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ORIGINAL PAPER

Quantification of extraradical soil mycelium and ectomycorrhizas of *Boletus edulis* in a Scots pine forest with variable sporocarp productivity

Herminia De la Varga · Beatriz Águeda · Fernando Martínez-Peña · Javier Parladé · Joan Pera

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Abstract The availability of most edible ectomycorrhizal mushrooms depends on their natural fructification. Sporocarp formation of these fungi is linked to habitat characteristics and climate conditions, but these data alone do not explain all the trends of fungal fruiting and dynamics. It could be hypothesized that the amount of soil mycelia could also be related to the production of carpophores. Soil samples (five cylinders of 250 cm^3 per plot) were taken monthly, from September to November, in five fenced permanent plots (5×5 m) in Pinar Grande (Soria, Spain), a *Pinus sylvestris* stand situated in the north of the Sistema Ibérico mountain range. Plots were chosen to establish a gradient of *Boletus edulis* productivity from 0 to 38.5 kg/ha year, according to the mean fresh weight of sporocarps collected during the last 10 years. *B. edulis* ectomycorrhizal root tips were identified in each soil sample according to its morphology and counted. DNA extractions were performed with the PowerSoil™ DNA Isolation Kit and quantification of extraradical soil mycelium by real-time polymerase chain reaction using specific primers and a TaqMan® probe. The concentration of soil mycelium of *B. edulis* (mg mycelium/g soil) did not differ significantly between plots (p=0.1397), and sampling time (p=0.7643) within the fructification period. The number of mycorrhizal short roots per soil volume showed significant differences between the plots (p=0.0050) and the three sampling times (p<0.0001). No significant correlation between the number of mycorrhizas and the productivity of the plot (kg of *B. edulis*/ha year) was detected (p=0.615). A statistically significant positive correlation (p=0.0481) was detected between the concentration of mycelia of *B. edulis* in the soil samples and the presence of short roots mycorrhizal with *B. edulis* in these samples. The productivity of the plots, in terms of sporocarps produced during the last 10 years, was not correlated either with the concentration of soil mycelium or with the presence or abundance of ectomycorrhizas.

Keywords *Boletus edulis* · Soil mycelium · Real-time PCR · *Pinus sylvestris* · Mushroom productivity

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Introduction

Wild edible fungi are valuable nonwood forest products around the world and have a clear potential for commercial expansion (Boa 2004). Some genera such as truffles (*Tuber* P. Micheli ex F.H. Wigg.), Saffron milk caps (*Lactarius* Pers.), or boletes (*Boletus* L.) are specially valued in markets of many countries, and its trade has become an important economic income in geographic areas where other benefits from forest products are difficult to obtain. These gastronomically appreciated mushrooms live as symbiotic fungi, forming ectomycorrhizal associations with the roots of trees, and depend on the host plant for fruiting. With the exception of truffles, cultivation of edible ectomycorrhizal mushrooms is difficult, and harvesting in natural forests is the usual way of obtaining this resource (Wang and Hall 2004, Cannon and Kirk 2007).

It has been demonstrated that harvesting of edible ectomycorrhizal fungi has declined during the past years

(Wang and Hall 2004). Climate change, habitat degradation and overexploitation of fungal resources are among the possible causes of this decline (Boa 2004). The development of methods for controlled inoculation of plants aimed at establishing productive plantations could be a sustainable way to exploit the collection and commercialization of these mushrooms without affecting the conservation of natural populations.

Among the edible ectomycorrhizal sporocarps *Tuber melanosporum* Vittad. and *Tuber aestivum* Vittad. have been cultivated commercially around the world (Bonet et al. 2009). Although some success has been achieved with other species, such as *Lactarius deliciosus* (L.) Gray (Guerin-Laguette et al. 2000; Parladé et al. 2004) and *Boletus edulis* Bull. (Águeda et al. 2008), we are still far from considering them as a feasible crop. Since the availability of the majority of these species depends, almost exclusively, on their natural and unpredictable fructification, it is important to establish forest management guidelines leading to maintain and increase the production of sporocarps, preserving at the same time the conservation and diversity of the fungal species (Ortega-Martínez and Martínez-Peña 2008; Egli et al. 2010).

Different factors are known to influence on sporocarp formation by fungi. Several studies have shown that fructification is linked to habitat characteristics (Bonet et al. 2004) and climate conditions, mainly soil moisture and temperature (Laganà et al. 2002, Salerni et al. 2002, Martínez de Aragón et al. 2007, Barroetaveña et al. 2008, Pinna et al. 2010, Bonet et al. 2010). But the same studies stated that these data alone do not explain all the trends in the dynamics of fungal fruiting. The appearance of mushrooms is obviously linked to the presence of mycelium in the soil. It can be hypothesized that the concentration of soil hyphae and/or ectomycorrhizas could also be related to the amount of sporocarps produced, but research done to date only offer contradictory results (Peter et al. 2001; Kjøller 2006; Suz et al. 2008; Rineau et al. 2010).

The mycelium of ectomycorrhizal fungi growing in the soil may represent 30–80% of the fungal biomass (Wallander et al. 2001; Högberg and Högberg 2002) and creates an extensive and dynamic mycelial network which plays a key role in the nutrient uptake by plants and in the reciprocal transfer of carbon and nutrients between plants of the same ecosystem (Read 1992; Simard et al. 2002). The distribution and density of extraradical fungal mycelium in the soil was poorly understood until appropriate methods for its study were developed (Anderson and Cairney 2004). The development of techniques based on direct nucleic acid extraction coupled with polymerase chain reaction (PCR) amplification has provided new insights to the ecology of these soil fungi (Guidot et al. 2002, 2003). Real-time PCR allowed for the relative or absolute quantification of fungal biomass and compared to other quantification techniques, as total hyphal length or biochemical markers, provides a species-specific measure for mycelial biomass estimations (Landeweert et al. 2003). This technique has been adapted for monitoring plant pathogenic fungi (Hietala et al. 2003, Gachon and Saindrenan 2004) and mycorrhizal fungi (Schubert et al. 2003, Parladé et al. 2007, Kennedy et al. 2007, van der Linde et al. 2009).

The objectives of the present work were: to design specific oligonucleotides for the detection and quantification of *B. edulis* extraradical mycelium in a Scots pine forest soil by real-time TaqMan PCR and to determine the concentration of soil mycelium and the abundance of mycorrhizal short roots during the fruiting season in different forest plots representing a gradient of expected mushroom productivity.

Materials and methods

Study site

The study site was located in Pinar Grande, a homogeneous *Pinus sylvestris* L. stand that covers a 12,533 ha area situated in the Sistema Ibérico mountain range, in the inner northeast zone of the Iberian Peninsula. The altitude ranges between 1,097 and 1,543 m with dominating west and east orientations. Soils are acidic brown earths or alluvial with marked acid pH (4–5), sandy loam to sandy texture, a low holding capacity, and low fertility. Average annual rainfall is 865 mm/year, 69 mm/year falling in July and August, and 132 mm/year in September and October. Medium annual temperature is 8.8°C, with July being the warmest month (17.4°C). The frost period begins in November and ends in April, with frequent frosts in late spring and early autumn. Scots pines are harvested every 100 years. The forests are managed by clear cutting with soil movement and sowing.

From 1995 to 2008, the autumnal average production of *B. edulis* in Pinar Grande was 30.3 kg/ha, being the most productive stands those belonging to the age class comprised between 31 and 70 years, with a 69% of the total production (Martínez-Peña 2009, Ortega-Martínez et al. 2010).

Ectomycorrhizas and soil sampling

For the present study, permanent plots established in Pinar Grande since 1995 for quantifying autumnal sporocarps production (Ortega-Martínez et al. 2010) were used. Ectomycorrhizas and soil were sampled in five 5 x 5 m plots selected by their average production of sporocarps

between 1995 and 2007 to represent a gradient between minimum and maximum productivity (Table 1).

Soil samples in each plot were taken three times in 2008: in mid-September, mid-October, and mid-November (before, during, and after the fruiting season peak, respectively). At each sampling time, five soil samples were extracted in each plot using a cylindrical (2 cm radius, 20 cm deep, 250 cm^3) soil borer (Taylor 2002). Samples were taken next to the angles and in the center of the squares plots with a minimum distance of 30 cm apart from any tree trunk.

The soil samples were let to dry at air temperature (20–25°C) to reduce the excess of water to constant weight and then sieved and homogenized. Ten grams of soil were separated for molecular analysis and the remaining was frozen at −23°C and stored until ectomycorrhizal identification and quantification.

Soil DNA extraction

DNA extraction was performed with the PowerSoil™ DNA Isolation Kit (MoBio laboratories Inc., Carlsbad, CA, USA) from 0.25 g of soil per sample according to manufacturer's instructions. Five extractions of each soil sample were processed in order to avoid the variability that entails working with a heterogeneous medium as the soil. A total of 375 samples were processed. The extracted DNA was stored at −20°C.

PCR amplification

PCR reactions were performed using the specific primer pair Bedu1F (ATGGAGGAGTCAAGGCTGTC) Bedu2R (TAGATTAGAAGCGATTCACT) developed by Mello et al. (2006); only on the first extraction made in each combination of date and sampling point in each plot, a total of 75 samples were processed. Amplifications were obtained by PCR reactions containing 2 μl (from a 10 μM stock) of each primer, 25 μl of 2XPCR Solution Premix Taq™ (*TaKaRa Ex Taq*™ Version; TAKARA BIO INC., Japan), 2 μl of DNA template (corresponding to 20–40 ng of DNA) and HPLC water (Scharlau-Chemie, Barcelona, Spain) to a final volume of 50 μl. PCR reactions were performed in a GeneAmp® 9700 thermocycler (Applied Biosystems, CA, USA) with an initial denaturation step at 95°C for 5 min, 35 cycles at 94°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 1 min, and a final extension step at 72°C for 7 min.

Quantification of extraradical soil mycelium by real-time PCR

Specific primers: FWD-Bedu (CTGTCGCCGGCAACGT), RVS-Bedu (TGCACAGGTGGATAAGGAAACTAG), and

Table 1 Mean production of *B. edulis* sporocarps (kg/ha) between 1995 and 2007 and top soil (0–20 cm) properties measured in the five sampling plots

Plot	*Boletus edulis* (kg/ha)	Top soil properties												
		Sand (%)	Silt (%)	Clay (%)	Coarse elements (%)	Water holding capacity (mm)	pH (H_2O)	Organic matter (%)	N%	K (ppm)	Mg (ppm)	Ca (ppm)	Fe (ppm)	P (ppm)	C/N
9A	38.49	84	11	5	0.1	183	4.2	4.3	0.13	85	18	103	6,650	6.55	19
4C	16.47	56	32	12	6.6	250	4.7	3.4	0.11	78	35	417	4,780	2.21	18
6D	10.97	43	45	12	0.1	369	4.5	7.2	0.20	76	70	920	4,160	2.89	20
5A	7.88	61	29	10	0.1	259	5.0	4.6	0.21	126	58	455	3,066	4.62	13
7B	0	54	31	15	0.3	313	4.7	5.6	0.27	95	93	520	8,014	13.97	12

TaqMan® probe STQBedu (6FAM-CCCTTTCTCTTT CGTGGAACCTCCCC-TMR), were designed with the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA) to amplify a 75 bp fragment, based in the variability of the rDNA internal transcribed spacer (ITS)1 region detected in the alignments among different *B. edulis* sequences. A search for highly similar sequences (megablast) was performed in the GenBank database to test the specificity of the designed oligonucleotides.

Standard curves for mycelium quantification by real-time PCR were generated using known amounts of mycelium from active growing colonies of *B. edulis* as described by Parladé et al. (2007) and Hortal et al. (2008). In short, 570 mg of *B. edulis* mycelium grown on a cellophane sheet on agarified biotin-aneurin-folic acid medium (Oort 1981) were added to 10 ml of distilled autoclaved water and fragmented in a Polytron (Kinematika GmbH, Kriens—Lucern, Switzerland) at medium speed for 5 s. An aliquot of 100 μl of the mycelial suspension was mixed with 0.5 g of a soil collected in Pinar Grande. The soil was previously checked for the absence of *B. edulis* DNA by conventional PCR with universal (ITS1F/ITS4) and specific primers (Bedu1F/Bedu2R). Soil with added mycelium was left to dry to constant weight at air temperature (20–25°C). DNA was extracted from the soil with the added mycelium, and control soil sample without added mycelium, using PowerSoil™ DNA Isolation Kit according to manufacturer's instructions. Tenfold serial dilutions from the extract were prepared until 10^{-6}, obtaining serial concentrations of: 11.40000, 1.14000, 0.11400, 0.01140, 0.00114, 0.00011, and 0.00001 mg of mycelium/g of soil.

DNA extracts were amplified by real-time PCR in a LightCycler® 480 real-time PCR system (Roche), with the LightCycler ®480 Probes Master (Roche) according to the manufacturer's instructions for a final reaction volume of 20 μl. The reaction mix contained 10 μl of 2×conc. LightCycler ®480 Probes Master PCR mix buffer, 1 μl of water LightCycler ®480 Probes Master PCR-grade, 1.6 μl of each primer, and 0.8 μl of probe. Primers and probe concentrations were 800 and 100 nM, respectively. Five microliters of DNA extracted from soil samples were added as a template.

The analyses were performed on 96-well plates (LightCycler® 480 Multiwell plate 96). Three replicates of each sample were included in the analysis, as well as a negative control (using de-ionized water instead of DNA template) and the standards (three replicates of each serial dilution) in each plate. PCR conditions were 10 min at 95°C, 45 cycles at 95°C for 15 and 50 s at 60°C, and a final step of 30 s at 40°C.

LightCycler® data were processed with the LightCycler® 480 Software Version 1.5. The results (microgram target DNA in 20 μl PCR mixture) were converted to milligrams of mycelium per gram of soil. Ct values (cycle number at which the fluorescence emission exceeds a fixed threshold established in the exponential phase of the amplification curve) for each concentration were plotted against the logarithm of the corresponding amount of mycelium to generate the standard curve. Absolute quantification of mycelium biomass *of B. edulis* in each soil sample was determined by interpolation of the Ct value in the standard curve.

Quantification of ectomycorrhizas

Each soil sample was gently washed with tap water for extracting root tips. Under the stereomicroscope, *B. edulis* ectomycorrhizal root tips were identified according to its morphology (Águeda et al. 2008) and counted. Ectomycorrhizal root tips representative of *B. edulis* morphotype, obtained from each plot and sampling date, were separated and frozen. Molecular confirmation was carried out by amplification of the rDNA ITS region by PCR reactions using the universal primers ITS1F and ITS4 (Gardes and Bruns 1993). The PCR products were purified with a Roche®High Pure PCR Product Purification Kit (Roche Applied Science, IN, USA) and sequenced with both primers using a 3730 DNA Analyzer (Applied Biosystems, CA, USA). The forward and reverse sequences obtained were aligned (BioEdit Sequence Alignment Editor v. 7.0.9.0.) and identified by selecting the closest matches of a megablast searching in the GenBank database. Sequences were deposited in the GeneBank database with the accession numbers JF728991 to JF729002 and JF739384.

Data analysis

Statistical significance of the differences in the number of mycorrhizal tips per volume of soil (analyzed using log transformed data) and soil mycelial concentration (mg mycelium/g soil) obtained in the different plots and sampling dates were determined by two-way analysis of variance. Differences between means were analyzed by Tukey's HDS test ($p=0.050$). The relationships between soil mycelial concentration, abundance of mycorrhizal short rots, and plot productivity were determined by Pearson correlation analysis. Statistical analyses were performed with JMP®, Version 7, SAS Institute Inc., Cary, NC, 1989–2007.

Results

Conventional PCR using specific primers allowed to detect the presence of *B. edulis* mycelium in 19 of the 75 soil samples. Real-time TaqMan PCR, using the specific primers and probe designed, allowed to detect *B. edulis* in

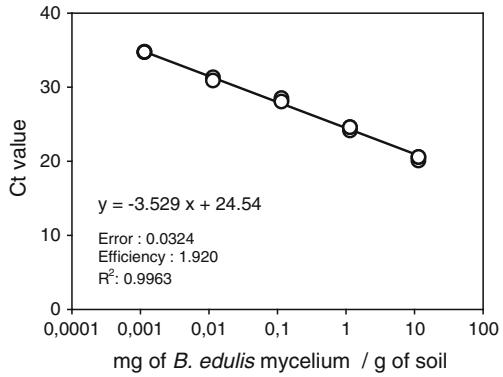

Fig. 1 Standard curve obtained for mycelium quantification of *B. edulis* in soil by real-time TaqMan PCR. The curve was generated by plotting the obtained Ct values against the logarithm of known amounts of mycelium added to soil

30 additional extractions, giving a total of 49 positive samples. The standard curve obtained for the quantification of extraradical mycelium of *B. edulis* in the soil is shown in Fig. 1. The lowest limit of detection using conventional PCR, with the specific primers Bedu1F and Bedu2R, was calculated to be around 0.039 mg of mycelium per gram of soil, according to the quantification by real-time PCR (Table 2). The results obtained by conventional PCR were not consistent when the concentration of soil mycelium ranged between 0.039 and 0.321 mg/g soil, founding either positive or negative amplifications in the given range. Samples with soil mycelial concentrations higher than 0.321 mg/g resulted always in positive amplification by conventional PCR with specific primers. Real-time PCR, using the oligonucleotides and conditions established in the present work, detected concentrations as low as 0.001 mg of mycelium per gram of soil.

The concentration of mycelium of *B. edulis* in the soil (mg mycelium/g soil) (Table 3) did not differ significantly between plots (Table 4), irrespective of their average productivity in terms of kg of sporocarps of *B. edulis* collected during the preceding 10 years. The correlation between the concentration of soil mycelium and the productivity of the plots reported between 1995 and 2007 was not significant (F=0.595, p=0.443). Also, there were no significant differences in soil mycelial concentration over time, from September to November, within the fructification period (Tables 3 and 4).

Table 2 Detection of *B. edulis* mycelia in soil samples by real-time PCR (RT-PCR) in mg mycelium/g soil and conventional PCR using specific primers

Plot	Sampling point	September		October		November	
		RT-PCR	PCR	RT-PCR	PCR	RT-PCR	PCR
9A	a	0.310	+	0.105	+	0.347	+
9A	b	0.000	−	0.000	-	0.000	−
9A	c	0.001	−	0.067	−	0.033	−
9A	d	0.026	−	0.000	−	0.006	−
9A	e	0.000	−	0.000	−	0.000	−
4C	a	0.149	+	0.077	−	0.039	+
4C	b	0.007	−	0.004	−	0.002	−
4C	c	0.001	−	0.000	−	0.000	−
4C	d	0.001	−	0.000	−	0.000	−
4C	e	0.000	−	0.000	−	0.000	−
6D	a	0.136	−	0.089	−	0.085	+
6D	b	0.000	−	0.000	−	0.000	−
6D	c	0.671	+	0.018	−	0.091	+
6D	d	0.139	+	0.070	+	0.032	−
6D	e	0.000	−	0.027	−	0.000	−
5A	a	0.951	+	0.592	+	0.853	+
5A	b	0.321	−	0.137	−	0.299	−
5A	c	0.387	+	0.088	+	0.041	+
5A	d	0.000	−	0.000	−	0.000	−
5A	e	0.138	+	0.019	−	0.049	+
7B	a	0.001	−	0.001	−	0.004	−
7B	b	0.016	−	0.150	−	0.119	+
7B	c	0.000	−	1.799	+	0.000	−
7B	d	0.004	−	0.005	−	0.001	−
7B	e	0.000	−	0.000	−	0.001	−

Positive (+) or negative (−) amplifications were determined by the presence or absence of a 750 bp band in a 2% agarose gel electrophoresis. Both conventional and real-time PCR were performed using the first extraction made in each of the five sampling points per plot

Table 3 Number of mycorrhizal short roots per 250 cm^3 of soil (analyzed using log transformed data) and soil mycelial concentration (mg mycelium/g soil) obtained in the different plots and sampling dates

Plot	nº of mycorrhizal short roots/250 cm^3 of soil	mg mycelium/g of soil
9A	14.87 b	0.085 a
4C	42.07 ab	0.019 a
6D	97.73 a	0.258 a
5A	23.93 ab	0.058 a
7A	14.33 b	0.140 a
Date		
September	79.84 a	0.129 a
October	27.44 b	0.128 a
November	8.48 b	0.080 a

Values within each analysis sharing the same letter are not different by Tukey's HSD test (α=0.050)

The number of mycorrhizal short roots per soil volume showed significant differences between the plots and the three sampling times. No interactions between the two parameters were detected (Table 5). The number of mycorrhizal tips varied significantly over time. The highest number of mycorrhizas per soil volume was detected in the samples taken in September (79.8 mycorrhizas/250 cm^3 of soil). These amounts were significantly reduced in October and November (Table 3). The number of *B. edulis* ectomycorrhizas differed significantly between plots (Table 3), but no significant correlation between the number of mycorrhizas and the productivity of the plot (kg of *B. edulis*/ha year) was detected (F=0.255, p=0.615).

A statistically significant positive correlation was detected between the concentration of mycelia of *B. edulis* in the soil samples and the amount of short roots mycorrhizal with *B. edulis* in these samples (Fig 2).

Discussion

Real-time TaqMan PCR allowed the quantification of the fungal mycelium, and compared to conventional PCR, increased the detection of mycelium of *B. edulis* in soil samples in a 158%. Real-time PCR detected amounts as low as 0.001 mg of *B. edulis* mycelium per gram of soil in the samples taken in the *P. sylvestris* forest of Pinar Grande. Specific primers: FWD-Bedu and RVS-Bedu, and probe STQBedu, used in this work, has been designed in the rDNA ITS1 region. The internal transcribed spacer region of ribosomal DNA has been widely used because different specific primers have been designed to identify *B. edulis* (Moor et al. 2002, Mello et al. 2006, Bin et al. 2008) and several group-specific primer sets exist (Gardes and Bruns 1993, Martin and Rygiewicz 2005). Large quantities of sequences are available for this gene region on a wide range of fungal species, making easier to design specific and efficient primers for real-time PCR. Nevertheless, ITS regions of the ribosomal DNA gene are multicopy regions of the genome (Gardes and Bruns 1993), known to be different in number between species (Debaud et al. 1999). Since copy numbers of ITS genes on the genome vary between fungal species, it would be most reliable to use a gene with a known and constant number of copies for quantification purposes. In our study, we quantified the presence of a single species, *B. edulis*, and the number of copies of the ITS region is assumed to be constant within a species (Cassidy et al. 1984, Raidl et al. 2005).

The conditions of both PCR reactions (conventional and real-time) are different and this affects the efficiency of the reaction resulting in a greater effectiveness of real-time PCR in order to detect the presence of *B. edulis* DNA when small quantities are present in the sample.

The size of the fragment amplified by the specific primers designed to real-time PCR was 75 bp, approximately 6.8 times shorter than the fragment amplified by conventional PCR using the primers Bedu1F and Bedu2R. Due to the size of the fragment amplified, real-time PCR

Table 4 Analysis of variance of the concentration of mycelium of *B. edulis* in the soil samples (mg of mycelium/g of soil) (Test of Brown-Forsythe: Prob>F=0.2063)

Source model	DF	Sum of Squares	Mean square	F ratio	Prob>F
Model	14	1.173	0.084	1.166	0.3245
Error	60	4.312	0.072		
C. Total	74	5.484			
Effect tests					
Plot	4	0.519		1.805	0.1397
Date	2	0.039		0.270	0.7643
Plot date	8	0.615		1.070	0.3962

Table 5 Analysis of variance of the number of mycorrhizal short roots (log transformed) per volume of soil (250 cm^3)

Source model	DF	Sum of squares	Mean square	*F* ratio	Prob>*F*
Model	14	128.471	9.176	3.195	0.0009
Error	60	172.334	2.872		
C. Total	74	300.805			
Effect tests					
Plot	4	47.500		4.134	0.0050
Date	2	63.589		11.070	<0.0001
Plot date	8	17.382		0.756	0.6491

Test Brown–Forsythe: Prob>*F*=0.9047

might be less sensitive than conventional PCR to DNA fragmentation that might occur during processing of samples, and it could contribute to improve the effectiveness of real-time PCR. But, at the same time, it must be taken in account that it would be possible that we were detecting the presence of DNA extracted from mycelium already dead, introducing an overestimation in the quantification of the active mycelium. Using a vital stain, it is possible to determine whether living and/or dead fungal hyphae are being quantified, but this distinction is not possible when analyzing fungal DNA extracted from soil (Landeweert et al. 2003). On the other hand, Lindahl et al. (2010) found that dead mycorrhizal mycelium in the soil is rapidly degraded by opportunistic saprotrophic microorganisms. Also, Parladé et al. (2007) showed that the extraction and quantification of DNA from mycelium growing in a Petri dish with nutrient medium is dramatically reduced with aging. A long persistence of dead mycelium, or their DNA, in a medium as a forest soil is unlikely, and DNA recovered in the extraction procedure is more likely to belong to actively growing mycelium.

The productivity of the plots, in terms of mean sporocarp production, was not correlated either with the soil mycelium concentration estimated by real-time PCR or with the abundance of ectomycorrhizas counted in soil samples. This apparent uncoupling between above- and belowground fungal components has been also observed in other ectomycorrhizal species (Gardes and Bruns 1996, Hynes et al. 2010). It has been demonstrated that the belowground mycelial system of *Suillus grevillei* (Klotzsch) Singer (Zhou et al. 2001), *Tricholoma matsutake* (S. Ito and S. Imai) Singer (Lian et al. 2006), *Hydnellum peckii* Banker, and *Phellodon tomentosus* (L.) Banker (van der Linde et al. 2009), is not always centered around sporocarps and there was no quantitative relationship between the belowground abundance of mycelium and the number or distribution of sporocarps. Suz et al. (2008) compared nonproductive and productive trees in a *T. melanosporum* orchard, finding apparently higher quantities of mycelium in soil samples taken around nonproductive trees. Peintner et al. (2007), studying the soil fungal communities in a *Castanea sativa* Mill. forest, demonstrated that the overlap between above- and belowground fungal communities was very low. In their study, *Boletus* mycelia, compared with other soil fungi, were rare and scattered, whereas their sporocarps were the dominant in the mushroom production of that forest.

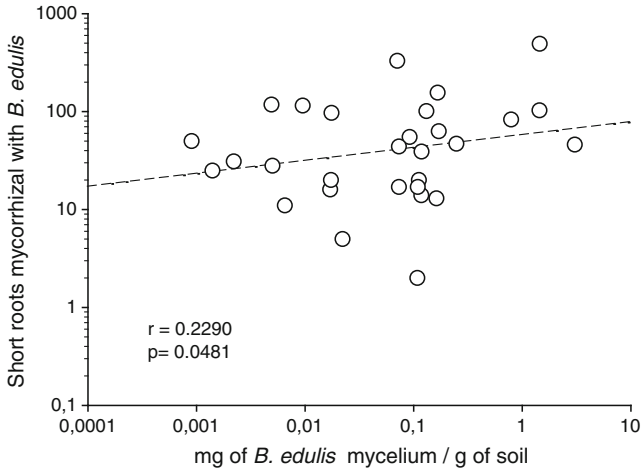

Fig. 2 Correlation between the number of *B. edulis* ectomycorrhizal rootlets in 250 cm^3 of soil and the concentration of *B. edulis* soil mycelium (mg/g) per soil sample

Soil DNA concentration is considerably heterogeneous. The application of molecular techniques based in DNA analyses in environmental samples strongly depends on the availability of reliable DNA extraction protocols for different types of soils (Feinstein et al. 2009). In our experiments, we used a commercial kit (PowerSoil™ DNA Isolation Kit) specific for soil DNA extraction, that has been reported as efficient to reduce de coextraction of PCR inhibitory substances (Dinnen et al. 2010). We focused on only one fungal species, *B. edulis*. So, great biases due to differences in efficiency during the extraction protocol were not expected. It is accepted that standard PCR amplifications were inhibited when DNA concentrations exceeded 1 μg/ml in the reaction mixture. DNA extracted in each soil sample was spectrophotometricaly quantified (A 260/280) rending a value of 1.92 in average. Total DNA extracted from a gram dry weight of soil ranged from 71.6 to 409.2 μg/g, and the mean concentration of total DNA in the real-time PCR reaction mixture was 0.12 μg/ml (±0.07 SD).

Guidot et al. (2002) found between 10 and 0.07 ng $g soil^{-1}$ of *Hebeloma cylindrosporum* Romagn. DNA in the vicinity of fruit bodies. Increasing the number of soil replicates extracted per treatment and testing each DNA extract in triplicate would possibly reduce the observed fluctuations (Landeweert et al. 2003). In our experiments, five sampling points per plot were used and DNA extractions were repeated five times per sample. Nevertheless, a high variability was still detected among mycelial concentrations. Agerer (2001) defined *Boletus* mycorrhiza as a long distance exploration type having their mycelia concentrated as rhizomorphs. This pattern of growth could cause a high degree of spatial heterogeneity and could explain the high variability encountered among soil samples. To minimize plot variability, the sampling design of ectomycorrhizal fungal mycelia could be optimized in future experiments collecting larger or pooled soil core samples (Guidot et al. 2002, Ranjard et al. 2003, Kang and Mills 2006). A further bias factor when quantifying mycelium by molecular techniques based in DNA analysis is the potential presence of both spores and mycelium in a single sample, which are likely to be co-extracted during DNA extraction process. The possibility of detecting basidiospore-derived DNA instead of mycelial DNA is a potential problem when using molecular methods on soil extracts (Guidot et al. 2002). Some authors tried to avoid the extraction and amplification of spores by collecting soil samples either before (Landeweert et al. 2005) or after (Dickie et al. 2002) the sporocarp production season at the field sites. Nevertheless, the persistence of fungal spores in soil is not well-known and could introduce a bias when considering soil samples, in particular during the fruiting season. In our experiment, the samples were taken in 2008 during the fruiting season; but during all the period of sampling in the studied plots, only one sporocarp of *B. edulis* was recorded in plot 4 C. Consequently, a great influence due to the presence of dispersed spores was not expected.

The presence of *B. edulis* mycorrhizal roots decreased clearly with time along the fruiting season. The highest concentration was detected in the samples taken at September, and dropped to almost one third in October and nearly to a tenth in November. The relationships between spatial distribution of mycorrhizas and sporocarps differed among fungal species; in some cases, an overlap of spatial distribution has been observed while in other cases sporocarps occurred where no mycorrhizas were detected (Gardes and Bruns 1996, Kikuchi and Futai 2003). Ectomycorrhizas in the soil would be patchily distributed, partly attributable to underlying patterns of root distribution, and the position of these patches changes over time (Pickles et al. 2009).

The spatial distribution of mycorrhizas and extraradical mycelium might not exactly match. Mycelium may proliferate in nutrient-rich patches independently of the location of mycorrhizas (Lilleskov and Bruns 2003). In our experiments, a statistically significant positive correlation was established between the concentration of *B. edulis* soil mycelium quantified by real-time PCR, and the number of *B. edulis* ectomycorrhizal tips counted in the same soil samples. Extraradical soil mycelium of *L. deliciosus* was also positively correlated with the percentage of mycorrhizal short roots in other experiments and fungus–plant combinations (Parladé et al. 2007). These results could open the possibility of using quantification of soil mycelium by real-time PCR as a good indicator for root colonization in field conditions, especially when a nondestructive sampling or less time consuming analysis were required.

Primers and TaqMan probe designed in this work are suitable for the specific detection and quantification of *B. edulis* mycelium in a forest soil by real-time PCR. Soil mycelium concentrations and the abundance of ectomycorrhizas counted in soil samples were not correlated with the productivity of the plots, considered in terms of mean sporocarp production in previous years. The number of *B. edulis* mycorrhizas and the concentration of mycelium in the soil decreased from September to November. Experiments are currently in progress to corroborate this fluctuation in a wider period of time.

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