Detection of *Tuber melanosporum* DNA in soil

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Received 10 August 2005; revised 20 October 2005; accepted 20 October 2005.
First published online 25 November 2005.

doi:10.1111/j.1574-6968.2005.00029.x

Editor: Geoffrey Gadd

Keywords
Tuber melanosporum; soil mycelium; PCR-RFLP; truffle orchard; ITS.

Abstract

Our objectives were (i) to develop a molecular method to detect mycelia of *Tuber melanosporum* (black truffle) in soil and (ii) to test for mycelial distribution around two truffle-bearing *Quercus ilex* trees in a truffle orchard. Isolation of total DNA from soil was performed, followed by PCR amplification with *T. melanosporum*-specific primers and restriction analysis. To address the detection sensitivity level, soil samples were inoculated with known amounts of gleba of *T. melanosporum*. The detection limit was ≥ 11.4 μg of hyphae g^{-1} of soil. Mycelium was detected primarily within the area defined by the truffle burn and within the top 35 cm of the soil in all directions from the trees.

Introduction

Truffles are ascomata of ectomycorrhizal hypogeous fungi belonging to the genus *Tuber* F. H. Wigg (Ascomycotina). More than 20 species of this genus thrive in the temperate and Mediterranean forests of Europe, where truffles have been collected for centuries. Annual world truffle production during the last 100 years has dropped from *c*. 1000 to 200 tons (Olivier, 2000). This drop has led to the establishment of truffle orchards, where an understanding of the biology and ecology of *Tuber* is required for successful management and economically sustainable production. Understanding is limited by difficulties in growing *Tuber* mycelium under axenic conditions (Pacioni & Comandini, 1999) and in studying the mycelium in its soil habitat.

The Perigord black truffle, *Tuber melanosporum* Vittad., is the most commercially valuable of the dark gleba truffles. For cultivation purposes, it is critical that the desired species be distinguished for seedling inoculation and monitoring of commercial plantations. Successful inoculation and colonization of plant seedlings with *T. melanosporum* is well documented (Chevalier & Grente, 1978; Bencivenga, 1982), but the factors that induce truffle formation are not known. Truffle production begins 5–10 years following orchard establishment, so confirmation of the presence of the fungus in the orchard prior to truffle production is important. The abundance and distribution of mycelium may be used as indicators of fungal activity and can provide information on nutritional requirements and on the fungal response to various cultivation treatments.

Tuber melanosporum hyphae are not visible to the naked eye, but its DNA can be selectively amplified by PCR from total soil DNA extracts using specific primers. Several authors have designed *T. melanosporum*-specific primers (Paolocci *et al*., 1997, 2000; Gandeboeuf *et al*., 1997a), but these primers were not adequate for our purposes because of weak or nonspecific amplifications. The objectives of this study were (i) to establish an accurate DNA isolation protocol applicable to soil samples (ii) to develop a PCR primer pair specific for *T. melanosporum*, and (iii) to test the ability of these primers to detect the presence and distribution of *T. melanosporum* mycelium in soil surrounding two *Quercus ilex* L. trees in a commercial truffle orchard.

Materials and methods

Source of fungal material

Fruit bodies from 12 *Tuber* species and from 12 different species belonging to other genera (Table 1) were collected from wild black truffle areas and orchards in different parts of Spain, or were obtained from the herbaria of the Centre Tecnològic Forestal de Catalunya (Solsona, Spain) and the MA-Fungi (Real Jardín Botánico de Madrid, Spain). Fresh fruit bodies were identified (Montecchi & Lazzari, 1993;

Table 1. Isolates used in this study, collection number (herbarium)* and province from which they were collected

Species	Collection
Tuber aestivum Vittad.	F059 and F060 (CTFC) Lleida; MA-Fungi 54690 to MA-Fungi 54693 Valladolid
Tuber albidum Pico	MA-Fungi 54686 Valladolid; MA-Fungi 54688 Palencia
Tuber brumale Vittad.	F061 to F071 (CTFC) Teruel; MA-Fungi 28373 Barcelona
Tuber excavatum Vittad.	F042 (CTFC) Huesca; MA-Fungi 35877 and 35878 Castellón; MA-Fungi 54695 Valladolid
Tuber maculatum Vittad.	MA-Fungi 46891 Albacete; MA-Fungi 57008 Asturias
Tuber malençonii Donadini, Riousset, G. Riousset & G. Chev.	F073 (CTFC) Teruel; MA-Fungi 28383 Castellón; MA-Fungi 28384 Lleida; MA-Fungi 35378 and MA-Fungi 46892 Albacete
Tuber melanosporum Vittad.	F046 to F058 and F079 (CTFC) Teruel; F080 (CTFC) Lleida; MA-Fungi 28386, MA-Fungi 28387A-B, MA-Fungi 28388B and MA-Fungi 28391 Huesca; MA-Fungi 28389 and MA-Fungi 35274 Castellón; MA-Fungi 28390 and MA-Fungi 33354 Guadalajara; MA-Fungi 28392 and MA-Fungi 28393 Tarragona; MA-Fungi 29269 and MA-Fungi 35380 Soria; MA-Fungi 33495 Segovia; MA-Fungi 35379 Albacete; MA-Fungi 41117 La Rioja
Tuber mesentericum Vittad.	MA-Fungi 26765 Soria; MA-Fungi 46893 to MA-Fungi 46895 Albacete
Tuber oligospermum (Tul. & C. Tul.) Trappe	F074 and F075 (CTFC) Teruel; MA-Fungi 39553 and MA-Fungi 41010B Madrid; MA-Fungi 47931B Valladolid
Tuber panniferum Tul.	MA-Fungi 46897 Albacete; MA-Fungi 40277 Navarra
Tuber rufum Pico	F076 and F077 (CTFC) Teruel; MA-Fungi 25122 Barcelona; MA-Fungi 28397 Girona
Tuber uncinatum Chatin	F078 (CTFC) Lleida; MA-Fungi 26760 and MA-Fungi 29390 Navarra
Balsamia vulgaris Vittad.	MA-Fungi 56974 Asturias
Ganoderma lucidum (Curtis) P. Karst	F081 (CTFC) Lleida
Genea fragrans Wallr.	MA-Fungi 40255 Navarra
Genea verrucosa Vittad.	MA-Fungi 46887 Albacete; MA-Fungi 56993 Asturias
Hymenogaster citrinus Vittad.	MA-Fungi 47722 Girona
Hymenogaster lycoperdineus Vittad.	MA-Fungi 47723 Girona
Hymenogaster luteus Vittad.	MA-Fungi 29639 León
Hymenogaster niveus Vittad.	MA-Fungi 54837 Valladolid
Melanogaster variegatus (Vittad.) Tul & C. Tul.	MA-Fungi 47721 Girona
Rhizopogon cf. roseolus (Corda) Th. Fr.	MA-Fungi 47724 Burgos
Russula fragilis (Pers.) Fr.	MA-Fungi 42067 Barcelona
Terfezia arenaria (Moris) Trappe	F082 (CTFC) Teruel

*CTFC, Centre Tecnològic Forestal de Catalunya (Solsona, Spain); MA-Fungi, Real Jardín Botánico de Madrid (Spain).

Riousset *et al.*, 2001), freeze-dried and stored at $-20\,^{\circ}C$ before use.

Soil samples

Four soils were used to test the extraction procedure: (1) from nursery pots of truffle-inoculated *Q. ilex* seedlings; (2) from a wild truffle bed; (3) from a productive truffle orchard; and (4) from a cereal field adjacent to the truffle orchard. All samples were stored at $-20\,^{\circ}C$ until use. Roots from nursery seedlings were examined under a light microscope to confirm the presence of *T. melanosporum* ectomycorrhizae, which were characterized by spinulae on the surface of the mantle and sheath ornamentations (Rauscher *et al.*, 1995). Soil samples from the wild truffle bed and the truffle orchard were collected close to the base of truffle-bearing trees.

Samples from the field soils (2–4) were collected from the upper 15 cm of the profile. The nursery substrate contained 60% composted peat, 15% perlite-vermiculite, and 25% nonsterilized soil from wild truffle beds (pH 8.0). The wild truffle bed soil is a loam [39.5% sand, 42.5% silt, 18% clay, 2.6% organic matter, (pH 8.2)]. The truffle orchard soil is a sandy-clay-loam [55% sand, 18% silt, 27% clay, 1.5% organic matter, (pH 8.3)]. The cereal field soil characteristics match those of the orchard which had been established within this field, and whose soil was used as the negative control for detection of *T. melanosporum* mycelium and for soil inoculation assays.

DNA isolation and PCR amplification

DNA from 93 fruit bodies was isolated using the E.Z.N.A® Fungal DNA miniprep kit (Omega Bio-Tek, Doraville, GA) following the manufacturer's instructions. The soil DNA isolation protocol was based on a described hexadecyltrimethylammonium bromide (CTAB) extraction method (Kårén *et al.*, 1999), in which the initial amount of sample was increased to 7 g (wet weight). All soil samples were examined to confirm the absence of ectomycorrhizae or spores of *T. melanosporum*. Small stones, roots and debris were removed. Samples were ground in a porcelain mortar

with 40 mL of CTAB-lysis buffer containing 0.5% polyvinylpyrrolidone. The slurry was transferred to 50 mL polypropylene tubes and incubated for 1 h at 65 °C. After incubation, samples were centrifuged at 9800 ***g*** for 5 min at 10 °C. Six hundred microliters of the supernatant was transferred to a 1.5 mL microcentrifuge tube and the published protocol (Kårén *et al.*, 1999) was followed for the remaining steps. The DNA content of fruit bodies and soil extracts was quantified spectrophotometrically. Contamination by proteins and humic acids in the DNA was evaluated by $OD_{260/280\,nm}$ and $OD_{260/230\,nm}$ ratios.

DNA extracted from fruit bodies and soils were amplified (Martín & Winka, 2000) with primers ITS1F/ITS4 (White *et al.*, 1990; Gardes & Bruns, 1993) to estimate the extraction efficiency and to confirm the presence of fungal DNA and the lack of *Taq* polymerase inhibitors. Three different *T. melanosporum*-specific primer pairs were tested to amplify DNA from mycelium of *T. melanosporum* in soil samples: $MELF_{926}/MELR_{926}$ (Gandeboeuf *et al.*, 1997a), SS14Fw/SS14Bk (Paolocci *et al.*, 2000) and ITSML/ITS4LNG (Paolocci *et al.*, 1997). Extractions from *T. melanosporum* ascomata and from nursery-pot soil were used as positive controls while extractions from ascomata from other *Tuber* species and from soil from the cereal field were used as negative controls. Controls with no DNA were included in every series of amplifications. Amplification reactions were carried out using puReTaq Ready-To-Go PCR Beads (Amersham, Buckinghamshire, UK), following the cycling conditions proposed by Paolocci *et al.* (1997, 2000) and Gandeboeuf *et al.* (1997a) and containing 10–30 pmol of each primer. Amplicons were analyzed on 2% agarose gels.

Design of specific primers

Oligonucleotide sequences specific to *T. melanosporum* were designed based on all available sequences (GenBank [NCBI]) of the internal transcribed spacer (ITS) regions including the 5.8S rRNA gene, for 12 *Tuber* species (Table 1). Two primers were designed: the forward primer ITS1TM (5′-GTATTCCCGAACACAAACCT-3′) in positions 24–43 of *T. melanosporum* ITS1 region sequence (accession number AF106877), and the reverse primer ITS2TM (5′-AGACTTGTGACTGATCCAGG-3′) that corresponds to the complement of positions 140–159 in the ITS2 region. Amplification reactions (Martín & Winka, 2000) were done with PCR Beads. Each 25 μL reaction included 10 pmol of each primer. Positive, negative and controls with no DNA were included in each amplification series.

Restriction fragment length polymorphism (RFLP)

Five microlitres of the amplification products obtained with ITS1TM/ITS2TM were analyzed by RFLPs generated by *Alu*I, *Eco*RI, *Hinf*I, *Mse*I (Invitrogen, Paisley, Scotland), and *Taq*I (Amersham). The fragments were resolved on 2% agarose gels. Fragments smaller than 100 bp were not recorded.

Sequencing and nucleotide accession numbers

To confirm the identity of amplification products obtained from soil extractions as *T. melanosporum*, amplicons were cleaned with the E.Z.N.A® Cycle Pure kit (Omega Bio-tek) and sequenced with ITS1TM/ITS2TM primers. Nucleotide BLAST searches were used to compare the sequences obtained in this study against other DNA sequences in the NCBI database (Altschul *et al.*, 1997). Sequences were deposited in GenBank as AJ786645 and AJ786646.

Detection limit of designed protocol

Tenfold dilution series (3.5 mL) of gleba from an immature *T. melanosporum* ascoma ranging from 800 to 8×10^{-4} mg of hyphae, were used to inoculate seven samples of the cereal field. Before inoculation procedures, DNA from two samples (7 g) of cereal field soil was extracted and amplified with ITS1F/ITS4 and with ITS1TM/ITS2TM (to confirm the absence of *T. melanosporum*). Deionized water was added to another cereal field soil sample as a negative control. To determine the detection limit without soil, the same dilutions of gleba were ground in 40 mL of extraction buffer and DNA extracted from all samples according to our developed protocol.

To confirm the amount of inoculated DNA, a 10-fold dilution series was freeze-dried and the DNA extracted in 1.5 mL tubes (Kårén *et al.*, 1999). PCR amplifications were made with ITS1F/ITS4 and ITS1TM/ITS2TM. To compare the detection threshold from these samples with that obtained from the DNA extractions carried out with 600 μL of the 40 mL of extraction buffer, we used an equivalent smaller volume from the 600 μL of supernatant after first centrifugation, for the extraction procedure. This small volume was increased to 600 μL by adding warm (65 °C) CTAB extraction buffer. Each procedure was performed at least twice.

Field-testing of the detection techniques

Extraction and amplification protocols were tested for their ability to detect mycelium of *T. melanosporum* in the soil surrounding two truffle-bearing trees from an 8-year-old commercial *Q. ilex* truffle orchard, in Sarrión, Teruel. The area surrounding both trees displayed mycelium-induced burns. The soil type was a typic calcixerept characterized by four distinct horizons. The upper horizon (0–30 cm) was an Ap with a loam structure, followed by a horizon Bw, with a loam–clay structure (30–50 cm), and a Bkm horizon

(50–65 cm) with aggregates of calcium carbonate, above the parent material (C, 65 cm) formed by a hard and continuous limestone layer. Samples were collected in November 2003 with a 7 cm diameter soil corer along two transects, one running north–south and the other east–west of the trees, at 40, 140 and 240 cm from the stem. At each distance, soil cores from the upper soil profile (depths of 5–10 cm), the middle soil profile (30–35 cm) and just above the rock layer (55–60 cm) were processed (a total of 72 samples) to determine the presence of mycelia. Burn extension (cm) from the stem of each tree at each cardinal point was measured. For all samples from which we did not observe amplification with ITS1TM/ITS2TM, PCR reactions with ITS1F/ITS4 were performed.

Image processing

Digital images of the electrophoresis gels were processed with NIH Image (U.S. National Institutes of Health, v.1.1.63) to measure DNA band intensities. We established five band intensities by comparing the percentage of the area of the intensity peak of each soil sample to the corresponding area of a known standard from an ascoma of *T. melanosporum*. These percentages were used to establish intensity gradients for our PCR products from the soil samples (Fig. 1, Table 2).

Results

DNA isolation and PCR amplification

DNA extracts from soils gave $OD_{260/280\,nm}$ ratios between 1.0 and 1.5 and $OD_{260/230\,nm}$ ratios between 0.4 and 1.0. DNA extractions from fruit bodies were successfully amplified with the fungal-specific primers ITS1F/ITS4, giving the expected ITS-size amplicons. Extractions from soils gave different bands after amplification with this primer pair, indicating the presence of multiple fungi.

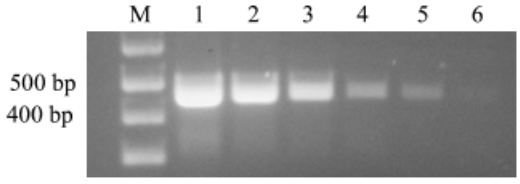

Fig. 1. Patterns of band-intensities[a] of *Tuber melanosporum* in soil extractions after amplification with ITS1TM/ITS2TM. Lane M, 1 kb Plus Ladder marker. Lane 1, DNA from a *T. melanosporum* ascoma (known standard), level [+++]; lanes 2–6, DNA from soil samples: lane 2, 70.92% = [+++]; lane 3, 41.44% = [++]; lane 4, 10.71% = [+]; lane 5, 6.96% = [(+)]; lane 6, nondetectable = [−]. [a]Intensity gradings correspond to percentages comparable with band intensity from the known standard. [+++] = 70–100%; [++] = 40–70%; [+] = 10–40%; [(+)] < 10%; [−] = nondetectable.

Polymerase chain reactions carried out with the primers ITSML/ITS4LNG resulted in non-specific amplifications from two ascomata of *T. brumale*. Parallel amplifications with ITS1F/ITS4 primers were consistent with the initial identification of those ascomata as *T. brumale*. Amplifications made with $MELF_{926}/MELR_{926}$ yielded the expected DNA fragment in *T. melanosporum* ascomata but amplified fragments of various lengths from ascomata of *T. brumale, T. excavatum* and *T. rufum* and gave weak amplifications from soil samples. Reactions with SS14Fw/SS14Bk gave weak amplifications for ascomata of *T. melanosporum* even when the primer concentration was increased to 20 and 30 pmol. Primers ITS1TM/ITS2TM did not amplify DNA in any extractions other than those containing *T. melanosporum*. We found the expected amplicon of 465 bp in *T. melanosporum* ascomata, in soil from nursery pots and in soils from commercial and wild truffle beds.

RFLP and sequencing

The RFLP patterns from ascomata and soil mycelia were identical: *Alu*I (< 100, 120, 250 bp), *Eco*RI (double band around 230 bp), *Hinf*I (219, 228 bp), *Mse*I (370 bp) and *Taq*I (170, 185 bp). Since amplicons had the same digestion patterns, only one from soil mycelium and one from an ascoma were sequenced. Both of these sequences were identical and also to those of 100 other *T. melanosporum* sequences in GenBank (GenBank accession numbers: AJ459543–AJ459559, AJ459573–AJ459577, AJ459582, AJ583559, AJ583567–AJ583636, AJ583825–AJ583826 and AJ548481–AJ548484).

Detection limit of designed protocol

DNA extractions from the noninoculated cereal field soil gave amounts of DNA of *c.* 16 μg g^{-1} of soil with $OD_{260/280\,nm}$ of 1.2 and $OD_{260/230\,nm}$ near 0.7. Amplification with ITS1F/ITS4 yielded several bands, but no *T. melanosporum* DNA was detected in the ITS1TM/ITS2TM amplifications. DNA extracted from inoculated soils could be amplified with ITS1F/ITS4 and ITS1TM/ITS2TM. DNA from samples with dilutions down to and including 8×10^{-2} mg of gleba had the expected 465 pb band (Fig. 2). DNA extracted from gleba ground alone in buffer solution had the expected band in samples down to and including 8 mg of gleba (Fig. 2). The amount of *T. melanosporum* DNA inoculated into the soil, inferred from the amount of DNA obtained from the extraction of freeze-dried dilutions, suggests ~310 μg of DNA g^{-1} of gleba. PCR results from the extractions corresponding to the small volumes of supernatants showed that the expected band could be detected in dilutions containing down to and including 8×10^{-2} mg of gleba. Extractions performed with the remaining large volume of supernatant after first centrifugation, gave amplification with the specific

Table 2. Intensities* of the amplified ITS1TM/ITS2TM fragment of DNA extractions from soil surrounding two black truffle-bearing trees in four directions at three distances and three depths

Tree 1						Tree 2					
			Distance (cm)						Distance (cm)		
Direction	Burn (cm)	Soil depth (cm)	40	140	240	Direction	Burn (cm)	Soil depth (cm)	40	140	240
North	243	5–10	–	++	–	North	152	5–10	+	++	+
		30–35	+++	+	–			30–35	+++	+	(+)
		55–60	–	–	–			55–60	(+)	–	–
South	230	5–10	++	+++	+++	South	165	5–10	+	+	–
		30–35	++	+	+++			30–35	–	(+)	(+)
		55–60	(+)	+	–			55–60	+	–	–
East	190	5–10	(+)	+	(+)	East	159	5–10	+	+	–
		30–35	(+)	(+)	(+)			30–35	(+)	–	–
		55–60	(+)	(+)	–			55–60	–	–	–
West	180	5–10	+	++	+	West	140	5–10	+	++	–
		30–35	+++	(+)	+			30–35	–	–	–
		55–60	–	–	–			55–60	–	–	–

*Intensity gradings correspond to percentages comparable with band intensity from the known standard. +++, 70–100%; ++, 40–70%; +, 10–40%; (+) < 10%; –, nondetectable. These intensities are visually explained in Fig. 1.
Burn extension, soil depth and distance from the tree to the edge of the burn are given for each direction.

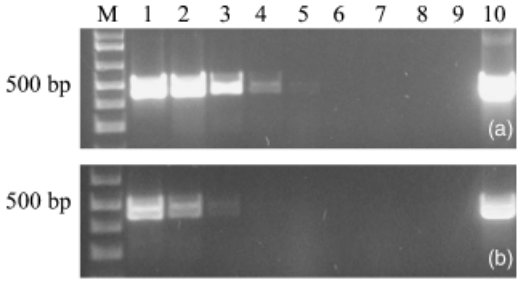

Fig. 2. (a) Sensitivity of PCR using primers ITS1TM/ITS2TM in DNA extractions from soil samples inoculated with serial 10-fold dilutions of 800 mg of *Tuber melanosporum* hyphae from gleba. Lane M, 1 kb plus ladder marker. Lanes 1–7, quantities of hyphae were 800, 80, 8, 8×10^{-1}, 8×10^{-2}, 8×10^{-3} and 8×10^{-4} mg; lane 8, soil inoculated with deionized water; lane 9, control with no DNA; lane 10, *T. melanosporum* ascoma. (b) Amplified products from the same dilutions of gleba without soil and extracted with 40 mL of buffer solution. Lane 8, deionized water. Three independent replications gave the same results.

primers down to and including 8×10^{-4}. Negative controls of soil yielded no amplification products.

Field-testing of the detection techniques

Optical density$_{260/230\,nm}$ ratios ranged between 1.0 and 1.3 and OD$_{260/280\,nm}$ between 0.4 and 1.0. The average amount of DNA recovered in crude preparations were of 24.68 ± 6.83, 17.57 ± 4.82 and $6.32 \pm 1.23\,\mu g\,g^{-1}$ of soil in 5–10, 30–35 and 55–60 cm of depth soil cores respectively. The final DNA extracts were suitable for PCR and no additional purification was required. PCR reactions with primers ITS1TM/ITS2TM detected the expected band. Results from PCR amplifications from soils surrounding both trees are listed in Table 2.

Discussion

Our first objective, to develop a molecular tool to detect *Tuber melanosporum* mycelium in soil, was achieved, providing a technique applicable for monitoring the presence of this fungus in truffle orchards even prior to truffle production. In our field testing, we did not obtain very pure DNA extracts, but the noninhibition of *Taq* polymerase indicated that our resultant DNA extractions were sufficiently pure for amplification without need for further purification, which is time-consuming and reduces the DNA yield (Kuske *et al.*, 1998). In addition, extracts with the lowest ratios of OD$_{260/280\,nm}$ and OD$_{260/230\,nm}$ (< 1.0) did not correspond to the lowest *T. melanosporum* detection levels after PCR reactions.

When using the *T. melanosporum*-specific primers designed by other authors (Paolocci *et al.*, 1997, 2000; Gandeboeuf *et al.*, 1997a), we encountered problems of weak or nonspecific amplifications. The low level of genetic variability found in *T. melanosporum* (Henrion *et al.*, 1994; Gandeboeuf *et al.*, 1997b; Bertault *et al.*, 2001; Mello *et al.*, 2002) and the high number of available sequences in GenBank, allowed us to design a specific primer pair for this fungus (ITS1TM/ITS2TM) and to test it with a relatively low number (31 collections) of *T. melanosporum* ascomata. The use of RFLP comparisons of the ITS region permits the confirmation of species-level identification (Henrion *et al.*, 1994; Amicucci *et al.*, 1996; Kårén *et al.*, 1997; Grebenc *et al.*, 2000). We obtained amplifications with enough DNA for RFLP or sequencing analyses. The similar RFLP patterns

from the soil mycelia and the ascomata amplicons allowed the identification of those from soil mycelium as *T. melanosporum*. Sequences obtained confirmed the RFLP identification.

To determine the sensitivity of our extraction protocol and designed primers, we tested soil samples inoculated with known quantities of sterile hyphae from the immature gleba. It was not possible to utilize hyphal mycelia because of growth difficulties in pure culture (Fasolo-Bonfante & Fontana, 1973), or hyphae from germinating spores because of the symbiotic nature of this fungus, which cannot grow well in the absence of a host. The minimum quantity of *T. melanosporum* DNA that we could detect from inoculated soil samples with our technique was 3.6 ng g^{-1} of soil corresponding to 11.4 μg of *T. melanosporum* hyphae g^{-1} soil. As only 1 μL of the 50 μL of the soil DNA extracts was used for PCR amplification, this value indicates that our PCR protocol has the potential to detect as little as 7.5 pg of *T. melanosporum* DNA. We observed a positive soil effect in DNA extraction procedures but a negative effect in PCR-amplifications. The positive effect of soil in extractions could be explained by additional abrasion of the soil particles increasing the lysis efficiency and subsequent DNA yield. The negative effect of soil in amplifications is probably because of the presence of *Taq* polymerase inhibitors. Differences in the minimum detectable DNA between samples from gleba without soil (ground in a standard mortar and pestle), and gleba ground in a 1.5 mL tube, also may be due to the effectiveness of cell disruption, which is greater with a pellet pestle in a 1.5 mL tube.

We did not expect to find *T. melanosporum* outside the burn but we detected mycelium in samples as far as 88 cm outside the burn. This suggests that the burn does not necessarily coincide precisely with mycelium distribution and could explain the occurrence of truffle ascomata outside the burns. Generally, we found that *T. melanosporum* hyphae were distributed throughout the first 35 cm of the soil profile, in the same depth range where ascomata grow, but below where ectomycorrhizae are usually found. Mycelium was infrequent at 60 cm but where found, it was detectable at very low levels.

To our knowledge, this is the first reported study of *T. melanosporum* mycelium distribution in soil. This detection method provides essentially qualitative results. Quantitative aspects, inferred by the establishment of an intensity gradient for amplicons, can be problematic with respect to the ratio of amplification products, which may or may not adequately reflect the initial ratio of template molecules, as band intensities do not continue to increase after a certain number of PCR cycles (Brüggemann *et al.*, 2000). Further work using DNA quantification techniques such as real-time PCR are needed to obtain a more precise measure of mycelial quantity. Using mycelium quantity as a response variable to cultivation techniques in truffle orchards, we could improve our knowledge of black truffle ecological requirements.

Acknowledgements

This work was supported in part by the DMAH-GENCAT, by scholarship 2002FI-00711 to L.M. Suz from the DURSI-GENCAT, and by the research project FMI-REN2002-04068-CO2-01. We are indebted to M. Doñate for the open access to his truffle orchard. The authors thank C.R. Fischer for helpful suggestions and English support.

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