0.01–0.02 mm^2) was removed and transferred directly into the PCR tube containing 20 µl of sterile water. The ITS gene of *T. melanosporum* from mycorrhizal root tips DNA was amplified using the specific primers ITSML/ITS4LNG (5'-TGG-CCATGTGTCAGATTTAGTA-3'), (5'-TGATATGCTTAAGTTCAGCGGG-3'), and primers ITS1F/4 (5'-CTTGGTCATTTAGAGGAAGTAA-3'), (5'-GCATATCAATAAGCGGAGGA-3') and TUBNESTREV (5'-GGATACCCGCTGAACTTAA-3') following previously described protocols for these primers [25, 26]. A phylogram was elaborated using the Blast tool to confirm the specificity of the target sequence, gene, and related truffle species. PCR amplification with specific primer pairs was performed in an OMNI-E (Hybaid, UK) thermal cycler using 0.3–5 ng of target DNA isolated from root tips: The reaction was carried out under the following conditions: initial denaturation was at 95 ℃ for 3 min, 23 cycles consisting of 30 s at 94℃, 30 s at 63℃, and 45 s at 72℃; and final extension for 7 min. The amplification was performed in a final aqueous volume of 50 µl containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 4 mM $MgCl_2$, 20 pmol of each specific primer, 200 mM of each dNTP, in the presence of 2.5 U of *Taq* Polymerase (Pharmacia Bio-tech, Sweden). Twenty

µl of PCR products were run on 2% agarose gel.

PCR amplification with ITS1F and ITS4 primers on the ectomycorrhizal fungi was performed as above, but the annealing temperature was decreased to 55℃. Positive and negative controls were included in the DNA experiment.

Results

The mycorrhizal synthesis of the Périgord black truffle, *Tuber melanosporum* was successfully performed in two out of three oak species native to Mexico (Figs. 1–5). A phylogenetic analysis tree of the ITS amplified with the specific primers ITSML/ITS4LNG (5'-TGG-CCATGTGTCAGATTTAGTA-3'), (5'-TGATATGCTTAAGTTCAGCGGG-3') of the mycorrhiza of the truffle used as inoculum confirmed the specificity of the primers for the sequences of *T. melanosporum*, genes, and origin (Périgord from France) in 100% identity (gene bank numbers GU979083.1, GU810153.1, and GU810152.1) and can be seen in Fig. 3, 5d.

On the other hand, in Fig. 4 and 5 can be seen the mycorrhizal formation of *T. melanosporum* with *Q. polymorpha* and *Q. fusiformis* which is characterized by having the tips of the rootlets with a swollen or inflated shape in a monopodial way. These results agree with those reported by other researchers [28–30], who point out that the multinucleate dark hyphae of the mycorrhizal fungus grow on the root surface in the cortical cells forming a mantle around the root but never penetrate the cells. Although recently, arbutoid mycorrhiza of black truffle *T. melanosporum* has been observed ultrastructurally [31]. In Fig. 4e and 5c the mantle can be seen forming a "puzzle" characteristic of *T. melanosporum* and the presence of cystidia in *Q. polymorpha* root tip (Fig. 4b–d). In contrast, all *Q. canbyi* seedlings inoculated with *T. melanosporum* died very likely due to high temperatures (38℃) prevailing in the place where the assay was done, indicating that this host is not suitable for truffle synthesis at this altitude, climate, and soil conditions and was excluded from the assay (data are no shown).

The best host for mycorrhizal synthesis was *Q. fusiformis*, its ectomycorrhiza ratio in the root system

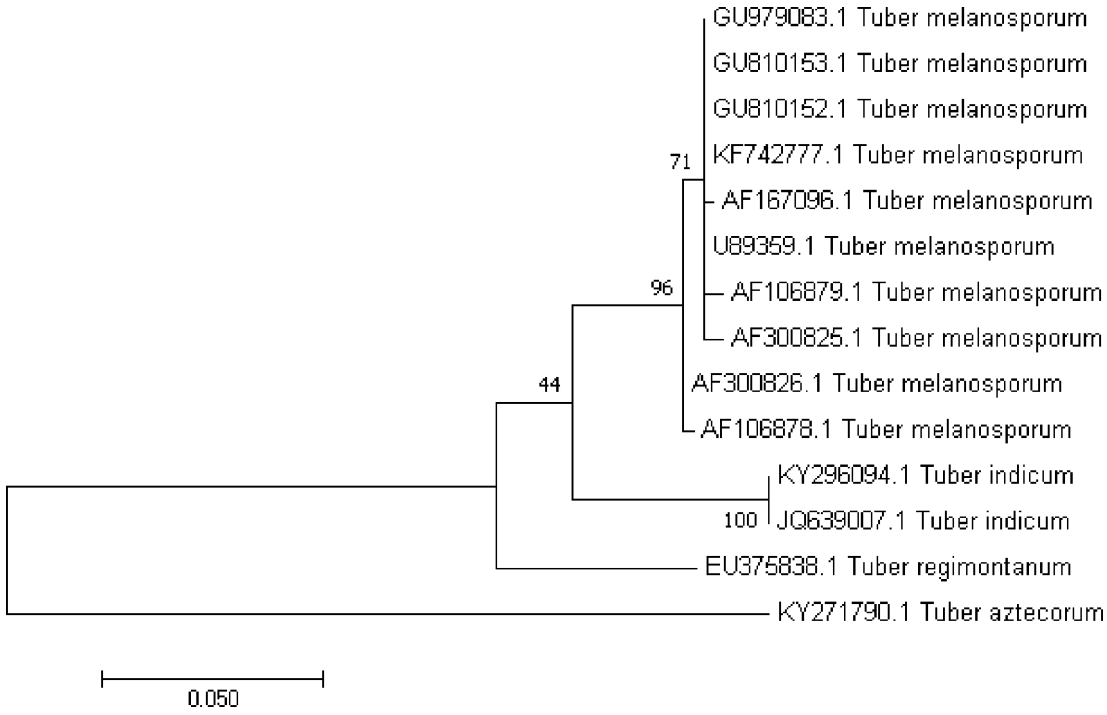

Fig. 3. Phylogenetic tree of the ITS amplified with the specific primers ITSML/ITS4LNG (5'-TGG-CCATGTGTCAGATTTAGTA-3'), (5'-TGATATGCTTAAGTTCAGCGGG-3) for the mycorrhizae of the truffle *T. melanosporum* used as inoculum retrieved from Blast NCBI data base. (gene bank numbers GU979083.1, GU810153.1, and GU810152.1)

was 30–35%. This result agrees with reports [32–34] indicating good percentage.

The results of the PERMANOVA analysis showed that the effect of the truffle *T. melanosporum* in the development of the two oak species is statistically significant different in both the inoculation treatments ($p < 0.01$), and between the oak species ($p < 0.01$) (Table 1). However, the interaction between treatments and oak

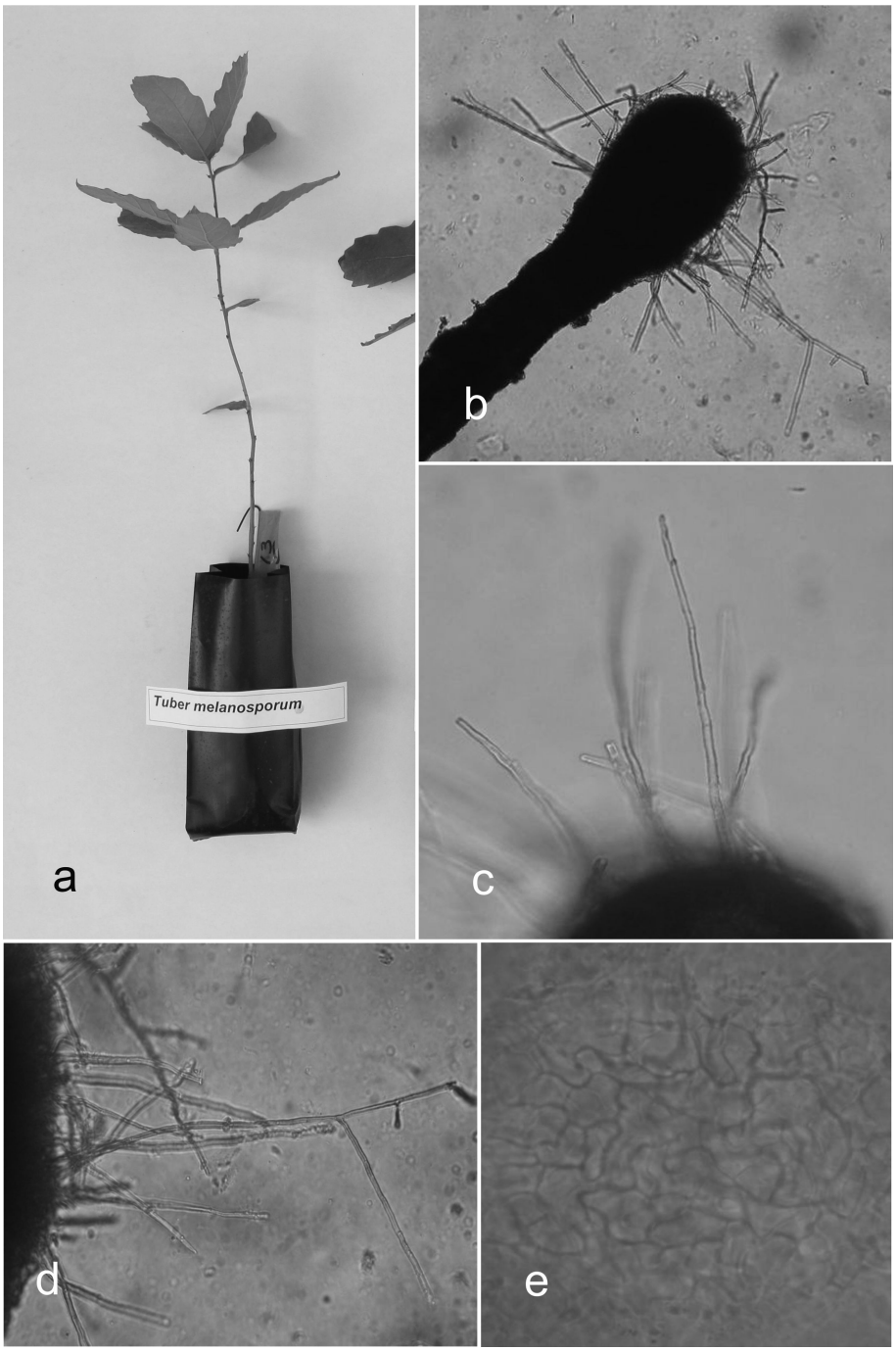

Fig. 4. (a) *Quercus polymorpha* seedling after a year of inoculation. Root tip colonized with *T. melanosporum* (b). Close up of *T. melanosporum* cystidia (c). Angular hyphae of *T. melanosporum*(d). Close up of mantle (puzzle shape) of *T. melanosporum* on root surface (e).

http://dx.doi.org/10.48022/mbl.2109.09012

species showed high significance values ($p = 0.99$), which indicates equality between the four combinations. Based on the Factor Analysis, the variable Initial Stem Diameter presented the highest contribution in the first factor (Fig. 1). While the Final Stem Height was the variable that explains the variation of the second factor in the greatest way. Considering the Kaiser and Cumulative variance criteria, only these factors are significant in explain the data variation.

Finally, the molecular analysis of the genomic DNA of the fungus growing in the root system of two oak species (*Quercus polymorpha* and *Q. fusiformis*) using specific primers for *T. melanosporum* can be seen in Fig. 5d. The primers amplified the ITS gene of the mycorrhizal fungus in the root tips except in line 87 that did not amplify. An ITS phylogenetic tree of the mycorrhizae truffle used as inoculum to trace its origin can be seen in Fig. 3. It can be concluded that the truffle *T. melanosporum* can be detected with molecular techniques using specific primers for this edible fungus that grows in ectomycorrhizal association with two native oak species from Northeast of Mexico.

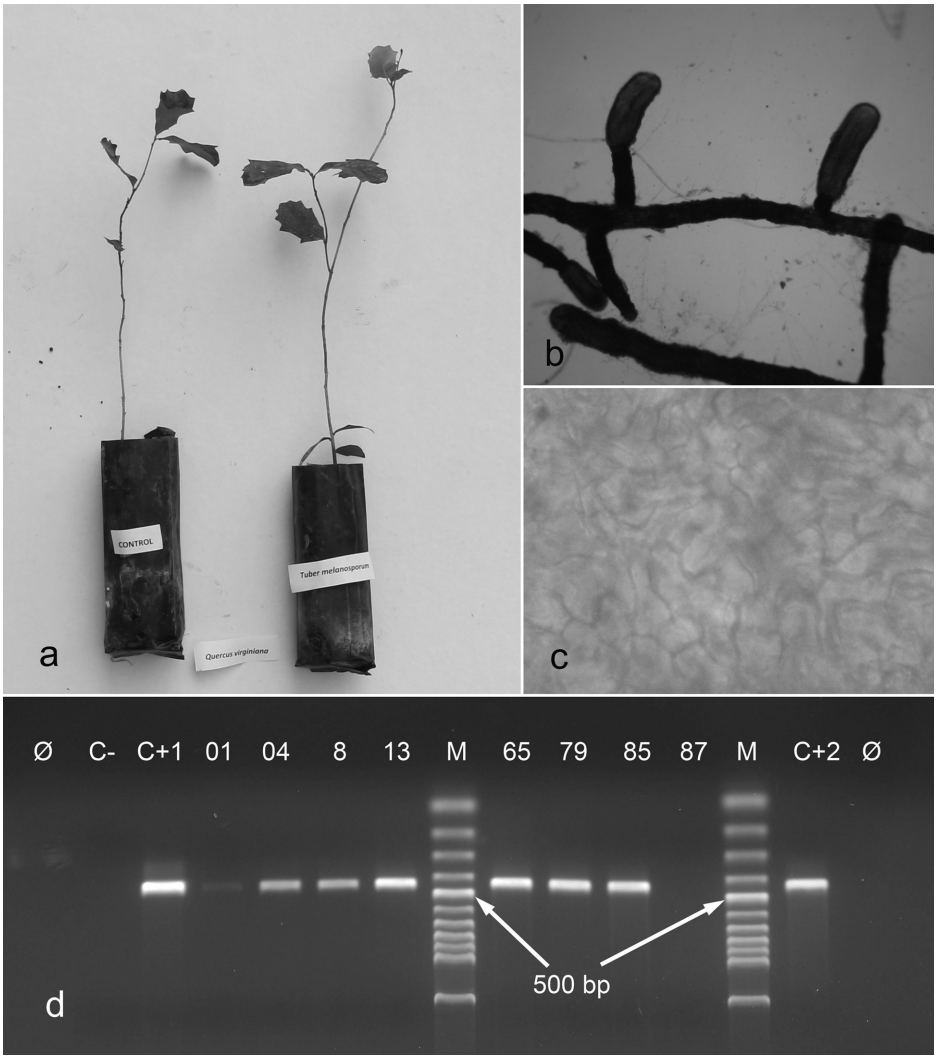

Fig. 5. (a) *Quercus fusiformis* seedlings inoculated with *T. melanosporum* after a year of inoculation. Monopodial mycorrhizae of *Q. fusiformis* and *T. melanosporum* (b). Close up of *T. melanosporum* mantle (puzzle) on *Q. fusiformis* root tip (c). Electrophoresis of mycorrhizae of *T. melanosporum* by primers ITSML/ITS4LNG on *Q. polymorpha* (lines 04, 8 &13) and *Q. fusiformis* (lines 01, 65, 79, 85, & 87), c- = negative control, c+1 and c+2 = positive control, M = molecular marker (Lader 5 prime Perfect Size), line 87 was contaminated (d).

Table 1. PERMANOVA test results.

Resourses	Sum of squares	Degree freedom	Mean square	*F*	*p*
Treatments	0.0043	1	0.0043	44.6890	**0.0001**
Oak species	0.0230	1	0.0230	239.1800	**0.0001**
Interaction	-0.0005	1	-0.0005	-5.7625	0.9999
Residual	0.0030	32	0.00009		
Total	0.0298	35			
Permutations	9999				

Discussion

The edible Périgord black truffle *T. melanosporum* is grown in all continents [1, 2, 35]. Its culture is a profitable sustainable alternative as an agroforestry activity; however, truffle farming does not exist in Mexico yet. The discovery of the Mexican black truffle *T. regimontanum* in 2008 (Fig. 2B) which similarity to the European black truffle or Périgord *T. melanosporum*, aroused the interest in its cultivation (Fig. 2a). In addition, the demand for the French Périgord black truffle by the haute cuisine and the potential of this million-dollar bioindustry worldwide encouraged the performance of this research on Mexican hosts tree [35].

Ecological conditions required for the cultivation of truffles, such as climate, topography, geology, type of soil, flora, associated fauna, and susceptible species for cultivation, among other recommendations in Europe are well known and must be considered to introduce the truffle culture in Mexico [3, 4, 14, 19, 36].

The results indicate that the inoculation of *T. melanosporum* induces greater development of the height in these native oak species used in the experiment, at least under laboratory/greenhouse conditions. In addition, the species *Q. fusiformis* showed a greater development of the stem diameter with respect to *Q. polymorpha* (Table 1, Fig. 1). This symbiotic research demostrated their association which could potentially be important as a new agroforestry alternative for México. In addition, the regional ecological conditions for its growth and susceptible host species for synthesis were suitable and promising for its cultivation. The finding of *Tuber regimontanum* in Higueras Nuevo León, Mexico (N.L.), and other native economic potential species such as *T. canaliculatum* Gilkey from N.L., *T. miquihuanense* from Tamaulipas and *T. bonitoi* Guevara & Trappe from Veracruz and *Tuber* spp. from other regions of Mexico [37–40], set up the possibility of its cultivation and might be confirmed by morphological characterization and molecular means [25–27, 41–43]. The features of the mycorrhizal synthesis of *T. melanosporum* with *Q. fusiformis* and *Q. polymorpha* are very similar to those hosts reported in Europe where the black Périgord truffle and other *Tuber* spp. are cultivated with oak (*Quercus* spp.) but can also be grown in *Corylus avellana* L., *Carpinus betulus* L., *Fagus sylvatica* L., *Betula pendula* Roth, *Populus alba* L., *P. nigra* L., *Tilia sylvestris* Desf., *Ostrya carpinifolia* S, *Salix viminalis* L., *Cedrus deodara* (D. Don), *Carya illinoinensis* (Wangenh.) K. Koch, *Pinus halepensis* Mill. and *Abies alba* Mill. [44–49]. *Alnus acuminata* Kunth and *Fagus grandifolia* var. *mexicana* (Martínez) E. Murray have been recently reported as a host for *Tuber* spp. as well [47]. Recently, a tripartite association has been reported including arbuscular mycorrhizal plants with oaks species and *T. melanosporum* [49].

The black diamond of gastronomy can be cultivated in agricultural soils, with a pH of 7.5–8.0. The topography of study region (Tamaulipas, Mex.) presents ideal pH (7–8.1) for *T. melanosporum* culture, although annual range temperature is (2℃ min) 13 a 37℃ (42℃ max) and an annual average temperature of 30.1℃. Furthermore, the Périgord black truffle has been reported successfully surviving under water stress condition [29, 32]. On the other hand, forest soils are not recommended because they contain many propagules of ectomycorrhizal fungi species that would compete with the truffle [3, 29, 30, 32]. *T. melanosporum* grows strictly in calcareous soil with a C/N ratio close to 10, the texture of the soil must be well structured (*i.e* plenty of spaces and entrances for the movement of water, gases, nutrients, roots and organisms), and it develops best at annual temperatures

of 11–14℃, with an annual rainfall of 500–900 mm, in deep soils, well aerated, drained and with water availability in dry periods [21, 36]. The value of the production of this edible truffle can exceed the value of the wood when inoculated trees mature, making it highly profitable [50]. Mycorrhizal formation must be ensured in seedlings in order to increase success for sporocarp yield. The inoculation must be performed by dispersing fresh ascospores on naked roots in 1–4 month-old seedlings, although applying directly to plantations (*e.g Corylus avellana*) may improve yields [11, 34]. In our assay, 6 months old, inoculated seedlings' roots did not showed any mycorrhizae formation but plantlets after one year from inoculation. Seedlings were taken at random, and the roots were observed for mycorrhiza formation and morphology to validate its mycorrhizal status, and also by species specific primers. Although 35% of the root system was colonized by the black truffle in this study, several aspects of its life cycle need to be addressed to obtain ascocarp production. *T. melanosporum* is a heterothallic fungus [51, 52]. The mating types of genes recognized are Mat 1-1 and Mat 1-2 and play a role in the recognition and compatibility among different strains and mating types genes by controlling pheromone production. The ectomycorrhiza in *T. melanosporum* is haploid. The ascospores after germinating produce haploid mycelium that colonizes the tips of the roots thus forming the ectomycorrhizae. The plant can be colonized by different haploid mycelium at the same time but can be stay separated (spatially) preventing contact, compatibility, and therefore fruiting body formation [51, 52]. In addition, the exclusion of one mating type by the opposite one in the old ectomycorrhizal root tips may prevent ascocarp formation and must be considered in planning a truffle orchard [51–53]. Thus, the experience of this research it is expected that the truffle farming introduction in México will open a new agroforest alternative for rural economic development (truffle culture) for this region producing certifying mycorrhized seedlings with root (tips) morphology/anatomy characterization and/or molecular markers.

Acknowledgments

Guevara-Guerrero thanks CONACyT and TecNM for economic and research support. The senior author acknowledges Ana Maria Mejia García for her encouragement to perform this research. Victor A. Vargas Vázquez is appreciated for research support.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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