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REGULAR ARTICLE

Study of nitrogen and carbon transfer from soil organic matter to *Tuber melanosporum* mycorrhizas and ascocarps using ^{15}N and ^{13}C soil labelling and whole-genome oligoarrays

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Abstract

Background and aims We previously showed by $^{13}CO_2$ host labelling that almost all of the constitutive carbon allocated to the truffles originated from the host. The objective of this present work was to determine the putative capacity of *T. melanosporum* ectomycorrhizas and ascocarps to use soil carbon and to uptake or assimilate soil nitrate.

Methods The current investigation involved ^{13}C and ^{15}N soil labelling by incorporating labelled leaf litter and expression of genes involved in carbon and nitrogen metabolism in ascocarps and ectomycorrhizas.

Results The ascocarps harvested in the labelled plots were highly enriched in ^{15}N but were almost never enriched in ^{13}C. The main source of soil mineral nitrogen was nitrate. A nitrate transporter, one nitrate reductase and a nitrite

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reductase were well expressed in ectomycorrhizas. Several genes involved in aminoacid synthesis or in transamination processes were also well expressed in ectomycorrhizas. No nitrate transporter was expressed in ascocarps where the CAZyme genes upregulated were mainly Glycosyltransferases involved in saccharide transfer.
Conclusion Ascocarps did not exhibit saprotrophic capacity for C, supporting previous results from $^{13}CO_2$ host labelling showing that C is provided by the host tree. The ^{15}N present in the ascocarps after soil labelling is supplied as ammonium or aminoacids by the ectomycorrhizas, which are able to uptake, reduce and metabolize nitrate.

Keywords Truffle · ^{13}C · ^{15}N · Ectomycorrhizas · Ascocarps · Gene expression

Introduction

Ectomycorrhizal fungi mainly rely on their host for carbon. For example, the Oak Ridge Reservation study (Tennesse, USA) demonstrated that less than 2 % of the ectomycorhizal biomass originated from the litter (Treseder et al. 2006). However, ectomycorrhizal fungi release in soil extracellular enzymes such as cellulases or polyphenol oxydases (Courty et al. 2005). Lindahl and Tunlid (2015) suggest that ectomycorrhizal fungi are soil organic decomposers, but not saprotrophs. They do not regularly use soil organic matter as carbon source, but contribute to degrade organic complexes and to release nitrogen (Lindahl and Tunlid 2015). Carbon derivation from the host depends on the ectomycorrhizal species and showed a range from 1 to 21 % of total net primary production (Hobbie 2006). However, most of the studies on carbon budgets for ectomycorrhizal fungi have not taken into account the fruitbodies production.

There exists experimental evidence that the development of ectomycorrhizal sporocarps depends on host carbon allocation (Last et al. 1979; Godbout and Fortin 1992; Lamhamedi et al. 1994). Under girdled or defoliated trees (*Pinus sylvestris*), the production of ectomycorrhizal sporocarps was shown to decrease considerably (Högberg et al. 2001; Kuikka et al. 2003). Teramoto et al. (2012) showed in rhizobox trials that *Laccaria amethystina* sporocarps were provided with recently assimilated carbon. We demonstrated recently through $^{13}CO_2$ pulse-labelling of the host tree that *Tuber* ectomycorrhizas provided host carbon to the ascocarps and that *Tuber* ascocarps were dependent on their hosts throughout their development (Le Tacon et al. 2013). In the latter study, the ascocarps continued to accumulate host carbon through complete maturity and several months after the end of ^{13}C assimilation by the host. This finding strongly suggests that carbon allocated to the truffle ascocarps is provided by compounds stored in the wood and hydrolyzed during the cool periods. This contrasts with *Laccaria* spororocarps, which are provided with carbon in the form of simple sugars only just synthethized by the host (Teramoto et al. 2012).

The transfer of nitrogen (N) from ectomycorrhizas to sporocarps is not well documented. Nitrate or ammonium concentrations in the soil solution represent a low percentage of nitrogen potentially available for ectomycorhizas. Nitrogen being supplied to ectomycorrhizas depends on the microbial transformations of organic N forms. Ectomycorrhizal fungi can increase the uptake of N-labelled NH_4^+ into seedling trees (Reid et al. 1983; Rygiewicz et al. 1984). Since ammonium is the dominant form of inorganic nitrogen in forest soils, ectomycorrhizal fungi tend to favor NH_4^+ uptake (Bledsoe and Zasoski 1983; Genetet et al. 1984; Martin et al. 1986; Botton and Chalot 1999a, b) and there is considerable variability in their ability to utilize NO_3^- (Plassard et al. 2000; Gobert and Plassard 2002). Inorganic nitrogen absorbed by ectomycorrhizas is incorporated on host carbon skeletons most likely by utilizing metabolic pathways that differ from those of the host plant (Martin et al. 1986, 1988; Finlay et al. 1988; Dell et al. 1989; Chalot et al. 1991). Some ectomycorrhizas can produce proteolytic enzymes which release N from various peptides (Abuzinadah and Read 1986; Courty et al. 2005; Näsholm et al. 2009), and they have the capacity to absorb aminoacids or small peptides (Chalot and Brun 1998; Abuzinadah and Read 1989). However, the significance of this fact relative to the uptake of mineral N in the field has not yet been quantified.

Nevertheless, whatever form of N is taken up by the ectomycorrhizas, it seems to be transferred as aminoacids or peptides to the host (Botton and Chalot 1999a, b). Whether these aminoacids, or other N forms, are provided by the ectomycorrhizas to the sporocarps or not remains to be investigated.

$^{\text{Thirteen}}C$ and ^{15}N natural abundance has frequently been used to differentiate sporocarps of ectomycorrhizal fungi from those of saprotrophic fungi (Hobbie et al. 2002). Sporophores of ectomycorrhizal fungi are more enriched in ^{15}N than those of saprotrophic fungi (Taylor et al. 1997, 2003; Hobbie et al. 1999, 2001; Kohzu et al. 1999; Trudell et al. 2004; Zeller et al. 2007). Although

the relative contribution of N sources and the different internal processes involved in the fractionation of ^{15}N remain unclear, these findings suggest that the nitrogen used by the ectomycorrhizal sporophores is mainly provided by the ectomycorrhizas.

The life cycle of the ectomycorrhizal species belonging to the genus *Tuber* is not completely known (Le Tacon et al. 2015). It takes up to 6 months for *T. melanosporum* ascocarps to fully develop (Le Tacon et al. 2015). For a long time, it was believed that truffles became independent of their host not long after the formation of the primordium and that the ascocarps were able to use dead host tissues or soil organic matter as main carbon and nitrogen sources (Callot 1999).

The first objective of this work was to characterize the potential of nitrogen mineralization (ammonium and nitrate) in the soil of two experimental sites producing ascocarps. The second was to determine how *T. melanosporum* ectomycorrhizas or ascocarps can uptake and assimilate soil nitrate and whether they are able to use soil carbon. The third was to assess the genes involved in the assimilation and transfer of nitrogen and carbon between the different compartments of the host and its associated fungus.

Our experimental strategy was first to carry out two parallel experiments of soil labelling using a ^{15}N and ^{13}C labelled leaf litter in two sites producing *T. melanosporum* ascocarps, the first in northeastern France under *Corylus avellana* L. and the second in southeastern France under *Quercus ilex* L.. In these experiments, we measured the mineralization of the labelled organic matter in the two soils and determined the absorption rates and the ^{15}N and ^{13}C transfers between host trees and their fungal associates (mycorrhizas, ascocarps, roots and leaves). The second part of our strategy was to use whole-genome oligoarrays to investigate the transcriptional reprogramming of genes involved in nitrogen or carbohydrate metabolism in free-living mycelium cultivated in pure culture in laboratory conditions, in ectomycorrhizas collected in greenhouse and in *T. melanosporum* ascocarps collected in field conditions at different stages of maturation.

Materials and methods

Experimental sites

The experiment was performed on two separate test sites, Rollainville and Pierre Blanche.

The first site, Rollainville, is located in the western part of the Vosges department in France on a limestone plateau of the Jurassic period (latitude 48° 18′ 42″; longitude 5° 44′ 13″; elevation 360 m; annual rainfall 941 mm with a maximum in July; mean annual temperature 9.5 °C). The soil is a brown calcisoil (WRB 2006) with a silty clay texture, a high alkaline pH (pH 7.97), a moderate content of organic matter (9.4 %) and a limestone content of 8.8 %. This soil is poor in available phosphorus and moderate in available potassium and magnesium. It is free-draining, highly granular and aerated. The site is not irrigated.

The Rollainville truffle orchard was established by one of the co-authors (Christophe Robin). Previously, it had been a cultivated site (mixed field of grass/alfalfa). Hazel trees (*Corylus avellana* L.) inoculated with black Perigord truffle (*T. melanosporum* (Vittad.) marketed by the Naudet nurseries (http://www.pepinieres-naudet.com/) were planted in 1991. The first truffles were harvested in November 2005.

The second site, Pierre Blanche, is situated at Visan, 600 km south of the first site, in the Vaucluse department of France, on calcareous alluvial deposits (longitude: 4.916°, latitude: 44.366°; elevation: 200 m; annual winter-dominant rainfall 759 mm; mean annual temperature 13 °C). The soil is a deep brown calcisoil with a sandy-silty texture, a high alkaline pH (pH 8.3), a moderate content of organic matter (8.6 %) and a limestone content of 24 %. The soil is very poor in available phosphorus and moderate in available K and Mg. It is also highly granular, aerated and free-draining. The site is ploughed each year (first 5 to 10 cm), but is not irrigated. Before becoming a truffle field, the site had previously been cultivated for cereals. Acorns of holm oak (*Quercus ilex* L.) were sown in 1982. The seedlings became mycorrhizal with undetermined naturally occurring fungi and *T. melanosporum*. The first black truffles were harvested in 1998.

Production of the labelled leaf litter

Sixty one-year-old hazel (*Corylus avellana*) seedlings were transferred in April 2010 into two 1 m^3 growth chambers (30 seedlings per chamber) of the CEA/Cadarache-DSV-DEVM (Saint-Paul-les-Durance, France). The photosynthetic photon flux density in the PAR range was 500 $\mu mol.m^{-2}.sec^{-1}$, with a photoperiod of 12/12 (D/N) from April 1, 2010 to May 16 of the same year, and then became 13/11 (D/N) through to

harvesting (September 30). The day temperature was set at 20 °C with a relative humidity of 70 % and the night temperature at 12 °C with a relative humidity of 80 %. The seedlings were fed continuously with a nutritive solution containing $K^{15}NO_3$ (isotopic excess of ^{15}N 10 %). From April 2 to June 1, the seedlings were grown in an atmosphere containing a $^{13}CO_2$ isotopic excess of 10 %. From June 3 to June 23, they were subjected to a 1-day pulse $^{13}CO_2$ labelling (Wednesday) once a week and then to labelling over two consecutive days per week up to the harvest. The last labelling took place on September 28 and 29, 2010. The total N and C content and the ^{13}C and ^{15}N excess were measured in June and at the end of September; measurements were taken on 3 samples per chamber. At the end of September, the litter was well enriched in ^{13}C (atom ^{13}C in %=3.768 and ^{13}C in g/kg of dry matter=15.68) and a bit less enriched in ^{15}N (atom ^{15}N in %=2.423 and ^{15}N in g/kg of dry matter=0.28) (Table 1) .

^{15}N and ^{13}C labelled senescent leaves were picked at the end of November 2010 from the sixty hazel seedlings formerly enriched in ^{15}N and ^{13}C. The litter was air-dried and thoroughly grounded. In all, 600 g of labelled litter were obtained .

Soil labelling

On each site, under one tree known to produce truffle ascocarps, labelled dried litter was introduced into 4 plots of 1 m^2 each on March 9, 2011 in Rollainville and on May 13, 2011 in Pierre Blanche. In Rollainville, 4 contiguous plots (three on a line and the fourth on the following one) were settled at the place where truffles were harvested the previous year. In Pierre Blanche, in the absence of information, the 4 plots were settled at the 4 cardinal points at a distance of one meter from the trunk. The litter of each plot and soil on 4 cm depth were first removed. Seventy-five g of labelled litter were then mixed with 2 kg of this soil. The mixture was homogeneously distributed on the 1 m^2 of each plot, and covered with about 4 kg of non-labelled soil. Finally, the original litter of the plot was re-positioned. At both sites, from each of the plots, about 50 g of soil were collected before and after being mixed with the labelled litter in order to measure total C, total N, $\delta^{13}C$, $\delta^{15}N$, atom% ^{13}C and atom% ^{15}N in the soil before and after addition of leaf labelled litter.

Sampling

Ascocarps harvest and identification

In both sites, ascocarps were collected using a truffle-hunting dog in the 4 labelled plots and in control plots under non-labelled trees. Ascocarps were sampled during the three consecutive harvest seasons following the supply of the labelled leaf litter (2011–2012, 2012–2013 and 2013–2014).

In Pierre Blanche, ascocarps were also collected outside the labelled plots under the labelled tree. They were immediately frozen after harvest (using dry ice) for transcriptome analyses.

These ascocarps were also confirmed as belonging to *T. melanosporum* using the molecular method described below.

Sampling of roots and ectomycorrhizas

In Rollainville, fine roots (≤2 mm diameter), roots of 2 to 5 mm diameter (medium roots) and ectomycorrhizas were carefully retrieved from the soil of each labelled plots, or outside the labelled plots under the labelled tree or under a non-labelled tree as control and washed in water under a dissecting microscope. There were four replicates per treatment or organs. *T. melanosporum* ectomycorrhizas were identified via morphotyping on

Table 1 Initial total C and N in %, C/N, atom ^{13}C excess in % and atom^{15}N excess in % of the labelled *Corylus* litter in September 2010. Natural abundance of non-labelled *Corylus* leaves: atom ^{13}C in % =1.1084; atom ^{15}N in %=0.366 (Means and standard deviations in brackets)

	Total C %	Total N %	C/N	atom ^{13}C in % (PDB=1.1111%)	^{13}C in g/kg of dry matter	atom ^{15}N in % (air=0.3663%)	^{15}N in g/kg of dry matter
Mean	41.7 (0.39)	1.17 (0.15)	35.5 (2.4)	3.768 (0.25)	15.68 (0.47)	2.423 (1.116)	0.28 (0.13)

the basis of color, mantle shape and surface texture. Fine roots and ectomycorrhizas were then treated for ten minutes with 1 N chlorhydric acid and then washed with water to eliminate soil calcium carbonate. Ectomycorhizas and roots were collected at three dates corresponding to 13, 21 and 29 months after the introduction of the labelled leaf litter (2012-04-23, 2013-01-04 and 2013-08-07).

In Pierre Blanche truffle orchard, it was not possible to collect sufficient *Tuber* ectomycorrhizas due to soil tillage and the morphology of the oak roots.

Ectomycorrhizas were confirmed as being associated with *T. melanosporum* using molecular methods. Genomic DNA was extracted with the DNeasy Mini Kit (Qiagen SA, Courtaboeuf, France) following the manufacturer's instructions. *T. melanosporum* mycorrhizas were checked using species-specific ribosomal-DNA internal transcribed-spacer (ITS) primers (Paolocci et al. 1999; Rubini et al. 1998).

Leaf sampling

In Rollainville, ten leaves were collected around the crown in the middle part of the labelled tree at the four cardinal points and then pooled together to obtain one sample per cardinal point. Leaves were also collected from an unlabelled tree.

There were four replicates of each compartment (leaves, roots, mycorrhizas), with the exception of ascocarps for which the number depended on the production.

The samples were first air dried, then dried at 60 °C for 48 h and ground to a fine powder using a shaker with steel beads.

Net soil N mineralization and nitrification

One year after labelling, soils of each of the 4 plots in both sites were sampled and incubated at 25 °C and at field moisture for 4 weeks. Soils were first pre-incubated during 2 weeks with respect to the initial mineralization peak. After these 2 weeks, the incubation took place for 4 weeks. Nitrate and ammonium concentration of the incubated soils were measured at the beginning of the incubation and after 2 and 4 weeks. Potential net mineralization and net nitrification rate was calculated as the difference between the final concentration (2 and 4 weeks) and the initial concentration. This difference was divided by the number of days between the two extractions. As the rates were similar after 2 and 4 weeks we decided to use the endpoint approach. In addition, soil respiration was measured in the same soils after 4 weeks of incubation. At each date, mineral nitrogen was extracted from 4 replicates of 12 g by shaking soil with 60 ml of 0.5 M K_2SO_4 for 1 h. NH_4^+ and NO_3^- concentrations were determined using continuous flow colorimetry (TRAACS, Bran and Luebbe).

To perform ^{15}N measurements in soil K_2SO_4 extracts, an aliquot of the solution was reduced to 100 µl using a freeze-dryer and dried in tin capsule.

Measurements of soil respiration

Soil respiration was measured for the two sites after 3 weeks of incubation under controlled conditions (25 °C and at field moisture) on the same samples than those used for nitrogen mineralization. Jars were connected to the $^{13}CO_2$ analyzer in order to monitor the respiration flux and the isotopic composition of the respired CO_2.

The isotope composition of CO_2 effluxes from the soil was calculated using [$^{12}CO_2$] and [$^{13}CO_2$] concentrations measured at the inlet and outlet of a flow-through chamber (Marron et al. 2009; Plain et al. 2009) using tunable diode laser absorption spectroscopy with a trace gas analyzer (TGA 100A; Campbell Scientific)

Total soil CO2 efflux (FCO_2) and its $\delta^{13}C$ ($\delta^{13}CF$) were calculated using the following equations:

$$F_{CO_2} = \frac{\left([CO_2]_{out} - [CO_2]_{in}\right) \times P \times F}{8.314 \times T \times W}$$

where $[CO_2]_{in}$ and $[CO_2]_{out}$ represent the CO_2 concentrations in the incubator influx and outflux, P is the atmospheric pressure, F is the flow inside the bottle, W is the weight of soil incubated in each bottle (kg), T is the temperature (°K), 8.314 J. mol^{-1} is the ideal gas constant.and

$$\delta^{13}C_F = \frac{\frac{[^{13}CO_2]_{out} - [^{13}CO_2]_{in}}{[^{12}CO_2]_{out} - [^{12}CO_2]_{in}} - 1}{R_{VPDB}}$$

(R_{VPDB}, the isotopic ratio of Vienna Pee Dee Belemnite (VPDB; 0.011179602)).

Isotopic analyses

The percentages of the total C and C isotopic compositions in the leaves, fine roots, mycorrhizas and ascocarps were determined by using an on-line continuous flow CN analyzer (Carlo Erba NA 1500) coupled with an isotope ratio mass spectrometer (Finnigan delta S). Values were reported using standard notation ($\delta^{13}C$ ‰) relative to Vienna Pee-Dee Belemnite (VPDB), employing PEF (IAEA-CH-7) as a standard.

$\delta^{13}C$ values were calculated with the standard formula:

$$\delta^{13}C = \left(R_{sample}/R_{VPDB}\right)-1) \times 1000$$

wherein R represents the molar ratio of $^{13}C/^{12}C$ and R_{VPDB} represents the molar ratio of Pee-Dee Belemnite.

$\delta^{15}N$ values were calculated in the same manner, R_{VPDB} being the molar ratio of atmospheric N_2 for N, using $(NH_4)_2SO_4$ (IAEA-N-1) as a standard.

Expression of genes involved in nitrogen and carbon metabolism in free-living mycelium, ectomycorrhizas and in the gleba of the ascocarps

Due to high tannin concentrations, we were unable to extract high quality RNA from field *T. melanosporum* ectomycorrhizas. Young ectomycorrhizas were sampled from 5 month-old hazel (*C. avellana* L.) seedlings inoculated by *T. melanosporum*. Mycorrhizal seedlings were grown in the greenhouse facilities of the AGRI-TRUFFE Company (Saint-Maixant, France) and fed at pH 7.5 with both ammonium and nitrate. Gene expression in ectomycorrhizas was compared to free-living *T. melanosporum* mycelium (strain Mel28) grown at 25°C on 1% malt agar (Cristomalt-D, Difal, Villefranche-sur-Saône, France) for 5 weeks before harvest. The strain Mel28 was first subcultured at Inra Clermont-Ferrand (France) from the gleba of an ascocarp collected at Saint-Rémy de Provence (France) in 1988 (strains Mel28) and then subcultured at Inra-Nancy (Martin et al. 2010). Mycelium and ectomycorrhizas were snap-frozen in liquid nitrogen.

To determine the expression of genes involved in nitrogen and carbon metabolism in ascocarps, truffles were harvested in Rollainville on four separate dates (September 28, 2010 and November, 17 2010 for non mature ascocarps, December 22, 2010 and January 27, 2011 for mature ascocarps). Ascocarps were frozen immediately after harvesting in the truffle orchard using dry ice.

Total RNA was isolated from ascocarps (gleba), ectomycorhizas or free-living mycelium of *T. melanosporum* using the Qiagen plant mini kit (Qiagen, Courtaboeuf, France) as previously described (Martin et al. 2010) and was submitted for DNase treatment (Qiagen). The sample quality was controlled using the Bio-Rad Experion analyser and RNA StdSens Kits (Bio-Rad, Marnes la Coquette, France). Total RNA preparations (four biological replicates for ectomycorrhizas, a variable number for ascocarps at different stages of maturation and seven for mycelium) were amplified using the SMART PCR cDNA Synthesis Kit (Ozyme, Saint-Quentin-en-Yvelines, France), according to the manufacturer's instructions, and used for hybridizations to NimbleGen oligoarrays (Martin et al. 2010).

Double-stranded cDNA was synthesized and amplified using the SMARTPCR cDNA Synthesis Kit

Table 2 Total C and total N in %, $\delta^{13}C$ and $\delta^{15}N$ in ‰, atom ^{13}C and atom ^{15}N in % of the soil before and after addition of leaf labelled litter at Rollainville and Pierre Blanche (Means and standard deviations in brackets)

		Total C0 in %	Total N in %	C/N	Atom ^{13}C In %	$\delta^{13}C$ in ‰	Atom ^{15}N in %	$\delta^{15}N$ in ‰
Rollainville	Soil	7.11 (0.26)	0.61 (0.02)	11.6 (0.01)	1.084 (0.004)	−24.46 (0.36)	0.3679 (0.001)	4.24 (0.37)
Rollainville	Soil+labelled litter	8.67 (0.36)	0.65 (0.02)	13.3 (0.1)	1.566 (0.014)	409.14 (12.54)	0.5057 (0.045)	386.88 (12.27)
Pierre Blanche	Soil	5.77 (0.21)	0.20 (0.01)	28.8 (0.2)	1.092 (0.004)	−17.01 (0.4)	0.3682 (0.002)	5.19 (0.43)
Pierre Blanche	Soil+labelled litter	7.33 (0.38)	0.24 (0.01)	30.2 (0.2)	1.660 (0.016)	494.25 (14.43)	0.7378 (0.128)	1014.15 (34.83)

(Clontech) according to the manufacturer's instructions. Single dye labelling of samples, hybridization procedures and data acquisition were performed at the NimbleGen facilities (NimbleGen Systems, Reykjavik, Iceland) following their standard protocol. The *T. melanosporum* custom-exon expression array (GPL8982) manufactured by Roche NimbleGen Systems (Madison, WI, USA) (http://www.nimblegen.com/products/exp/index.html) contained five independent, non-identical, 60-*mer* probes per gene model coding sequence. Included in the oligoarray were 7,496 annotated protein-coding gene models, 5,736 TE sequences, 3,913 random 60-*mer* control probes and labelling controls. For 1,876 gene models, technical duplicates were included in the array. Microarray probe intensities were quantile normalized across chips and average expression levels were calculated for each gene from the independent probes on the array and were used for further analyses (Hacquard et al. 2013). Raw array data were filtered for non-specific

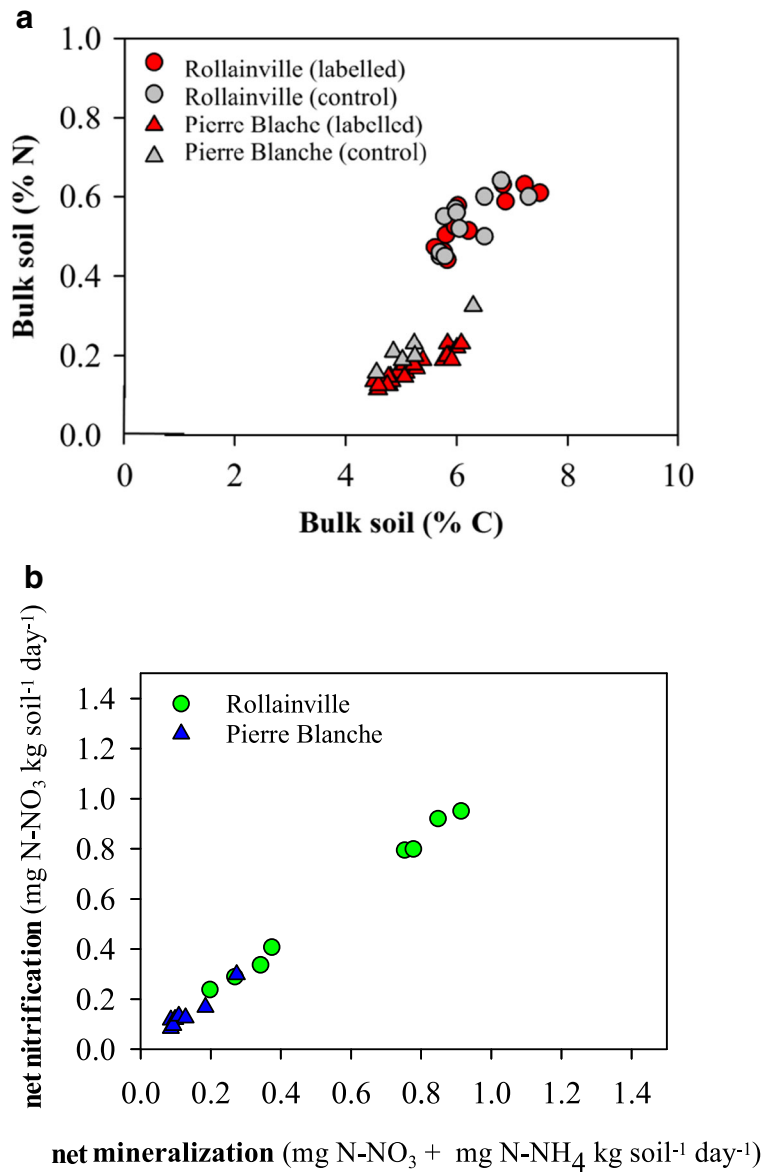

Fig. 1 Percentage of total carbon and nitrogen in the soils at Rollainville and Pierre Blanche before and after the addition of ^{13}C and ^{15}N labelled hazel litter (Fig. 1a). Net mineralization rate and net nitrification rates in the soils from Rollainville and Pierre Blanche (Fig. 1b). Soils were collected from two soils depths, 0–5 cm and 5–10 cm (n=4; per site and per depth)

probes (a probe was considered as non-specific if it shared more than 90% homology with a gene model other than the gene model it was made for) and renormalized using the ARRAYSTAR software (DNASTAR, Madison, WI, USA). For 1,015 gene models, no reliable probes remained. A transcript was considered to be expressed when its signal intensity was threefold higher than the mean signal-to-noise threshold (cut-off value) of the random oligonucleotide probes present on the array.

Statistical analyses

For labelling experiments, analyses of variance were conducted using the R software (R project for Statistical computing, http://www.R-project.org) using the aov function or a Kruskal-Wallis test for small sample sizes. Post hoc tests were performed using Tukey's honestly significant difference test. The statistical significance was set at $p<0.05$. For each set of results, a table of variance analysis is given to investigate the effects of treatments (soil labelling or not), harvest years, sites and interactions on $\delta^{15}N$ and $\delta^{13}C$ of ascocarps or other organs. Moreover, for each graph, letters or asterisks indicate the statistical significance between treatments.

For gene expression, natural log-transformed data was calculated and incorporated into the CyberT statistical framework (http://cybert.ics.uci.edu/) using the standard *t*-test unpaired data module. Transcripts presenting a 5-fold change in transcript level (FDR-corrected $P<0.05$) were considered as significantly differentially expressed. Complete expression data sets are available at the Gene Expression Omnibus (NCBI) as series GSE17529 and GSE36870.

Results

Potential net carbon and nitrogen mineralization of the two soils 1 year after the incorporation of the labelled litter

The soil of each plot was well enriched in ^{13}C and ^{15}N in the two sites (Table 2). The Pierre Blanche site greatly differed from the Rollainville site by its high C to N ratio (28.8 against 11.6) and correlatively by its low content of organic nitrogen (Fig. 1a). Accordingly, the potential net mineralization was significantly far lower in Pierre Blanche than in Rollainville ($p<0.001$) where it was high (Fig. 1a). All of the mineralized soil nitrogen was transformed to nitrate at both sites and on all plots (Fig. 1b and Table 3). The respiration rate was significantly higher in Rollainville soil than in Pierre Blanche soil ($p=0.05$) (Table 3).

The $\delta^{13}C$ of the respired CO_2 from soil (48.2 ‰ in Rollainville and 29.7 ‰ in Pierre Blanche) was very high compared to the ^{13}C natural abundance of atmospheric CO_2 (− 8.2 to − 9. 2‰, Longinelli et al. 2005). The $\delta^{15}N$ of the nitrate was significantly different on both sites. Its value was higher in Pierre Blanche than in Rollainville in the first soil layer, 1 year after the supply of leaf litter, and much higher than the ^{15}N natural abundance of soil nitrate.

^{15}N and ^{13}C ascocarp enrichment

Statistic analysis revealed no significant differences between the values of $\delta^{15}N$ and $\delta^{13}C$ of ascocarp peridium and gleba, as well for those harvested in control plots (natural abundance) as those harvested in labelled plots (Fig. 2 and Table 4).

Table 3 Potential C and N mineralization rates of soils collected in Rollainville and Pierre Blanche at the depth 0–5 cm in the plots labelled with ^{13}C and ^{15}N one year before; means of 4 replicates and standard deviations in brackets. NS: non significant between the two sites, **: significant at $p<0.01$, ***: significant at $p<0.001$

	CO_2 (μmol kg soil^{-1} h^{-1})	$\delta^{13}C$ of the respired CO_2 $_{in}$ ‰	Net mineralization (mg N kg soil^{-1} day^{-1})	Net Nitrification (mg N kg soil^{-1} day^{-1})	$\delta^{15}N$ of NO_3^- $_{in}$ ‰
Rollainville	16.9	48.2	0.823	0.866	50.4
	(1.73)	(15.7)	(0.036)	(0.040)	(3.4)
Pierre Blanche	14.72	29.7	0.121	0.124	103.4
	(4.46)	(29.3)	(0.024)	(0.017)	(12.6)
ANOVA	F(1,6)=0.309 NS	F(1,6)=0.209 NS	F(1,6)=262.4 ***	F(1,6)=284.6***	F(1,6)=17.39 **

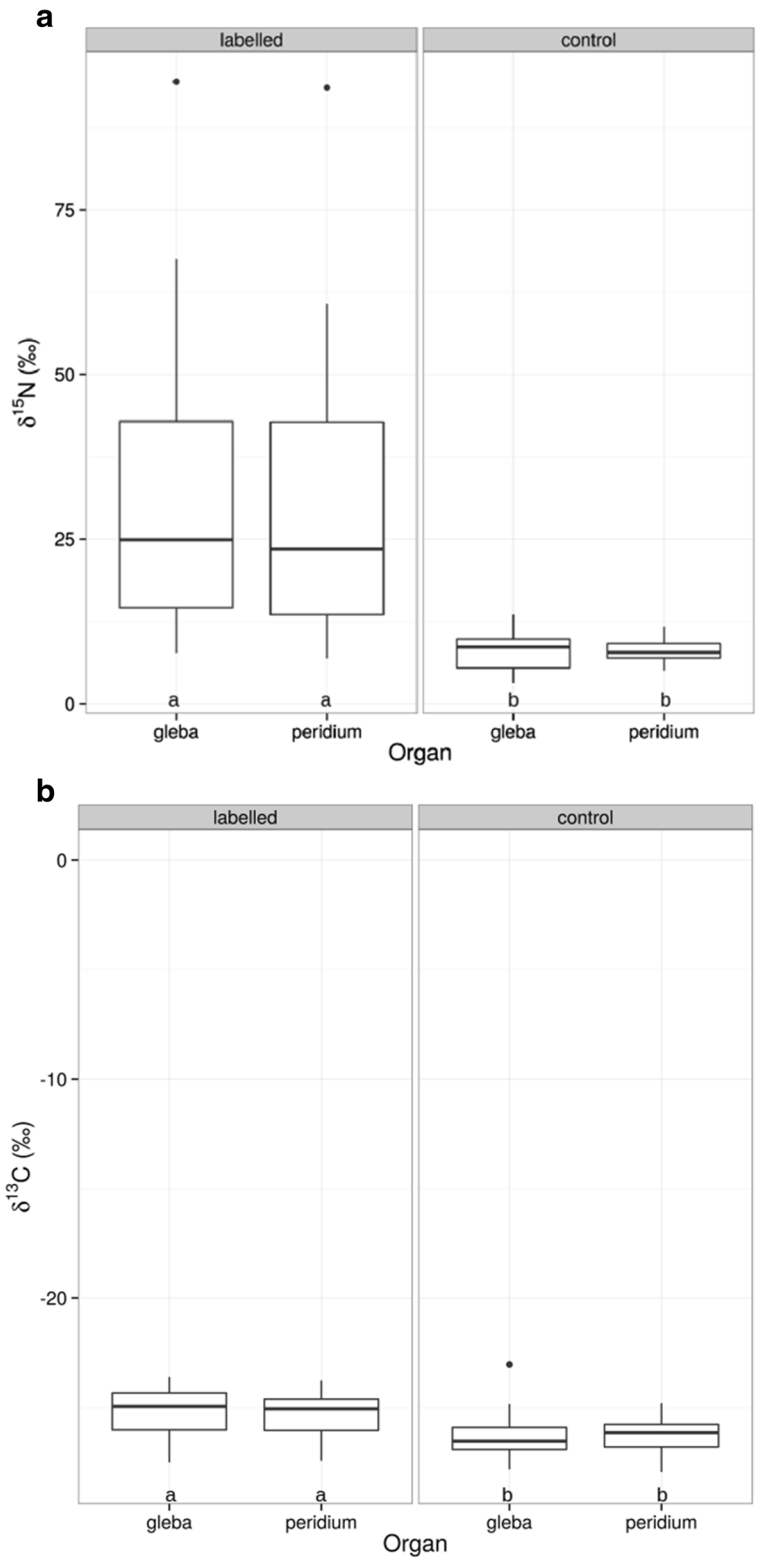

Fig. 2 Box plots of comparison of $\delta^{15}N$ (Fig. 2a) and $\delta^{13}C$ (Fig. 2b) between the pericarp and the gleba of all the ascocarps collected in Pierre Blanche and in Rollainville during 3 years in labelled plots and under control trees. The letters (**a** and **b**) indicate the statistical significance between treatments

Table 4 Analysis of variance table investigating the effects of tissues (pericarp or gleba) and treatments (soil labelling or not) on $\delta^{15}N$ (Fig. 2a) and $\delta^{13}C$ (Fig. 2b) of all the ascocarps collected in Pierre Blanche and in Rollainville during 3 years in labelled plots and under control trees

	$\delta^{15}N$	$\delta^{13}C$
Tissues (pericarp or gleba)	F(1,104)=0.03; NS	F(1,104)=0.22; NS
Treatments (soil labelling or not)	F(1,104)=55.56; $p<0.05$	F(1,104)=33.76; $p<0.05$
Interaction	F(1,104)=0.02; NS	F(1,104)=0.10; NS

^{15}N and ^{13}C ascocarp enrichment (peridium and gleba) according to the distance to the labelled plots (Pierre Blanche, Fig. 3a and b, Table 5)

During the season 2011–2012, in the controls (4 replicates), far from the labelled tree, the $\delta^{15}N$ of the ascocarp was 9.5‰ and the $\delta^{13}C$ −24.8‰. In the labelled plots (12 ascocarps collected), the $\delta^{15}N$ of the ascocarp was 29.4‰ and the $\delta^{13}C$ −24.4‰. Under the labelled tree at a distance of less than 1 m from the labelled plots (5 ascocarps collected), the $\delta^{15}N$ was 27.9‰ and the $\delta^{13}C$ −24.4‰. Under the labelled tree at a distance greater than 1m from the labelled plots (9 ascocarps cropped), the $\delta^{15}N$ was 11.6‰ and the $\delta^{13}C$ −23.7‰.

The $\delta^{15}N$ of the ascocarps was significantly higher in the labelled plots (29.4‰) and at a distance of less than one meter from the labelled plots (27.9‰) than in the controls (9.5‰) and under the labelled tree situated at more than 1 m from the labelled plots (11.6‰). The difference was not significant between the $\delta^{15}N$ of the ascocarps collected within the labelled plots and outside the labelled plots at distances of less than one meter from them (29.4‰ and 27.9‰ respectively). The standard deviation of the $\delta^{15}N$ was always higher in the enriched ascocarps than that of the control.

The $\delta^{13}C$ did not differ between the labelled plots and the controls regardless of the distance from the labelled area ($\delta^{13}C$=−24.4‰ in labelled plots, −23.4‰ less than 1 m from labelled plots, −23.7‰ more than 1 m from labelled plots, and −24.8‰ for controls).

Pierre Blanche and Rollainville $\delta^{15}N$ and $\delta^{13}C$ gleba enrichment one, two and three years after labelling (Fig. 4a and b, Table 6)

Regardless of the year and site considered, significant enrichment of the gleba was observed with ^{15}N from the leaf litter ($p<0.05$).

In 2011–2012, the 12 ascocarps collected in Pierre Blanche showed in their gleba a higher $\delta^{15}N$ than controls (29.4 ‰ and 9.5‰ respectively). The pattern was the same in 2012–2013 (7 ascocarps cropped) and in 2013–2014 (only one ascocarp cropped in the labelled plots).

In 2011–2012 and 2013–2014, the $\delta^{13}C$ of the gleba in the labelled plots of Pierre Blanche did not significantly differ from that of the control. The gleba was slightly enriched in ^{13}C in 2012–2013 (−26.3 and −27.7‰, less than 1.5 δ in labelled plots and controls respectively).

In 2011–2012, the 3 ascocarps cropped in Rollainville showed a $\delta^{15}N$ in gleba significantly higher than that of the 4 ascocarps harvested in the controls (45.8 and 4.8‰ respectively). The differences remained significant during the two following seasons but to a lower extent. The standard deviation of the gleba $\delta^{15}N$ was also higher in the enriched ascocarps of Rollainville than that of the controls.

Additionally, there was a slight (less than two δ) but significant difference in 2011–2012 and 2012–2013 between the $\delta^{13}C$ of the gleba of labelled plots and the controls, while there was no difference in 2013–2014.

^{15}N and ^{13}C enrichment of roots, ectomycorrhizas and leaves (Fig. 5a and b, Tables 7 and 8)

This work was performed only in Rollainville because it was not possible to sample enough *T. melanosporum* ectomycorrhizas in Pierre Blanche.

^{15}N natural abundance of mycorrhizas was significantly higher than the other organs (+4.6 vs −0.1, −1.8 and −3.4 for fine root, medium roots and leaves respectively in year 1). One year after the addition of labelled litter (spring 2012), the *T. melanosporum* ectomycorrhizas sampled in the labelled plots were highly and significantly enriched in ^{15}N ($\delta^{15}N$=81.8 ‰) compared to the controls ($\delta^{15}N$=4.6 ‰). They were also more

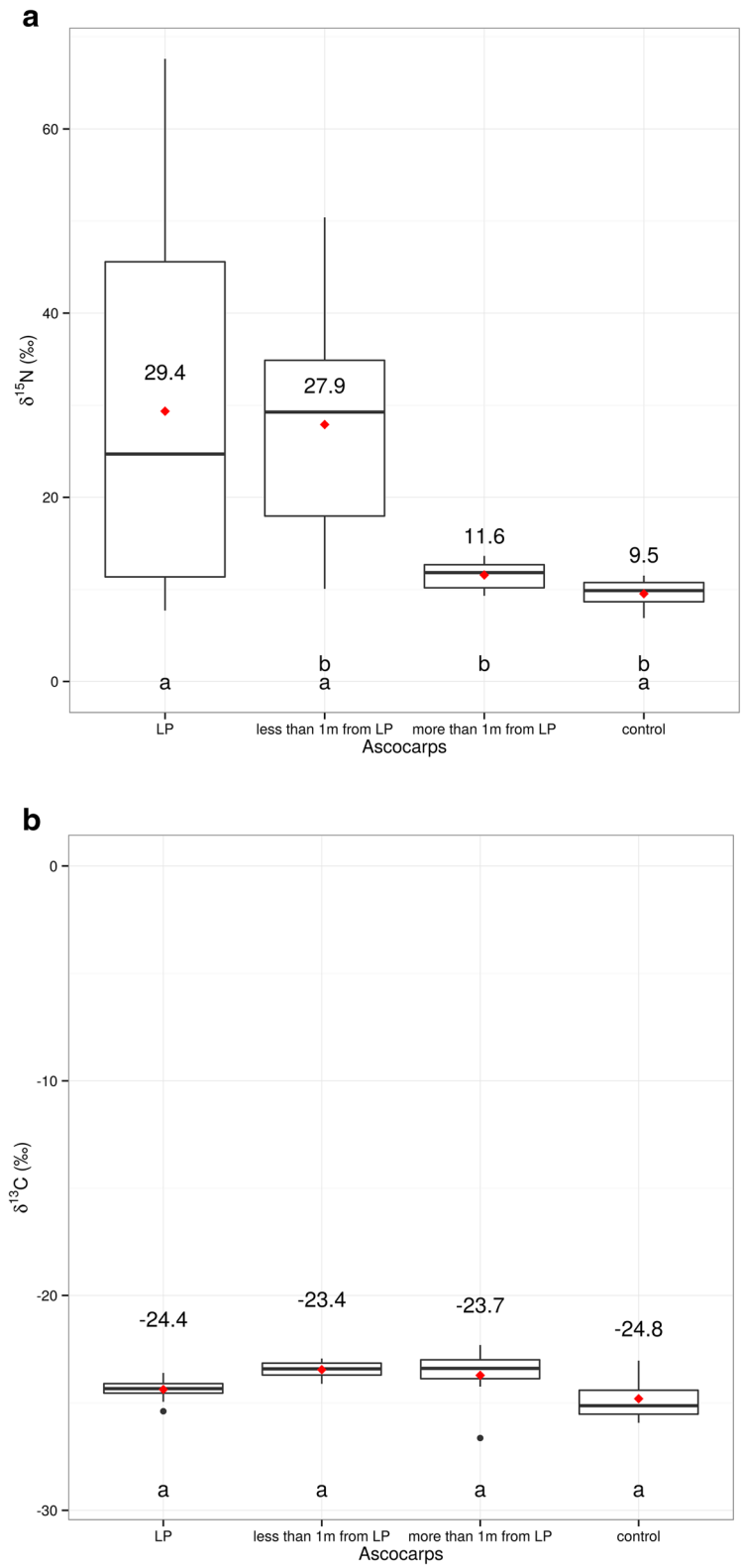

Fig. 3 $\delta^{15}N$ (Fig. 3a) and $\delta^{13}C$ (Fig. 3b) box plots of the ascocarps (gleba+pericarp) collected in 2011–2012 (6 to 9 months after soil labelling) in Pierre Blanche (LP: labelled plots, n=12; labelled tree less than 1m from the labelled plots, n=5; labelled tree more than 1m from the labelled plots n=9; controls, n=4). The letters (**a** and **b**) indicate the statistical significance between treatments

Table 5 Analysis of variance table investigating the effects of treatments (soil labelling or not, distance from the labelled plots) on $\delta^{15}N$ (Fig. 3a) and $\delta^{13}C$ (Fig. 3b) of ascocarps (pericarp+gleba) collected in 2011–2012 (6 to 9 months after soil labelling) in Pierre Blanche

	$\delta^{15}N$	$\delta^{13}C$
Treatments (distance to the labelled plots)	$F(3,60)=8.43$; $p<0.05$	$F(3,60)=4.05$; $p<0.05$

labelled than the fine or medium roots (57.0 ‰ and 37.3 ‰ respectively) but this was not significant. There was slight but significant ^{15}N enrichment of labelled tree leaves ($\delta^{15}N=1.1$ ‰, mean of the 2 years) compared to that of the controls ($\delta^{15}N=-2.5$ ‰, mean of the 2 years).

In winter of 2013, the ^{15}N enrichment of the ectomycorrhizas and roots was observed as very weak in the labelled plots but still statistically different from the control.

More than 2 years after the addition of labelled leaf litter (07 08 2013), there again occurred a higher ^{15}N enrichment of ectomycorrhizas, roots and leaves than in the corresponding controls.

Whatever the treatment, the date or the type of material used, the $\delta^{13}C$ remained almost constant. We can only note in winter 2013 a weak increase of the $\delta^{13}C$ in the labelled plots. For example, $\delta^{13}C$ was −25.9 ‰ for the mycorrhizas in the labelled plots and – 27.2 ‰ in the controls.

Expression of genes involved in nitrogen metabolism in free-living mycelium, ectomycorrhizas and gleba of ascocarps (Table 9 and 10)

In ectomycorrhizas of young seedlings grown in greenhouse in a medium provided with both nitrate and ammonium, the high affinity nitrate transporter NrtB and two of four ammonium transporters were highly expressed and more than in free-living mycelium (Table 9). Inversely, one ammonium transporter was overexpressed in free-living mycelium versus ectomycorrhizas. One nitrate reductase and the nitrite reductase [NAD(P)H] NirB were also well transcripted both in ectomycorrhizas and in free-living mycelium. We can underline that in *T. melanosporum* genome, these nitrate and nitrite reductase genes (GSTUMT00010229001 and GSTUMT00010228001) form a cluster on scaffold 68 as in *T. borchii* genome.

Several genes involved in aminoacid synthesis (glutamate synthase [EC:1.4.1.13], NAD-specific glutamate dehydrogenase, NADP-specific glutamate dehydrogenase, glutamine synthetase, asparagine synthase) or in transamination processes (aspartate transaminase) were also well expressed in *T. melanosporum* ectomycorrhizas of seedlings grown in greenhouses.

Four genes involved in aminoacid transport were upregulated in ectomycorrhizas versus free-living mycelium.

During ascocarp development in Rollainville truffle orchard, the nitrate transporter NrtB was poorly transcripted in the gleba and there was no expression of nitrate, nor of nitrite reductases (Table 10). Five transcripts were differentially expressed in the gleba throughout maturation, one of the four putative ammonium transporters, one of the two glutamine synthetase (GS), the NAD-specific glutamate dehydrogenase (GDH) and the two aspartate transaminases.

One gene involved in aminoacid transport was upregulated in mature ascocarps versus non-mature ascocarps.

Expression of genes involved in Cazyme metabolism in free-living mycelium, ectomycorrhizas and gleba of ascocarps (Table 11 and 12)

In young ectomycorrhizas collected in greenhouses, the CAZyme transcripts upregulated in comparison to the transcript concentration in free-living mycelium were mainly glycoside hydrolases belonging to several families and involved in degradation of hemicellulose and fungal cell wall polysaccharides (β-1,3-glucans, β-1,6-glucans, chitine). (Table 11).

The CAZymes with higher transcript concentrations in the gleba of mature ascocarps compared to younger truffles were mainly Glycosyltransferases belonging to different families and involved in the transfer of saccharides from glycosyl donor to acceptor (Table 12).

Discussion

The C to N ratio of soil was higher in Pierre Blanche than in Rollainville (28.8 against 11.6).

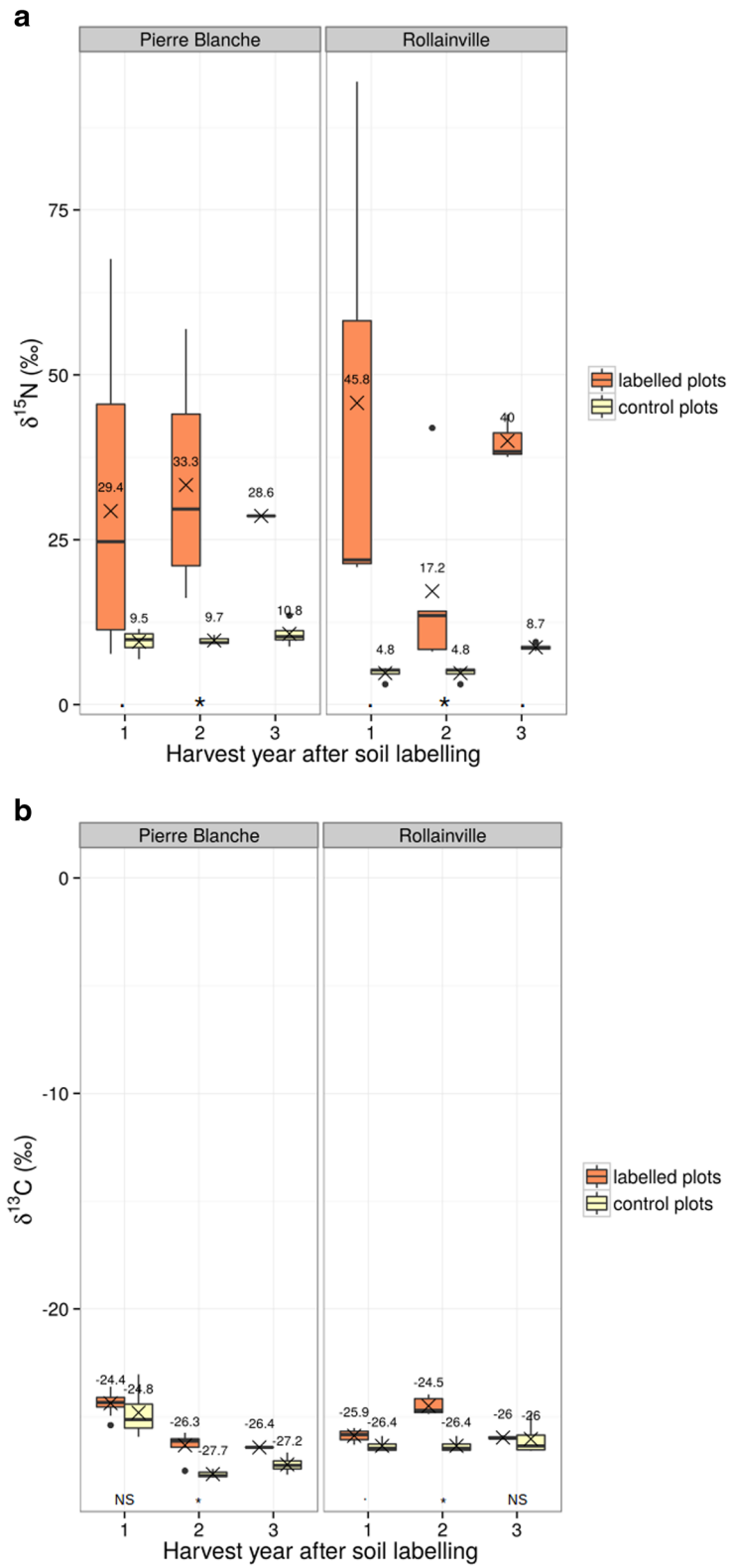

Fig. 4 $\delta^{15}N$ (Fig. 4a) and $\delta^{13}C$ (Fig. 4b) box plots of the ascocarps (gleba) collected in the labelled plots in 2011–2012 (year 1: 6 to 9 months after soil labelling, n=12 in Pierre Blanche and n=3 in Rollainville), in 2012–2013 (year 2: 18 to 21 months after soil labelling, n=7 in Pierre Blanche and n=4 in Rollainville) and in 2013–2014 (year 3: 30 to 33 months after soil labelling, n=1 in Pierre Blanche and n=3 in Rollainville). Pierre Blanche (**a**) and Rollainville (**b**). Control ascocarps (n=4) were taken outside the labelled trees (no indications, number of samples insufficient; ., significant at 0.5%; *, significant at 0.1%; **, significant at 0.01%)

Table 6 Analysis of variance table investigating the effects of treatments: soil labelling or not, harvest years, sites and interactions on $\delta^{15}N$ (Fig. 4a) and $\delta^{13}C$ (Fig. 4b)

	$\delta^{15}N$	$\delta^{13}C$
Sites	F(1,42)=1.43; NS	F(1,42)=1.06; NS
Treatments (soil labelling or not)	F(1,42)=25.87; $p<0.001$	F(1,42)=52.56; $p<0.001$
Harvest years	F(2,42)=0.66; NS	F(2,42)=24.71; $p<0.001$
Interactions Sites/treatments	F(1,42)=0.19; NS	F(1,42)=0.003; NS
Interactions Sites/harvest years	F(2,42)=1.52; NS	F(2,42)=33.36; $p<0.001$
Interactions Treatments/harvest years	F(2,42)=0.52; NS	F(2,42)=6.13; $p<0.01$
Interactions Treatments/sites/harvest years	F(2,42)=1.17; NS	F(2,42)=0.87; NS

Accordingly, the potential carbon mineralization was lower in Rollainvile, despite the lack of statistical difference between the two sites. The $\delta^{13}C$ of the respired CO_2 was similarly high in the first cm (about 30‰) for both sites, illustrating the ^{13}C soil enrichment by the litter and the mineralization activity of the microbial biomass.

The two sites differed by their potential nitrogen net mineralization, which was almost ten times higher in Rollainville than in Pierre Blanche. We can assume that this significant difference is due to the presence of *Quercus ilex* in Pierre Blanche. It is well known that the holm oak litter is characterized by a high C/N ratio, recalcitrant to mineralization (Ferran and Vallejo 1992; Sardans and Peñuelas 2010). We know also that *Q. ilex* is one of the least efficient Mediterranean species for recovering nitrogen from senescent leaves (Escudero et al. 1992).

The main source of soil mineral nitrogen was nitrate in both sites

Despite their differential rate of nitrogen mineralization, the two sites exhibited a high rate of nitrification. This is in agreement with their soil pH (7.97 for Rollainville and 8.30 for Pierre Blanche), which showed optimal levels of nitrification (Robertson 1982). In both sites, the soil nitrate exhibited high ^{15}N enrichment. The significantly higher ^{15}N enrichment of nitrate in Pierre Blanche than in Rollainville could be explained by the small pool of mineral nitrogen in Pierre Blanche and thus a less dilution of the mineralized ^{15}N nitrate.

Nitrate assimilation by *T. melanosporum* ectomycorrhizas

Ammonium being the dominant N form in forest soils (Bledsoe and Zasoski 1983), most of the studies on N uptake by ectomycorrhizal fungi have been devoted to NH_4^+ uptake and translocation. Relatively few studies have been devoted to NO_3^- uptake and reduction by ectomycorrhizal fungi and the outcomes are controversial. Very little information is available on the ability of *Tuber* species to use nitrate. Guescini et al. (2007) demonstrated that the expression level of *T. borchii* nitrate reductase is eight times higher in ectomycorrhizas than in free-living mycelium. These authors suggested that glutamate, glutamine and asparagine are the main aminoacids transfered to the host tree. In the association *T. borchii*/*Tilia platyphyllos*, fungal nitrate transporter, nitrate reductase and nitrite reductase genes were up-regulated, while the same host genes were down-regulated (Guescini et al. 2007); they also suggested that *T. borchii* ectomycorrhizas allow for better nitrate uptake and assimilation by the host, especially when nitrogen availability is low.

According to our results, we can assume that *T. melanosporum* ectomycorrhizas also play a fundamental role in nitrate uptake and assimilation. In Rollainville, in spring of the first year and in summer of the second year after soil labelling, the *T. melanosporum* ectomycorrhizas exhibited a high ^{15}N enrichment, indicating that they used nitrate, the only source of mineral nitrogen available in the soil. During winter of the second year after labelling, there were also differences between ectomycorrhizas sampled in

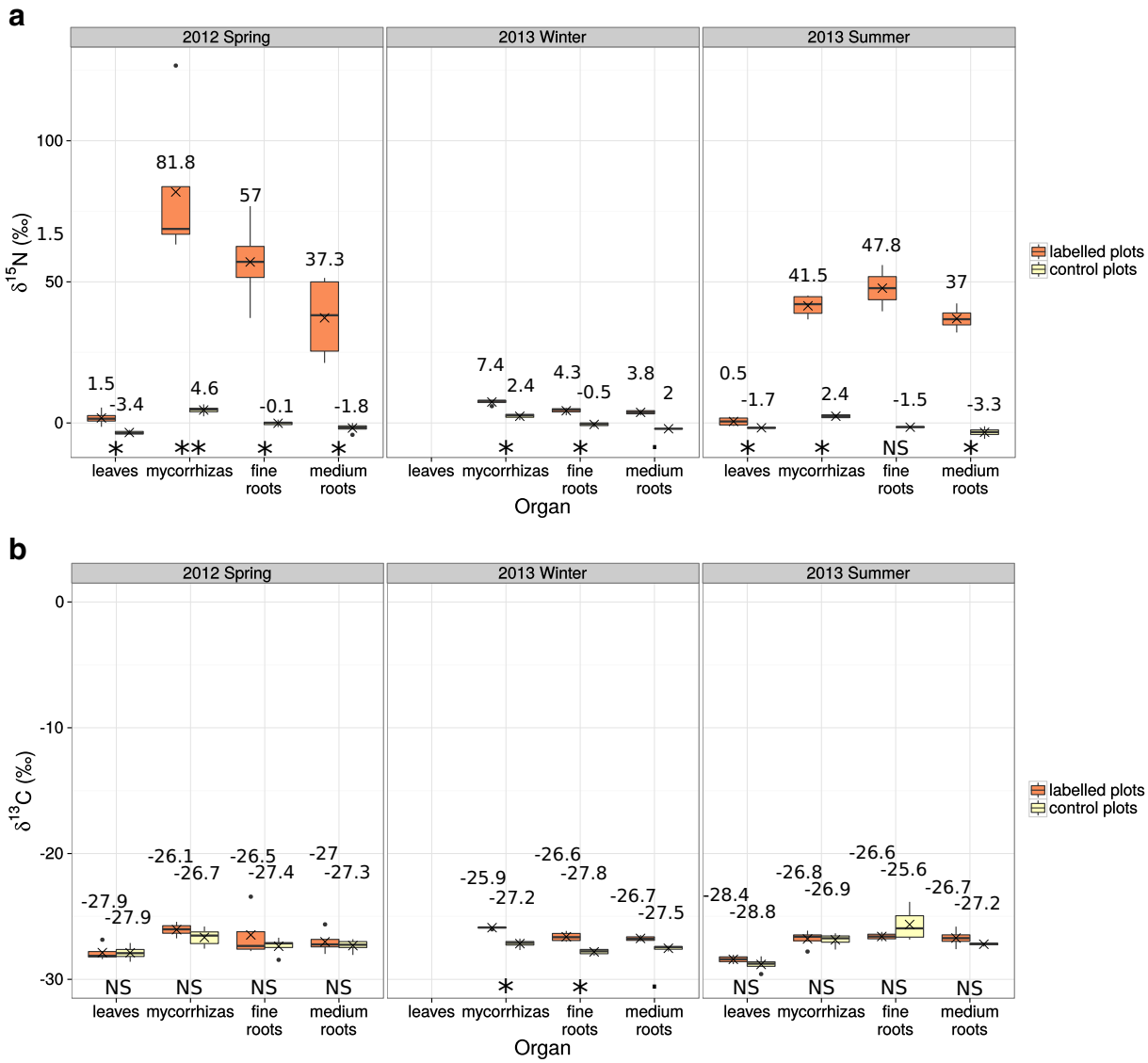

Fig. 5 Rollainville. Influence of the date of sampling on $\delta^{13}N$ (Fig. 5a) and $\delta^{13}C$ (Fig. 5b) of *T. melanosporum* ectomycorrhizas, fine roots, medium roots and leaves, (box plots). The ectomycorrhizas were collected at three dates 13, 21 and 29 months after the introduction of the labelled leaf litter, (23 04 2012, 04 01 2013 and 07 08 2013) and the leaves collected at two dates, 13 and 29 months after the introduction of the labelled leaf litter (23 04 2012 and 07 08 2013) (controls and labelled plots, n=4 for each organ at each date except for control medium roots in the third year, n=2)

the labelled plots and those sampled in the controls, but the enrichment in ^{15}N was weaker than in spring or summer, due to low nitrogen mineralization at low temperature.

The fine roots were less enriched in ^{15}N than the ectomycorrhizas and the mean roots less than the fine roots, suggesting that ^{15}N was transferred from the mycorrhizas to the fine roots and from the fine roots to the mean roots. However, this assumption is moderated by the fact that there is a natural ^{15}N enrichment of the mycorrhizas compared to the roots.

Due to a dilution effect, the ^{15}N enrichment of the leaves was weak, showing that the ^{15}N introduced in the soil was transferred in whole tree from the ectomycorrhizas via the roots and the vascular network. Indeed, a soil patch of 4 m^2

Table 7 Analysis of variance table investigating for Fig. 5a and b: the effects of treatment (labelling or not) and dates of sampling on $\delta^{15}N$ and $\delta^{13}C$

	$\delta^{15}N$	$\delta^{13}C$
Treatments (labelling or not)	F(1,84)=64.60; $p<0.001$	F(1,84)=3.96; $p<0.05$
Dates of sampling	F(2,84)=9.49; $p<0.001$	F(2,84)=0.31; NS
Interactions	F(2,84)=9.62; $p<0.001$	F(2,84)=2.28; NS

was labelled for a total soil surface colonized by roots of about 28 m^2. Moreover, the soil was labelled only in the first cm.

The studies of gene expression involved in nitrate uptake and nitrogen assimilation in young *T. melanosporum* ectomycorrhizas were in agreement with these field results. The nitrate transporter NrtB, one nitrate reductase and the nitrite reductase [NAD(P)H] NirB were well expressed, confirming that *T. melanosporum* ectomycorrhizas are able to both uptake and reduce nitrate. Similarly, several genes involved in aminoacid synthesis or in transamination processes also were well expressed in *T. melanosporum* ectomycorrhizas, suggesting strongly that glutamate, glutamine and asparagine are the main forms of nitrogen susceptible to be transferred to the host as suggested by Guescini et al. (2007) for *T. borchii*.

Origin of the nitrogen provided to the ascocarps

The ascocarps collected in the labelled plots of both experimental sites incorporated nitrogen from the litter during the three production seasons. But the amplitude of enrichment was highly variable from one truffle to another; we attribute this to the fact that the labelled leaf litter introduced as a fine layer at the upper part of the soil had been redistributed in a heterogeneous and random way by the soil microfauna at least in the first 20 cm where the ascocarps develop.

Table 8 Analysis of variance table investigating for Fig. 5a and b: the effects of treatment (labelling or not) and organs on $\delta^{15}N$ and $\delta^{13}C$

	$\delta^{15}N$	$\delta^{13}C$
Treatments (labelling or not)	F(1,82)=59.88; $p<0.001$	F(1,82)=5.84; $p<0.05$
organs	F(3,82)=6.45; $p<0.001$	F(3,82)=10.48; $p<0.001$
Interactions	F(3,82)=3.97; $p<0.05$	F(3,82)=0.21; NS

$^{Fifteen}N$ was found in truffles outside the labelled areas as well as in leaves of the host tree, demonstrating that the transfer of nitrogen through the ectomycorrhizas and the roots was systemic. The fact that in most cases no simultaneous enrichment of ^{13}C and ^{15}N was observed in ectomycorrhizas and ascocarps could indicate that only mineral nitrogen was taken up by the fungus, despite the fact that four genes coding four aminoacid transporters were overexpressed in ectomycorrhizas versus free-living mycelium. As stated below, we make the assumption that these transporters are involved in the transfer of aminoacids to the host or eventually to ascocarps, but not in aminoacid uptake from the soil.

These three facts demonstrate that *T. melanosporum* ectomycorrhizas take up the soil mineral nitrogen and seem to have not directly access to organic nitrogen contrarly to ectomycorrhizal fungi, which colonize leaf litter (Pena et al. 2013).

The study of the gene expression involved in nitrate metabolism in ascocarps during their development provides some insight on the origin of nitrogen. The nitrate transporter NrtB was not expressed in ascocarps, strongly suggesting that they are unable to take up nitrate directly from the soil. Moreover, no nitrite reductase was expressed in ascocarps. Consequently, the most likely process to have occurred is that N present in the ascocarps was supplied by the ectomycorrhizas, able to take up, reduce and metabolize nitrate. The expression of one of the putative ammonium transporters in the gleba could suggest that nitrogen was transferred to the ascocarps in part as ammonium after nitrate reduction in the ectomycorrhizas. According to Chalot et al. (2006), ammonium is considered as a candidate for nitrogen transfer at the mycorrhizal interface.

The expression of the two forms of glutamine synthetase (GS), of NAD-specific glutamate dehydrogenase (GDH) and of the two aspartate transaminases, suggests that the ascocarps were able to

Table 9 Expression of genes involved in the nitrogen metabolism in young *T. melanosporum* ectomycorrhizas (ECM) and free-living mycelium (FLM). The Gene ID of the *T.melanosporum* gene model (http://mycor.nancy.inra.fr/IMGC/TuberGenome) and the putative function are given. Expression levels are the mean of seven and four biological replicates for free-living mycelium and ectomycorrhizas respectively. Significantly regulated transcripts (FDR *p*-value <0.05) are in bold; FLM transcripts up-regulated tin ectomycorrhizas are highlighted in grey

Gene ID	Putative function	ECM mean transcript level	FLM mean transcript level	ECM vs FLM FDR corrected p-value
GSTUMT00010227001	high affinity nitrate transporter NrtB	25344	27363	0.799
GSTUMT00005025001	Putative Ammonium transporter	**3587**	**27865**	**0.0148**
GSTUMT00002376001	Putative Ammonium transporter	20540	22587	0.613
GSTUMT00008550001	Putative Ammonium transporter	11	87	0.145
GSTUMT00008501001	Putative Ammonium transporter	21602	27015	0.366
GSTUMT00010098001*	Amino Acid-Polyamine-Organocation (APC) transporter	**16324**	**547**	**0.0002**
GSTUMT00009497001*	APC transporter	**8801**	**388**	**0.0007**
GSTUMT00001488001*	APC transporter	**31807**	**2486**	**0.002**
GSTUMT00004647001*	APC transporter	**12574**	**7406**	**0.007**
GSTUMT00010228001	nitrate reductase	13857	13933	0.905
GSTUMT00010229001	Nitrite reductase [NAD(P)H] NirB	28185	18912	0.626
GSTUMT00002450001	glutamate synthase [EC:1.4.1.13]	9541	5870	0.0874
GSTUMT00002451001	glutamate synthase [EC:1.4.1.13]	3232	1727	0.285
GSTUMT00006745001	NAD-specific glutamate dehydrogenase	35512	19503	0.126
GSTUMT00004021001	NADP-glutamate dehydrogenase	22941	21899	0.841
GSTUMT00004641001	glutamine synthetase	9921	12039	0.394
GSTUMT00001013001	glutamine synthetase	37487	37176	1
GSTUMT00003903001	asparagine synthase	**12883**	**19775**	**0.0146**
GSTUMT00003001001	asparagine synthase	104	147	0.594
GSTUMT00006745001	glutamate dehydrogenase	35512	19503	0.126
GSTUMT00004021001	glutamate dehydrogenase (NADP+)	22941	21899	0.841
GSTUMT00003243001	aspartate transaminase	**9012**	**20587**	**0.0169**
GSTUMT00006350001	aspartate transaminase	20796	21448	0.821
GSTUMT00004718001	glutamate decarboxylase	11608	32712	0.0579

*There are 14 Amino Acid-Polyamine-Organocation superfamily proteins in *T. melanosporum* genome. Shown are four selected transporters that are either significantly regulated in ECM compared to FLM or in mature truffles compared to non-mature truffles (Table 10)

metabolize in aminoacids the ammonium which could be supplied by the ectomycorrhizas. This assumption does not exclude that nitrogen could also be partly supplied by the ectomycorrhizas as aminoacids. The overexpression of 4 genes coding for aminoacid transporters in ectomycorrhizas versus free-living mycelium suggests that mycorhizas are able to supply aminoacids to their host and probably also to ascocarps. In the same way, the fact that one aminoacid transporter was overexpressed in mature ascocarps versus non-mature ascocarps could mean that ascocarps in full

Table 10 Expression of genes involved in the nitrogen metabolism of *T. melanosporum* ascocarps cropped at two stages of maturity in Rollainville

Gene ID	Putative function	Non-mature mean transcript level	Mature mean transcript level	Non-mature vs mature FDR corrected p-value
GSTUMT00010227001	high affinity nitrate transporter NrtB	51	137	0.714
GSTUMT00005025001	Putative Ammonium transporter	17	54	0.342
GSTUMT00002376001	Putative Ammonium transporter	**2152**	**22893**	**0.004**
GSTUMT00008550001	Putative Ammonium transporter	21	20	0.865
GSTUMT00008501001	Putative Ammonium transporter	217	146	0.425
GSTUMT00010098001*	Amino Acid-Polyamine-Organocation (APC) transporter	853	265	0.192
GSTUMT00009497001*	APC transporter	52	442	0.208
GSTUMT00001488001*	APC **t**ransporter	167	60	0.08
GSTUMT00004647001*	APC transporter	**782**	**5079**	**0.027**
GSTUMT00010228001	nitrate reductase	24	34	0.748
GSTUMT00010229001	Nitrite reductase [NAD(P)H] NirB	47	99	0.600
GSTUMT00002450001	glutamate synthase [EC:1.4.1.13]	48	219	0.558
GSTUMT00002451001	glutamate synthase [EC:1.4.1.13]	26	64	0.580
GSTUMT00006745001	NAD-specific glutamate dehydrogenase	**157**	**17995**	**0.0004**
GSTUMT00004021001	NADP-glutamate dehydrogenase	54	38	0.678
GSTUMT00004641001	glutamine synthetase	198	115	0.343
GSTUMT00001013001	glutamine synthetase	1694	8823	0.324
GSTUMT00003903001	asparagine synthase	**36**	**4147**	**0.001**
GSTUMT00003001001	asparagine synthase	47	36	0.425
GSTUMT00006745001	glutamate dehydrogenase	**157**	**17995**	**0.0004**
GSTUMT00004021001	glutamate dehydrogenase (NADP+)	54	38	0.678
GSTUMT00003243001	aspartate transaminase	**2542**	**10385**	**0.034**
GSTUMT00006350001	aspartate transaminase	**1700**	**29658**	**0.001**
GSTUMT00004718001	glutamate decarboxylase	70	3842	0.097

The Gene ID of the *T. melanosporum* gene model and the putative function are listed. Expression levels are the mean of 3 and 9 replicates for the non-mature ascocarps (two dates) and the mature ascocarps (two dates). Significantly up-regulated transcripts (FDR p-value <0.05) in mature ascocarps versus non-mature ascocarps are in bold

There are 14 Amino Acid-Polyamine-Organocation superfamily proteins in *T.melanosporum*. Shown are four selected transporters that are either significantly regulated in ECM compared to FLM (Table 9) or in mature truffles (gleba) compared to non-mature truffles (gleba)

growth are also able to play an active role in the transport of aminoacids provided by the ectomycorrhizas.

T. melanosporum does not exhibit saprotrophic capacity for carbon

T. melanosporum ectomycorrhizas and ascocarps cropped in the labelled plots exhibited no or almost no ^{13}C enrichment, which confirms that ascocarps are mainly provided in carbon by the host (Le Tacon et al. 2013). The very slight ^{13}C enrichment of truffles observed the second year after soil labelling and during winter in ectomycorrhizas and roots can be interpreted in two different ways. It might be possible that during winter time a reduced C allocation of hosts as well as reduced mineral N availability may drive an organic C and N uptake by ectomycorrhizas in form of aminoacids directly released from the labelled leaf litter or indirectly from the turn-over of the microbial biomass that incorporated ^{13}C.

Table 11 Expression of CAZyme genes in young *T. melanosporum* ectomycorrhizas (ECM) and free-living mycelium (FLM). The Gene ID of the *T.melanosporum* gene model (http://mycor.nancy.inra.fr/IMGC/TuberGenome) and its Cazyme annotation (http://www.cazy.org/) is listed. In addition, for each Cazyme its putative substrate is given. Expression levels are the mean of seven and four biological replicates for free-living mycelium (FLM) and ectomycorrhizas (ECM) respectively. A gene was considered as significantly up-regulated if: *t*-test $P<0.05$ (Cyber-T) and expression ratio ECM versus FLM>5 (fold change). FLM transcripts up-regulated in ectomycorrhizas are highlighted in grey

Gene ID	Cazyme annotation	ECM mean transcript level	FLM mean transcript level	ECM vs FLM FDR corrected p-value	Putative substrate
Plant cell wall degrading					
GSTUMT00009298001	Glycoside Hydrolase Family 12	11028	216	0.015	cellulose
GSTUMT00011089001	Glycoside Hydrolase Family 45	5304	253	0.015	cellulose
GSTUMT00012789001	Carbohydrate Esterase Family 8	2628	125	0.033	pectin
GSTUMT00012011001	Glycoside Hydrolase Family 28	17909	1537	0.011	pectin
Fungal cell wall degrading					
GSTUMT00012353001	Carbohydrate-Binding Module Family 18	2293	132	0.014	chitin
GSTUMT00004733001	Carbohydrate-Binding Module Family 18	3486	36576	0.001	chitin
GSTUMT00011326001	Glycoside Hydrolase Family 18	1754	85	0.029	chitin
GSTUMT00011529001	Glycoside Hydrolase Family 18	13602	1102	0.001	chitin
GSTUMT00011889001	Glycoside Hydrolase Family 55	885	41	0.007	β−1,3-glucan
Both plant and/or fungal cell walls					
GSTUMT00007318001	Glycoside Hydrolase Family 36	987	30	0.007	Hemicellulose; α-galactan
GSTUMT00008333001	Glycoside Hydrolase Family 31	6347	285	0.002	α-glucan
GSTUMT00004616001	Glycoside Hydrolase Family 31	7689	987	0.002	α-glucan
GSTUMT00010862001	Glycoside Hydrolase Family 31	1752	304	0.002	α-glucan
GSTUMT00002130001	Glycoside Hydrolase Family 16	2387	4	0.001	β-glucan
GSTUMT00008973001	Glycoside Hydrolase Family 5	14686	8	0.0002	β-glucan
GSTUMT00008530001	Glycoside Hydrolase Family 5	6730	230	0.013	β-glucan
GSTUMT00005599001	Glycoside Hydrolase Family 3	171	8866	0.001	β-glucan ; α-glucan
GSTUMT00007083001	Glycoside Hydrolase Family 3	5189	1098	0.032	β-glucan ; α-glucan
Others					
GSTUMT00005701001	Glycosyltransferase Family 8	41856	7819	0.003	retaining NDP-sugar α-glycosyltransferase

However, the very slight ^{13}C enrichment of truffles observed the second year in the two sites also could be attributed to the assimilation by the leaves of the $^{13}CO_2$ coming from the mineralization of the labelled leaf litter.

In young ectomycorrhizas, the CAZyme genes overexpressed versus free-living mycelium were glycoside hydrolases involved in hemicellulose degradation, which could reflect their ability to degrade host cell walls. In addition, we know that

Table 12 Expression of CAZyme genes in *T. melanosoprum* ascocarps cropped at two stages of maturity in Rollainville. The Gene ID of the *T. melanosporum* gene model (http://mycor.nancy.inra.fr/IMGC/TuberGenome) and its Cazyme annotation (http://www.cazy.org/) are listed. In addition, for each Cazyme its putative substrate is given. Expression levels are the mean of 3 and 9 replicates for the non-mature (two dates) and mature ascocarps (two dates). A gene was considered as significantly upregulated if: *t*-test $P<0.05$ (Cyber-T) and expression ratio mature truffles versus non-mature truffles>5 (fold change). Mature truffle transcripts up-regulated in non-mature ascocarps are highlighted in grey

Gene ID	Cazyme annotation	Non-mature mean transcript level	Mature mean transcript level	Non-mature vs mature FDR corrected p-value	Putative substrate
Fungal cell wall degrading					
GSTUMT00004366001	Glycoside Hydrolase Family 15	529	13519	0.002	α-glucan
GSTUMT00007845001	Carbohydrate Esterase Family 4	455	5365	0.017	esterase related to cyclic imide hydrolase
Both plant and/or fungal cell walls					
GSTUMT00007484001	Glycoside Hydrolase Family 17	365	7373	0.033	β-glucan
Others					
GSTUMT00000402001	Carbohydrate-Binding Module Family 21	382	9474	0.022	distantly related to protein phosphatase glycogen-binding regulatory subunit
GSTUMT00011849001	Glycosyltransferase Family 2	102	5119	0.004	chitin
GSTUMT00005561001	Glycosyltransferase Family 2	878	7604	0.021	chitin
GSTUMT00007493001	Glycosyltransferase Family 48	801	13928	0.009	1,3-β-glucan
GSTUMT00004829001	Glycosyltransferase Family 32	387	8306	0.005	N-glycans; O-glycans
GSTUMT00006689001	Glycosyltransferase Family 22	48	627	0.031	N-glycans; O-glycans
GSTUMT00011376001	Glycosyltransferase Family 15	715	7895	0.005	N-glycans; O-glycans
GSTUMT00001845001	Glycosyltransferase Family 20	621	17506	0.002	trehalose
GSTUMT00000797001	Glycosyltransferase Family 4	752	86	0.017	trehalose
GSTUMT00009971001	Glycosyltransferase Family 4	351	30	0.003	trehalose
GSTUMT00011488001	Glycosyltransferase Family 4	3061	241	0.020	trehalose
GSTUMT00000799001	Glycosyltransferase Family 4	6129	390	0.012	trehalose
GSTUMT00006332001	Glycosyltransferase Family 4	1619	46	0.003	trehalose

T. melanosporum genome displays a restricted repertoire of genes coding for CAZymes that are able to degrade dead organic matter (Martin et al. 2010), which corresponds with the fact that no or very few labelled carbon was found in *T. melanosporum* ectomycorrhizas in Rollainville,

where it was possible to sample them, nor in ascocarps in the two sites. As mentioned above, the very slight ^{13}C enrichment of truffles observed the second year in the two sites could be attributed to the assimilation by the host of the $^{13}CO_2$ released after mineralization of the labelled leaf litter.

In mature ascocarps, the CAZyme genes upregulated compared to those of non-mature ascocarps were mainly Glycosyltransferases involved in saccharide transfer and more precisely trehalose, a fungal metabolite. This result supports the previous $^{13}CO_2$ host labelling showing that carbon is provided to the ascocarps by the host via the ectomycorrhizas until the end of their growth and maturation process (Le Tacon et al. 2013).

Conclusions

Despite their differential rate of nitrogen mineralization, the main source of soil mineral nitrogen for both sites was nitrate. The ascocarps, cropped in the labelled plots, were highly enriched in ^{15}N. According to gene expression in *T. melanosporum* ectomycorrhizas, we can assume that the $^{15}NO_3^-$ uptaken by the ectomycorrhizas in the labelled plots was largely reduced in the fungal tissues and then transformed in aminoacids by incorporation of host-specific carbon skeletons. These aminoacids, most likely glutamate, glutamine and asparagine as for *T. borchii,* are then either transferred to the fine roots or transferred to the ascocarps via aminoacid transporters well expressed in ectomycorrhizas. But it also appears that *T. melanosporum* ascocarps could in part be supplied in ammonium by the ectomycorrhizas and could be able to metabolize it through their own glutamine synthetase or NAD-specific glutamate dehydrogenase.

The ascocarps cropped in the labelled plots showed almost no enrichment in ^{13}C. These results support results from earlier $^{13}CO_2$ host labelling and confirms that carbon used by ascocarps is mainly provided by the host. Moreover, in mature ascocarps, the CAZyme genes upregulated in the gleba were mainly Glycosyltransferases involved in fungal saccharide transfer. In young ectomycorrhizas, the glycoside hydrolases genes overexpressed versus those of the free-living mycelium could reflect their ability to degrade host cell walls.

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Author contributions François Le Tacon: conceived and supervised the work, wrote the first draft and edited the manuscript. Christophe Robin: provided the truffle orchard, harvested the truffles and contributed to correct, to rewrite and to edit the manuscript. Bernd Zeller: conducted the soil labelling experiment and contributed to the correction of the manuscript. Annegret Keller and Francis Martin: conducted the study on gene expression and contributed to correct or to rewrite this part of the work. Caroline Plain: conducted the soil respiration measurements and contributed to the correction of the manuscript. Christian Hossann and Claude Bréchet conducted the isotopic analyses and contributed to the correction of the manuscript. Jean Villerd: designed the statistic analyses and contributed to correct and to rewrite parts of the manuscript.

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