Mycelial abundance and other factors related to truffle productivity in *Tuber melanosporum–Quercus ilex* orchards

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Abstract

Relative quantification of DNA from *Tuber melanosporum* mycelia was performed by conventional and real-time PCR in soil from trees in three truffle orchards of different ages to determine: (1) whether *burn* appearance is related to the amount of *T. melanosporum* mycelium in soil, and (2) whether productivity onset and truffle production are related to (a) the amount of *T. melanosporum* mycelium in soil, (b) tree height and diameter, (c) *burn* extension and (d) surface rock cover. The *burn* seems to appear only after a certain amount of mycelium has formed. Precociously productive trees presented higher quantities of mycelium than nonproductive trees in the productivity onset study, while highly productive trees presented less quantities of mycelium than nonproductive trees in the productivity study. Trees with high but not excessive surface rock cover showed greater truffle production. Larger trees tended to display a *burn* earlier than smaller trees.

Introduction

Truffles are edible fruitbodies of hypogeous ascomycetes that establish an ectomycorrhizal relationship with trees, mainly oaks and hazelnuts. The Black truffle (*Tuber melanosporum* Vittad.) is a gastronomically prized species, with a high market value. It grows naturally in calcareous zones of Spain, France and Italy, although it has been introduced in other countries by planting seedlings inoculated with this fungus. Wild production of truffles has decreased sharply in the last century, and production from truffle orchards is increasing with keen interest in understanding production factors.

The onset of truffle production in orchards is unpredictable (4–12 years) and highly variable among trees in the same orchard. Tree basal diameter has been shown to be an important predictor of truffle production (Shaw *et al*., 1996), possibly because with a larger host tree, more photosynthetic tissue becomes available for carbon assimilation by the fungal partner. Surface rock cover is an important feature of truffle orchards because it favors soil aeration and drainage, reducing compaction and erosion by rain, and can explain 24% of production variability (García-Montero *et al*., 2007a).

An early indicator of Black truffle activity in soil is the appearance of a zone with diminishing vegetation surrounding the stem of the tree, known as the *burn*, caused by the phytotoxic effect of *T. melanosporum* mycelium (Fasolo-Bonfante & Fontana, 1971). The *burn* usually appears before truffle production begins but it does not guarantee that the tree will produce truffles. Fungal species that fruit abundantly are not necessarily abundant belowground in their mycorrhizal form (Gardes & Bruns, 1996; Horton & Bruns, 2001; Taylor, 2002). This lack of correspondence between ascoma and mycorrhiza abundance has been observed for *Tuber magnatum* and *T. melanosporum* (Murat *et al*., 2005; Baciarelli-Falini *et al*., 2006; Bertini *et al*., 2006). Other than confirming the presence of the fungus in the soil, observations of *T. melanosporum* mycorrhizae provide no predictive information regarding ascomata production.

Because growth of mycorrhizal fungi depends on the uptake of carbon from the plant (Smith & Read, 1997; Hampp & Schaeffer, 1999), there should exist an equilibrium between carbon allocated to the fungus and the investment of this carbon in the different fungal structures, including mycorrhizae, mycelium and fruitbodies. We know that mycelium of ectomycorrhizal fungi may comprise 30–80% of fungal biomass (Wallander *et al*., 2001; Högberg & Högberg, 2002). We hypothesize that the quantity of soil hyphae, inferred from the quantity of DNA detected from

this truffle species in soil, should be related to the production of truffles, which represent one of the belowground structures dependent on plant carbon allocation.

DNA-based techniques have only recently been applied to study fungal ecology in soil. Detection of DNA from *T. melanosporum* mycelium in soil, carried out by Suz *et al.* (2006), showed that increasing amounts of Black truffle hyphae in soil gave proportionally greater relative band intensities using conventional PCR with specific primers. Quantification of DNA from hyphae in soil through real-time PCR allows detection and monitoring of the distribution and abundance of a particular fungus (Landeweert *et al.*, 2003; Kennedy *et al.*, 2007; Parladé *et al.*, 2007).

The purposes of this work were: (1) to test the reliability of the *T. melanosporum*-specific primer pair ITS1TM/ITS2TM (Suz *et al.*, 2006) to perform real-time PCR, applying this quantitative technique to detect and quantify the DNA of this fungus in soil and to validate results obtained by conventional PCR; (2) to determine whether *burn* appearance is related to (a) differences in the amount of *T. melanosporum* mycelium in soil, and (b) tree height and diameter; and (3) to determine whether the onset of truffle production and differences in truffle production among trees are related to (a) differences in the amount of *T. melanosporum* mycelium in soil, (b) tree height and diameter, (c) *burn* extension and (d) percentage of surface rock cover.

Materials and methods

Field sites and experimental design

This study was carried out in three Holm oak (*Quercus ilex* L.) Black truffle orchards. Site and soil characteristics are

described in Table 1. The parameters included in this table allow us to compare the sites with respect to defined features of Black truffle habitat (Colinas *et al.*, 2007). Although they differ in their calcium carbonate content, they all have well-aerated calcareous soils, and all three sites are located in areas where truffles have been found in naturally occurring truffle beds. Production of *T. melanosporum* truffles was monitored during the winter season (November 2004–March 2005) in the three orchards before soil sampling.

The three orchards in our study represent three critical phases in truffle cultivation, which can be observed aboveground and may reflect key belowground biological activity. After establishment of inoculated seedlings in the field, the appearance of the first *burn* signals that the fungus is thriving belowground; the onset of production signals that mycelium has reached a critical maturity and environmental conditions are adequate to permit fruiting; and the productivity phase reflects that trees are physiologically capable of supporting truffle production with the successful completion of the fungus's life cycle.

Burn onset study

The orchard in Santorens was sampled to study the relationships between *burn* presence and abundance of Black truffle mycelium in the soil and tree height and diameter. Ten out of 49 trees in this orchard displayed a clear *burn* and six trees displayed no *burn* in the spring of 2005. The remaining 33 trees showed incipient or unclear *burns*. We selected the six trees with no *burn* and randomly selected six out of the 10 trees with *burns* (totalling 12 trees) for tree measurements and soil collections.

Table 1. Site and soil characteristics for each experimental orchard

	Study (orchard)		
	Burn onset	Productivity onset	Productivity
Locality	Santorens (Huesca, Spain)	La Pinareja (Teruel, Spain)	San Agustín (Teruel, Spain)
Plantation establishment year	1999	1997	1995
Altitude (m)	1049	949	965
pH	8.1	8.4	8.3
Organic material (%)	4.0	4.56	1.45
Calcium carbonate (%)	46	17	5
Nitrogen (Kjeldahl) (%)	0.23	0.26	0.1
Phosphorus (Olsen) (ppm)	4	8	10
Potassium (% of potassium oxide)	263	270	419
Exchangeable magnesium (%)	–	94	90
Total sand (0.05 < D < 2 mm) (%)	40.6	47.0	54.9
Coarse silt (0.02 < D < 0.05 mm) (%)	13.4	13.1	7.5
Fine silt (0.002 < D < 0.02 mm) (%)	21.6	25.6	10.3
Clay (D < 0.002 mm) (%)	24.4	14.3	27.3
USDA Classification	Loam	Loam	Sandy-clay-loam

Productivity onset study

The orchard in La Pinareja was sampled to study the relationships between productivity onset and relative abundance of *T. melanosporum* mycelium in soil, tree height and diameter, surface rock cover and *burn* extension. Forty-two out of 247 trees had started to produce truffles in the winter season 2004–2005. Trees were classified into two groups: (1) nonproductive trees and (2) trees that had recently produced truffles. Twenty trees of each class (totalling 40 trees) were randomly selected for tree measurements and soil collections.

Productivity study

The orchard in San Agustín was sampled to study the relationships between truffle productivity and all variables analyzed in the productivity onset study. Trees in this orchard had started to produce truffles in 2001. According to their truffle production, measured in approximated ranges of kg truffles per tree from the winter 2004 to 2005, 190 trees were classified into four classes: (1) nonproductive (0 kg), (2) low production (0–0.5 kg), (3) intermediate production (0.5–1 kg) and (4) high production (> 1 kg). Twelve trees from each class (totalling 48 trees) were randomly selected.

Soil sampling and variable measurement

Burn presence and extension, tree height and diameter and surface rock cover were recorded from April to July 2005. *Burn* extension was calculated as an average of two perpendicular diameter measurements per tree. In the truffle productivity orchard, surface rock cover was estimated visually by comparison with a template of surface cover. Because surface rock cover was very high in the productivity onset orchard, rock cover here was estimated by measuring presence/absence of rocks in each centimeter of a 1-m transect randomly positioned on the *burn*. In both studies, surface rock cover was recorded as a percentage. Two soil samples were taken from each tree in opposite, randomly selected directions: at 50 cm from the stem in the *burn* onset and productivity onset studies, and at 1 m from the stem in the productivity study, due to the greater size of the trees. Soil samples were collected from 5 to 15 cm depth using a 4-cm diameter soil corer. The two samples from each tree were combined and a 7-g subsample was stored at $-20\,^{\circ}C$ for DNA extraction. A soil sample from a cereal field adjacent to each of the truffle orchards was collected and analyzed as a negative control. One soil sample from a tree in class 3 of the productivity study was lost, leaving 11 observations for that class.

DNA extraction

Twelve soil samples from the *burn* onset study, 40 from the productivity onset study and 47 from the productivity study

plus 3 negative control samples were processed (102 soil samples). All samples were examined under the stereoscope and microscope to avoid the presence of spores and mycorrhizae of *T. melanosporum* before DNA extraction. Total soil DNA was extracted from 7-g soil samples following Suz *et al.* (2006) with slight modifications (see supplementary Appendix S1). DNA extractions from the *burn* and productivity onset studies were purified using the commercial EZNA® Cycle Pure kit (Omega Bio-Tek) following the manufacturer's instructions. For real-time PCR, aliquots of 35 DNA extractions from soil samples from the productive orchard were also purified. DNA from fungal sporocarps used to test the specificity of the primers in real-time PCR (Table 2) was extracted previously by Suz *et al.* (2006).

Conventional and real-time PCR

All PCR reactions were performed as described in Suz *et al.* (2006) using the primer pairs ITS1F/ITS4 (White *et al.*, 1990; Gardes & Bruns, 1993) and ITS1TM/ITS2TM. DNA from *T. melanosporum* ascomata CTFC-F080&F047 was used as the standard. The relative intensity value for each sample was obtained by dividing the area of the intensity peak of the PCR band from the sample by the area of the intensity peak of the PCR band from the standard.

Quantitative assays were performed using the ABI Prism® 7700 Sequence Detection System (Applied Biosystems). Primers ITS1TM/ITS2TM were tested to amplify DNA extractions from species listed in Table 2 and from aliquots of 35 nonpurified and the same 35 purified DNA extractions from soil samples of the productivity study.

Table 2. DNA isolates used in this study with corresponding collection number (herbarium)*

Species	Collection
Tuber aestivum Vittad.	MA-Fungi 54690
T. albidum Pico	MA-Fungi 54688
T. brumale Vittad.	MA-Fungi 28373
T. excavatum Vittad.	MA-Fungi 35878
T. melanosporum Vittad.	CTFC-F046 to F048; CTFCF079-F080
T. oligospermum (Tul. & C. Tul.) Trappe	MA-Fungi 39553A
T. panniferum Tul.	MA-Fungi 40277
T. rufum Pico	MA-Fungi 25122
Balsamia vulgaris Vittad.	MA-Fungi 56974
Genea fragrans Wallr.	MA-Fungi 40255
G. verrucosa Vittad.	MA-Fungi 46887
Hymenogaster lycoperdineus Vittad.	MA-Fungi 47723
H. luteus Vittad.	MA-Fungi 29639
H. niveus Vittad.	MA-Fungi 54837
Rhizopogon cf. roseolus (Corda) Th. Fr.	MA-Fungi 47724
Russula fragilis (Pers.) Fr.	MA-Fungi 42067

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

Three replicates of each aliquot from purified DNA were used for quantitative runs. PCR reactions were performed in 96-well Optical Reaction plates. Twenty-five microliter reactions were pipetted in each well, containing 1 μL of DNA template, 0.2 μM of each primer, 12.5 μL of SYBR® Green PCR Master Mix (Applied Biosystems) and 10.5 μL of sterile Milli-Q water. DNA-free controls were run for each experiment. Amplification conditions were: 95 °C for 10 min, 35 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. A melting curve temperature profile for each sample was obtained at 95 °C for 1 min, 60 °C for 1 min and 95 °C for 20 min. Data were analyzed using the software packages SDS 1.9.1 and DISSOCIATION CURVES 1.0 (Applied Biosystems). A 10-fold dilution series from 10^0 to 10^{-8} containing known amounts of DNA from *T. melanosporum* were used to construct a calibration curve. To confirm that only one target sequence was amplified, the PCR products were run in 2% agarose gels. DNA from *T. melanosporum* in soil samples was converted to ng *T. melanosporum* DNA g^{-1} soil (wet weight), based on the calibration curve.

Statistical analyses

Standard ANOVA and LSD ($P < 0.05$) were used to compare and separate means, and linear regression to correlate relative band intensity with *burn* extension. Some variables were transformed to meet assumptions of ANOVA; their means were back transformed to the original scale and reported as medians (Ramsey & Schafer, 1996).

Results

DNA extraction and PCR

DNA was successfully extracted from 101 of the 102 soil samples. Extracted DNA was amplifiable with fungal ITS1F/ITS4 primers, showing mainly a wide band ranging between 600 and 1000 bp. One sample from a nonproductive tree in the productivity onset study could not be amplified with ITS1F/ITS4. When amplified with ITS1TM/ITS2TM, samples presented the expected *T. melanosporum* amplicon of 465 bp, with varying band intensities, except those from soils of three recently productive trees and three nonproductive trees from the productivity onset study. Negative controls from the cereal fields gave amplifications for ITS1F/ITS4 but not for ITS1TM/ITS2TM.
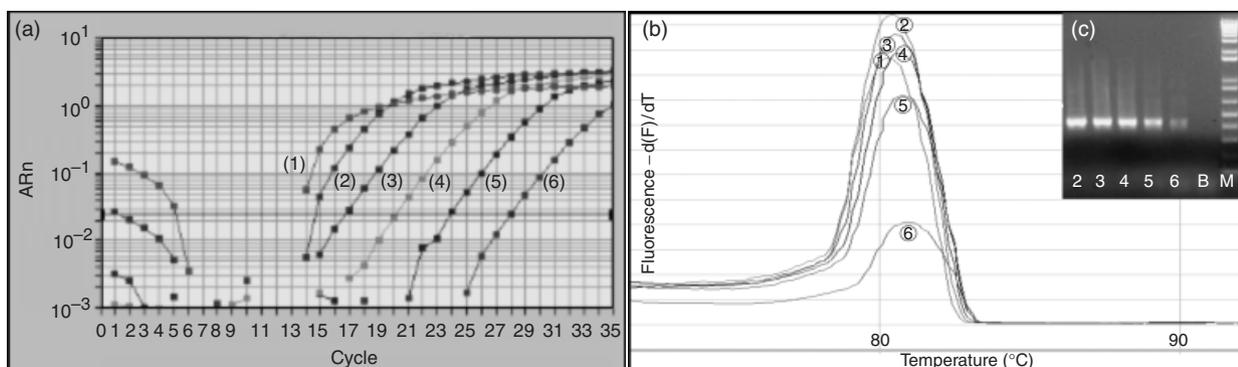
After real-time PCR, the 10-fold dilution series of DNA from a *T. melanosporum* ascomata, containing up to 4.55 fg DNA $μL^{-1}$, showed the expected amplicon of 465 bp (Fig. 1) as determined by either melting curve analysis or agarose gel electrophoresis. DNA extractions from other fungal species and nonpurified soil samples showed neither the melting peak nor the expected band in the gel. Soil collected from nonproductive trees presented an average of 124 ng *T. melanosporum* mycelium DNA g soil^{-1}, while low, intermediate and highly productive trees showed 64, 91 and 48 ng *T. melanosporum* mycelial DNA g soil^{-1}, respectively. We did not find clear differences ($P = 0.12$) among the four classes of truffle productivity.

Burn onset – abundance of Black truffle mycelium in soil and tree growth

In the *burn* onset study, only one soil sample (of 12), corresponding to one of the six trees lacking a *burn*, did not present DNA from *T. melanosporum* mycelium. Trees displaying a *burn* presented higher relative DNA band intensities (0.29 vs. 0.1; $P = 0.016$) and tended to have greater tree diameter (6.3 vs. 4.1 cm; $P = 0.07$) and were taller (1.97 vs. 1.37 m; $P = 0.013$) than trees without a *burn*.

Truffle productivity onset and productivity – abundance of Black truffle mycelium in soil

In the productivity onset study, recently productive trees showed greater relative band intensities than nonproductive

Fig. 1. Diagrams of (a) amplification plot of normalized fluorescence (Rn) of 10-fold dilution series of DNA from *Tuber melanosporum* obtained after real-time PCR; (b) melting curves, and (c) gel analysis after the PCR reaction. (1): undiluted simple; (2)–(6) dilutions 10^{-1}–10^{-5}; (B) blank; (M) 1-Kb Plus DNA Ladder.

trees (0.17 vs. 0.07; $P = 0.014$). However, in the productivity study and as observed in real-time PCR probes, differences in relative band intensity among the four classes of trees were not clear ($P = 0.1$), but the relative band intensity of nonproductive trees was significantly greater than the average of the grouped classes of productive trees (0.51 vs. 0.39; $P = 0.036$) and, when comparing nonproductive with highly productive trees, the former presented greater relative band intensities (0.51 vs. 0.35; $P = 0.04$). The ranking of the four productivity classes of trees according to *T. melanosporum* mycelium in soil coincided in both real-time and conventional PCR. The average relative band intensity from soils of the productivity study was greater than that of the productivity onset study (0.42 vs. 0.14, respectively; $P < 0.0001$).

Truffle productivity onset and productivity – *burn* extension, surface rock cover and tree growth

In the productivity onset study, recently productive trees tended to have greater *burn* extensions (2.45 vs. 2.1 m; $P = 0.087$) than nonproductive trees. In the productivity study, we found no significant differences in the extension of the *burn* (4.0 ± 0.12 m) among the four classes, but we observed that *burn* extension was negatively correlated ($P = 0.002$) with the relative band intensity (Fig. 2).

In the productivity onset study, nonproductive trees tended to have greater surface rock cover than recently productive trees (83.7 vs. 76.4%; $P = 0.08$) while in the productivity study, surface rock cover was clearly ($P = 0.007$) lower in nonproductive trees (22%) than in low (45.7%), intermediate (52.8%) and high (53.3%) productivity trees.

In the productivity onset and in the productivity studies, there were no significant differences between productive and nonproductive trees either in height or in diameter.
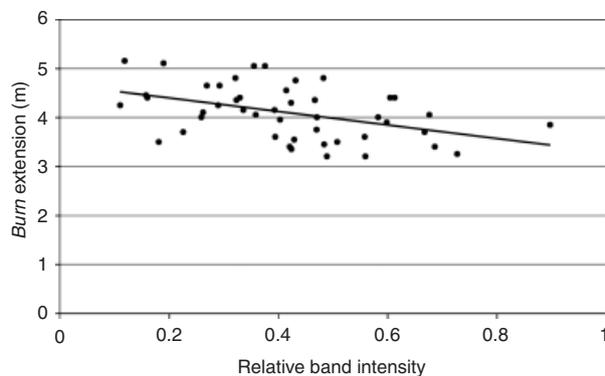
Fig. 2. Correlation between relative band intensity and extension of the *burn* (m) in the productivity study.

Discussion

The belowground habit of truffles engenders difficulties for understanding their biology and ecology. Because conventional PCR only allows relative quantifications, we compared the results obtained applying conventional PCR with those obtained with real-time PCR. We observed that both techniques provided similar results. Owing to the high sensitivity of real-time PCR, only purified DNA extractions gave amplifications. Even though ITS1TM/ITS2TM amplify a fragment of 465 bp, longer than is usually recommended, these primers were suitable for real-time PCR probes. These results confirm, at least in the range of quantities of DNA found in our soils, the reliability of conventional PCR for relative quantification and the applicability of ITS1TM/ITS2TM primers.

In the *burn* onset study, we detected greater quantities of DNA from *T. melanosporum* mycelium in soil from trees that had developed *burns* than beneath trees with no *burn* development, although *T. melanosporum* mycelium was detected in 5 out of 6 trees lacking a *burn*. The trees with *burns* were larger, with potentially more carbon available for the fungus (Wallander, 2006). In the productivity onset study, recently productive trees presented greater quantities of mycelium and greater *burn* extensions than nonproductive trees. This suggests that, for a given site, there may be a certain quantity of mycelial biomass at which the fungus is able to develop the phytotoxic activity that produces the *burn* or shift from vegetative growth to fruitbody production.

In the productivity study, we observed an equilibrium developing in the maturation of a truffle orchard. Among the four classes of trees, there were no differences in *burn* extension or tree diameter and height, and there were no strong differences with respect to quantities of mycelium. However, when comparing nonproductive with productive trees in this orchard, we found higher quantities of mycelium in soil from nonproductive trees. We hypothesize that this reversal in the comparative quantity of mycelium detected beneath the productive vs. nonproductive trees in the more mature orchard (10-year-old) is a reflection of the shift in resource allocation by the fungus to ascomata formation. In our study, this effect may be outstanding because soils were sampled in April, shortly after the truffle fruiting season. In the 10-year-old orchard we also found a negative correlation between *burn* extension and relative band intensity, suggesting a lower density of the mycelial network as the *burn* expands. Contrary to other authors (García-Montero *et al.*, 2007b), we did not observe a relationship between *burn* size and truffle productivity.

In the productivity onset study, we detected a greater relative band intensity from productive than nonproductive trees, although the amount of mycelium from both groups

was lower than from the highly productive trees in the productivity study. This difference may be related to the overall stage of fungal development in the truffle orchards: the orchard of the productivity onset study (8-year-old) had just produced its first truffles the previous winter, while the orchard of the productivity study had been producing truffles for 4 years. Studying replicates of orchards in similar phases of development would be a logical next step to see whether we can estimate the ranges of mycelial biomass at which the fungus can potentially shift to fruitbody production.

In the producing orchard, all three classes of productive trees had higher surface rock cover than the nonproductive trees. Lower surface rock cover was associated with earlier productivity in the productivity onset study, but this apparent contradiction could have been caused by the fact that the mean surface rock cover in the productivity onset study orchard (78.8%) was much higher than that in the productivity study orchard (43.3%) (data not shown). The optimal surface rock cover may lie somewhere in between. Furthermore, the chemical properties of the rock, particularly the calcium carbonate content (García-Montero *et al.*, 2007a), may be interactively influential with the physical properties of rock cover.

Further studies are also needed to record seasonal and temporal changes of mycelium abundance to better elucidate its development and distribution patterns in soil throughout a single year and over several years.

Acknowledgements

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Supplementary material

The following supplementary material is available for this article online:

Appendix S1. Procedure for soil DNA extraction method.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2008.01213.x (This link will take you to the article abstract).

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