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Original article

Saprotrophic versus symbiotic strategy during truffle ascocarp development under holm oak. A response based on ^{13}C and ^{15}N natural abundance

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Abstract –
• The development of truffles in the soil is not well understood. It is not known if a direct transfer of carbohydrates takes place between the host tree and the developing ascocarps through ectomycorrhizal structures or whether sporophores become independent from their hosts after several weeks or months and are able to use dead host tissues or soil organic matter as carbon (C) and nitrogen (N) sources.
• To study saprophytic or symbiotic capacities of truffle ascocarps the natural abundance of ^{15}N and ^{13}C in foliage, wood, fine roots, mycorrhizae, fungal sporophores and soil were determined in a truffle orchard.
• The processes of carbon and nitrogen allocation remained unchanged during the entire period of ascocarp development of *Tuber melanosporum*. From ^{13}C and ^{15}N natural abundance measurements, *T. melanosporum*, *T. brumale* and *T. rufum* did not exhibit saprotophic strategy during ascocarp development, which is contradictory to common statements found in handbooks regarding truffle cultivation.

^{13}C / ^{15}N / *Tuber melanosporum* / *Tuber brumale* / *Tuber rufum* / ascocarps development

Résumé – Stratégie saprophyte ou symbiotique durant le développement d'ascocarpes de truffes dans une truffière à chêne vert. Une réponse basée sur l'abondance naturelle du ^{13}C et du ^{15}N.
• Le développement des truffes dans le sol n'est pas encore bien compris. Les connaissances actuelles ne nous permettent pas de savoir s'il existe un transfert direct de sucres entre l'arbre hôte et les ascocarpes en développement via les structures ectomycorhiziennes, ou si les ascocarpes utilisent le carbone et l'azote directement issu de la matière organique du sol.
• Nous avons mesuré l'abondance naturelle du ^{15}N et du ^{13}C dans le sol, les feuilles, les mycorhizes, le bois et les carpophores d'une truffière naturelle à chêne vert afin de déterminer la stratégie de la nutrition carbonée des ascocarpes.
• Les processus d'allocation du carbone et de l'azote restent identiques pendant toute la phase de développement des ascocarpes de *Tuber melanosporum*. De ces mesures d'abondance naturelle du ^{15}N et du ^{13}C, il apparaît que *T. melanosporum, T. brumale* et *T. rufum* ne développent pas de stratégie saprophytique pendant le développement des ascocarpes, ce qui est en contradiction avec les idées habituellement véhiculées par les manuels de trufficulture

^{13}C / ^{15}N / *Tuber melanosporum* / ascocarpes / développement

1. INTRODUCTION

The most valuable truffles according to their aromatic and gustative qualities are two European species of Ascomycetes, *Tuber magnatum* Pico, the Piemont white truffle, and *T. melanosporum* Vittad., the Périgord black truffle or black diamond. In 2006, the Périgord black truffle could fetch 700 €/kg on the local market of Carpentras (south-east of France). In December 2003, after a long drought period, 1 kg of this truffle was valued at 1500 € on the local market of Périgueux (south-west of France) and 3000 € in Paris.

Despite their renown, the life cycle of truffles is poorly known. They form ectomycorrhizae, which originate from primary monokaryotic mycelia (Paolocci et al., 2006). From ectomycorrhizae, the primary extramatricial mycelium develops in the soil and gives birth to hypogeous sporophores (ascocarps). Ascogenous heterokaryotic tissues, resulting from an unknown fertilisation process, seem to be surrounded by homokaryotic maternal tissues.

The development of *Tuber* sporophores in the soil is not well understood. It is not known if a direct transfer of carbohydrates takes place between the host tree and the developing ascocarps through ectomycorrhizal structures or whether sporophores become independent from their hosts after several weeks or months and are able to use dead host tissues or

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soil organic matter as carbon (C) and nitrogen (N) sources. According to several studies, the ascocarp becomes independent from the host very early (Barry et al., 1994; Callot et al., 1999; Pradel, 1914). The young ascocarp appears to develop alongside a non-mycorrhizal root and could immediately to use soil carbon sources through external mycelium independently of the mycorrhizae. In pure cultures the mycelium of *T. melanosporum* is able to use cellulose as a source of carbon (Mamoun and Olivier, 1991). Similarly, in pure culture the mycelium of *T. melanosporum* could use cellobiose, lignin, chitin and tannins as sources of carbon (Barry, 1992). During its development, the truffle ascocarp develops from its peridium external mycelium and can colonize dead cells from living roots, dead roots, other dead organic tissues or mineral structures (Callot et al., 1999). These external hyphae absorb, water, orthophosphates and simple sugars, which are then transferred inside the ascocarp (Barry, 1992; Barry et al., 1993).

However, no convincing in situ ^{13}C or ^{15}N experiments have been carried out proving the independent saprophytic development of the truffle ascocarps. Natural abundance of ^{13}C and ^{15}N has frequently been used to study fungal sources of carbon and nitrogen (Hobbie et al., 2001). Gebauer and Dietrich (1993) found that positive ^{15}N values of fungal sporophores indicated that fungi can utilize organic soil nitrogen. Högberg et al. (1996) showed that ectomycorrhizae of Norway spruce and beech, collected across Europe, were 2‰ more enriched in ^{15}N than non-mycorrhizal fine roots. Fungal sheaths were 2.4 to 6.4‰ enriched compared to the root core. Other studies have confirmed that sporophores of ectomycorrhizal fungi are often more enriched in ^{15}N than sporophores of saprotrophic fungi, whereas sporophores of saprotrophic fungi are almost always enriched in ^{13}C compared to sporophores of ectomycorrhizal fungi (Hobbie et al., 1999; 2002; Kohzu et al., 1999; 2000; Taylor et al., 2003; Trudell et al., 2004). Lilleskov et al. (2002) demonstrated that isotope signatures reflect ecophysiological functions. Ectomycorrhizal fungi that utilise organic sources of nitrogen exhibit higher $\delta^{15}N$ than ectomycorrhizal species using mineral nitrogen. Emmerton et al. (2001a; 2001b) measured ^{15}N natural abundance in ectomycorrhizal fungi growing in pure culture and using high concentrations of ammonium, nitrate or glycine. They concluded that ^{15}N uptake was coupled with an internally fractionating process. However, Hobbie et al. (1999; 2001; 2002), Hobbie and Colpaert (2003; 2004), Hobbie et al. (2004; 2005) considered that in natural conditions with low nitrogen availability there is no fractionation upon uptake.

Although the relative contribution of the C and N sources and the different internal processes involved in the fractionation of ^{13}C and ^{15}N remain unclear, it appears that the analysis of natural abundance of C and N isotopes could provide insight into the respective trophic roles of saprotrophic versus ectomycorrhizal fungi (Gebauer and Taylor, 1999; Hobbie et al., 2004; 2005; Högberg et al., 1996; Kohzu et al., 1999; Taylor et al., 2003; Trudell et al., 2004; Zeller et al., 2007).

We hypothesised that the difference in $\delta^{13}C$ and $\delta^{15}N$ between saprophytic and mycorrhizal fungi reflects their source of C and N. The first aim was to establish in a truffle orchard of holm oaks in the south east of France the variations in the natural abundance of ^{13}C and ^{15}N of hypogeous sporophores formed by *Tuber melanosporum*, *T. brumale* and *T. rufum,* and other components of the ecosystem (epigeous ectomycorrhizal fungi, mycorrhizae, saprotrophic fungi, leaves and wood of holm oak, and soil). Using these information's the final aim was to determine the saprophytic or symbiotic capacities of truffle ascocarps.

Table I. Characteristics of the soil (A_1 horizon, 0–15 cm) of the Châteauvert stand (means and standard deviation, $n = 5$).

	Average	Standard deviation
Clay (< 2 mm) %	23.6	1.45
Fine silt (2–20 mm) %	16.4	0.66
Coarse silt (20–50 mm) %	10.5	0.46
Fine sand (50–200 mm) %	25.0	1.74
Coarse sand (200–2000 mm) %	24.6	0.78
Water pH	8.5	0.00
Total limestone %	41.0	1.14
Available P_20_5‰	0.02	0.02
CEC cmol+/kg	11.4	1.37
K^+ cmol+/kg	0.15	0.04
Mg^{++} cmol+/kg	0.20	0.03
Total C % (Carlo Erba)	1.4	0.27
Total N % (Carlo Erba)	0.2	0.01
C/N (Carlo Erba)	8.1	1.2
Total C % (Anne)	1.6	0.17
Total N % (Bremmer)	0.2	0.02
C/N (Anne, Bremmer)	9.7	0.70
$\delta^{13}C$ (‰)	–26.1	0.67
$\delta^{15}N$ (‰)	–4.6	1.18

2. MATERIAL AND METHODS

2.1. Field sampling

Samples were collected at Châteauvert, Visan, Vaucluse, France, in a holm oak truffle orchard (longitude: 4.916° E, latitude: 44.366° N). The altitude of the Châteauvert site is 200 m, the annual winter-dominant rainfall is 759 mm and the mean annual temperature 13 °C. Meteorological data were provided by a meteorological station (Météo France) located near the site.

The bedrock consists of calcareous alluvial deposits. The soil is a deep brown calcarisol with a sandy-silty texture, high alkaline $pH_{(water)}$ (8.5) and a limestone content of 41% (Tab. I). It is very low in available phosphorus and moderate in available K and Mg. It is a highly granular and aerated free-draining soil. Each year, the site is superficially ploughed (10 to 15 cm deep) and irrigated (100 mm of water supplied in summer).

The holm oak (*Quercus ilex* L.) stand (10000 m^2) was planted in 1976 on a vineyard site. The seedlings were naturally mycorrhizal with *T. melanosporum* and other naturally occurring ectomycorrhizal fungi.

In 2002, five trees which had been observed to have produced *T. melanosporum* ascocarps over many years were selected for sampling. Leaves, fine roots, branches, mycorrhizae, soil samples and *Tuber* ascocarps were collected in August 2002 (summer) and January 2003 (winter) underneath these five trees. In January 2003,

sporophores of epigeous fungi were also collected throughout the site.

In August 2002, non mature ascocarps of three *Tuber* hypogeous ectomycorrhizal fungi, *T. melanosporum* Vittad., *T. brumale* Vittad. and *T. rufum* Pico were located underneath the five selected trees by observing the cracks at the surface of the soil or by using a well trained dog. In January 2003, ripe ascocarps of *T. melanosporum* were all located by sniffing using the same well trained dog. The mature or non mature ascocarps were carefully retrieved from the soil using a small scratcher. Sporophores of eight epigeous fungi, two saprotrophic species (*Stereum hirsutum* (Willd.) Pers. and *Tubaria hiemalis*, var. *hiemalis* Romagn. ex. Bon) and six ectomycorrhizal species, (*Clavulina cristata* (Bull.) J. Schröt., *Russula maculata* Quél. & Roze, *Russula pallidopsora* J. Blum ex Romagn., *Inocybe fastigiata* (Schaeff.) Quél., *Amanita strobiliformis* Paulet ex Vittad. and *Xerocomus porosporus* Imler) were collected at random on the plot. Among the two saprotrophic fungi, *S. hirsutum* developed on dead wood and *T. hiemalis* developed mainly on the soil. Traditional mycological identification methods were used for taxonomic determination of the hypogeous sporophores collected underneath the five selected trees and the epigeous sporophores collected throughout the site (Courtecuisse, 2000).

Twenty leaves of the year were collected all around the crown in the upper part of each tree and pooled in order to have one sample per tree. Leaves visibly contaminated by insects or fungi were eliminated. One hundred grams of soil (A1 horizon, 0–15 cm) and 5 g of fresh fine roots ($\leqslant$ 2 mm diameter) were collected underneath each tree. Fine roots, mycorrhizae and soil were sampled from a 0.25 m^2 plot in the A1 horizon (0–15 cm) at equal distance from the trunk to the edge of the crown underneath each tree. Five samples of dead branches (diameter >10 cm) not still colonized by fungi were collected throughout the stand.

Oak fine roots ($\leqslant$ 2 mm diameter) and mycorrhizae were carefully retrieved from the soil using a small scratcher, washed in water under a dissecting microscope and treated with chlorhydric acid (HCl) in order to eliminate calcium carbonate and to only get the ^{13}C natural abundance of organic matter. For some fine root samples, the bark was carefully separated from the central cylinder using a scalpel. Mycorrhizae were tentatively identified by morphotyping on the basis of colour, mantle shape, surface texture and the presence of cystidia. They belonged mainly to the *Inocybe*, *Amanita* and *Suillus* genera. *Tuber* mycorrhizae were extremely rare despite the proximity of *Tuber* ascocarps. It was not possible to get sufficient material for each type of mycorrhizae. The samples used for isotopic analysis were a mixture of *Inocybe*, *Amanita, Suillus* and *Tuber* mycorrhizae. For isotopic analysis, like fine roots or mycorrhizae, soil samples were treated with chlorhydric acid in order to avoid the signal of calcium carbonate and to only get the ^{13}C natural abundance of the organic matter.

There were four or five replicates for each type of material (leaves, fine roots, mycorrhizae, wood, soil or sporophores). The samples were first air-dried, then dried at 60 °C for 48 h and ground to a fine powder using a shaker with an agathe mortar and agathe beads. After cleaning, elimination of sporophores contaminated by worms, drying and grinding, 63 fungal samples were kept for stable isotope analysis: 28 *Tuber* samples and 35 other fungal samples. With the exception of *S. hirsutum*, each sample of Basidiomycetes submitted to analysis comprised several different sporophores including the stipe, cap and gills. The small, wavy, leathery shelves of *S. hirsutum* were carefully detached from the dead branches and mixed together. *Tuber* ascocarps were individually subjected to analysis.

Forty-four other samples (leaves, fine roots, mycorrhizae and soils) were subjected to isotopic analysis.

2.2. Soil analysis

Soil samples (A1 horizon, 0–15 cm) were gathered underneath the five selected trees as previously described and analysed. The variables were: particle size distribution using wet sieving and flotation-sedimentation (Robinson method); organic C content was measured using a wet oxidation method (Anne, 1945); total N content was measured by the Kjeldahl method (Bremmer, 1960); pH (soil:water ratio = 1:25); cationic saturation and cationic exchange capacity were measured using 0.5 M NH_4Cl (Rouiller et al., 1980); "available" phosphorus was extracted consecutively with 0.004 N H_2SO_4 and 0.02N NaOH (Duchaufour and Bonneau, 1959).

2.3. Greenhouse samples

In order to obtain well-identified *Tuber* mycorrhizae, we used one-year-old holm-oak seedlings artificially inoculated with *T. melanosporum* in containers (300 mL) with a peat and vermiculite substrate added to calcium carbonate ($pH_{(H2O)}$ 8.05). Each seedling was inoculated with 1 g of fresh *T. melanosporum* ascocarp crushed with sand and suspended in 100 mL of water. The suspension containing spores was mixed with 300 mL of substrate in a cement mixer. Seedlings were grown in a greenhouse (16 h photoperiod of 50 Wm^{-2}, day-night temperatures 20–24 °C) for one year (February 2003 to February 2004).

Mycorrhizae and fine roots ($\leqslant$2 mm diameter) were collected individually under a dissecting microscope in February 2004, washed and treated with chlorhydric acid in order to eliminate calcium carbonate. *Tuber* mycorrhizae were initially sorted to genus level on the basis of colour, mantle shape, surface texture and presence of cystidia. Mycorrhizae were then confirmed as being associated with *T. melanosporum* using molecular methods. Genomic DNA was extracted by using the DNeasy Mini Kit (Qiagen SA, Courtabœuf, France) following the manufacturer's instructions. Polymerase chain reactions "PCR" were performed with ITS1f (Gardes and Bruns, 1993) and ITS4 primers (White et al., 1990). Amplification reactions were performed in a PE9700 thermal cycler (Perkin-Elmer, Applied Biosystems) in a 25 µL reaction mixture using the following final concentrations or total amounts: 5 ng DNA, 1× PCR buffer (20 mM Tris/HCl pH 8.4, 50 mM KCl), 1 µM of each primer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 unit of *Taq* polymerase (Promega). Amplification was conducted under the following conditions: an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s and extension at 72 °C for 2 min for each cycle. The last cycle was followed by a final extension at 72 °C for 10 min. PCR products were resolved on a 1.0% agarose gel and visualized by staining with ethidium bromide. For sequencing, the PCR products were purified with a MultiScreenTM PCR kit (Millipore, Molsheim, France) and quantified with a Low DNA Mass Ladder (Invitrogen, Cergy Pontoise, France) by electrophoresis on a 1.0% agarose gel. Sequencing was performed in CEQ 2000 DNA Analysis System (Beckman, Fullerton, CA) in the INRA Centre of Nancy (France) according to manufacturer's protocol. Similarities of ITS

Table II. Total C, total N, $\delta^{13}C$ and $\delta^{15}N$ in leaves, fine roots, mycorrhizae and dead wood of *Quercus ilex* L. in the Châteauvert stand (means and standard deviation, $n = 5$)

Sampling date	Material	C (%)	N (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
August 2002	Leaves	53.5 (4.46)	1.3 (0.26)	–29.7 (0.75)	1.6 (0.91)
	Fine roots (bark)	54.2 (2.29)	0.8 (0.08)	–27.4 (0.75)	2.0 (1.09)
	Fine roots (central cylinder)	51.3 (4.42)	0.6 (0.06)	–26.6 (0.94)	3.7 (0.91)
	Mycorrhizae	36.1 (1.99)	3.3 (0.19)	–26.3 (0.39)	3.2 (0.46)
January 2003	Leaves	49.4 (0.47)	1.4 (0.37)	–30.0 (0.57)	–1.6 (1.10)
	Dead wood	47.9 (0.82)	0.1 (0.03)	–22.7 (0.13)	0.6 (0.67)

sequences in GenBank database were determined by using BLAST (NCB).

Five leaves were collected on each tree and mixed together. There were five replicates for each type of material (leaves, fine roots and *T. melanosporum* mycorrhizae). All samples were first air- dried, then dried at 60 °C for 48 h and ground to a fine powder using a shaker with an agathe mortar and agathe beads. Fifteen samples (leaves, fine roots and mycorrhizae) were subjected to isotopic analysis. Together with the field samples a total of 123 samples were subjected to isotopic analysis.

2.4. Isotopic analysis

Percentages of C and N plus isotopic composition were determined in the INRA centre of Nancy-Champenoux using an online continuous flow CN analyser (Carlo Erba NA 1500) coupled with an isotope ratio mass spectrometer (Finnigan delta S). Values were reported using the standard notation ($\delta^{13}C$‰ and $\delta^{15}N$‰) relative to Pee-Dee Belemnite for C, using PEF (IAEA-CH-7) as a standard, and relative to atmospheric N_2 for N, using $(NH_4)_2SO_4$ (IAEA-N-1) as a standard. $\delta X = (R_{sample}/R_{standard}) - 1) \times 1000$, where R is the molar ratio $^{heavy}X/^{light}X$.

2.5. Statistical analysis

All data sets (C, N, $\delta^{15}N$, $\delta^{13}C$) were first tested separately for normal distribution and homogeneity of variances. The analysis of variance for experimental data was conducted using the Sigmastat 3.0 software. Student's t-tests were employed to test for significant differences between saprotrophic and ectomycorrhizal fungi, and One-Way-ANOVA for differences among the different species. When effects of groups on the dependent variables were significant at the 0.05 level, the least-significant difference test ($LSD_{0.05}$) was used to compare means. When mean values of groups or species are given the standard deviation is mentioned in brackets.

3. RESULTS

3.1. Field experiment

3.1.1. Total C and total N (Tabs. I and II)

The soil in Châteauvert is low in organic matter. The total C content averaged from 1.4% to 1.6% according to the method used (Carlo Erba NA1500 or Anne). The average total N content was 0.17% according to the two methods used (Carlo Erba NA1500 or Kjeldahl) (Tab. I) and the C/N ratio was 8.
For the trees under field conditions, the total N content was significantly lower in fine roots than in leaves and significantly higher in mycorrhizae than in fine roots or leaves.

3.1.2. $\delta^{13}C$ (Tabs. I and II, Fig. 1a)

Under field conditions, the $\delta^{13}C$ values of living oak leaves did not differ statistically between the summer (average –29.7‰) and the winter (average –30.0‰). $\delta^{13}C$ was significantly higher in leaves that in fine roots, but did not statistically differ between fine roots and mycorrhizae. In soil (A_1 horizon), $\delta^{13}C$ varied between –25.3‰ and –27.1‰ (average –26.1‰). Dead wood of Holm-oak displayed a very different $\delta^{13}C$ (–22.7‰) from A1 horizon.

3.1.3. $\delta^{15}N$ (Tabs. I and II, Fig. 1a)

Under field conditions, the average $\delta^{15}N$ values of *Quercus ilex* leaves were statistically identical in the summer and the winter (–1.6‰). $\delta^{15}N$ was significantly higher in fine roots and mycorrhizae than in leaves. $\delta^{15}N$ displayed positive values in root bark (2.0‰), in root central cylinder (3.7‰) and in naturally-occurring mycorrhizae (3.2‰). Dead wood of holm-oak also displayed a positive $\delta^{15}N$ (0.6‰). $\delta^{15}N$ remained highly negative in the A1 horizon of the soil (average –4.6‰).

3.1.4. Total carbon, total nitrogen, $\delta^{13}C$ and $\delta^{15}N$ of sporophores (Tab. III, Figs. 2a, 2b, 2c, and Fig. 3)

In January 2003, the average concentration of total C and total N of sporophores was respectively 41.7% and 3.2% respectively. The total nitrogen concentration ranged from 1.1% to 5.6% and the carbon concentration from 39.6% to 44.0%. Total N and total C could not be used to differentiate between saprotrophic fungi and ectomycorrhizal fungi. The two saprotrophic fungi displayed a total N concentration ranging from 1.1% (*S. hirsutum*) to 4.6% (*T. hiemalis*), while ectomycorrhizal fungi displayed a total N concentration ranging from 2.0% (*R. pallidospora*) to 5.6% (*A. strobiliformis*).

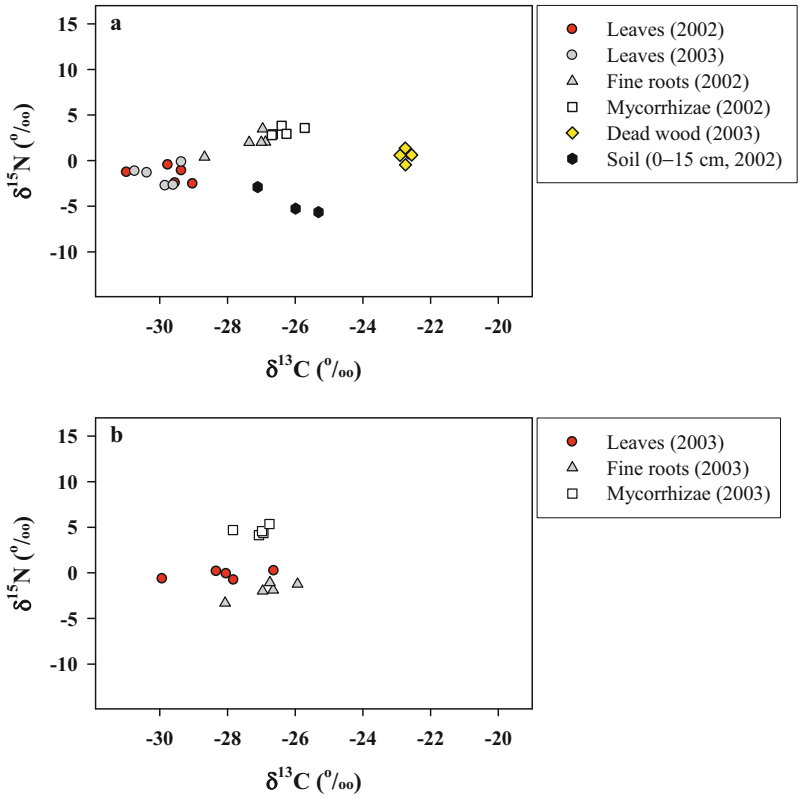

Figure 1. (a) Discrimination among leaves, fine roots, mycorrhizae, dead wood of *Quercus ilex* L. harvested in 2002 or 2003 and soil sampled in 2002, Châteauvert stand, according to $\delta^{13}C$ and $\delta^{15}N$ (individual values, average and standard deviation for each type of material). There were no statistically significant differences for $\delta^{13}C$ and $\delta^{15}N$ between fine roots and mycorrhizae, or between leaves harvested in 2002 and 2003. $\delta^{13}C$ differed significantly ($P < 0.001$) between leaves and fine roots or mycorrhizae, while $\delta^{15}N$ did not. $\delta^{13}C$ of dead wood differed significantly from all the other values ($P < 0.001$). **(b)** Discrimination among leaves, fine roots and mycorrhizae of one-year-old seedlings of *Quercus ilex* L. inoculated with *T. melanosporum* (nursery experiment 2003–2004) according to $\delta^{13}C$ and $\delta^{15}N$ (individual values, average and standard deviation for each organ). $\delta^{15}N$ differed significantly between mycorrhizae and leaves or fine roots ($P < 0.001$).

Among the three *Tuber* species, *T. rufum* displayed a significantly lower total N concentration (3.2%) than the two others, *T. melanosporum* (4.4%) and *T. brumale* (4.8%).

The immature sporophores of *T. melanosporum*, cropped in August, showed no significant differences in total N and total C compared to mature sporophores cropped in January.

$\delta^{13}C$ and $\delta^{15}N$ of sporophores differed significantly between the two saprotrophic fungi and the nine ectomycorrhizal fungi ($P < 0.001$).

The two saprotrophic sporophores displayed $\delta^{13}C$ values ranging from –24.2‰ (*T. hiemalis*) to –20.3‰ (*S. hirsutum*), while the sporophores of ectomycorrhizal fungi displayed $\delta^{13}C$ values ranging from –24.6‰ (*Inocybe fastigiata*) to –26.3‰ (*Russula maculata*). The $\delta^{15}N$ of the sporophores displayed a more striking difference between the two groups. The sporophores of the two saprotrophic fungi displayed a negative $\delta^{15}N$ (–1.1‰ for *S. hirsutum* and –4.4‰ for *T. hiemalis*), while all of the sporophores of ectomycorrhizal fungi displayed a positive $\delta^{15}N$, ranging from 2.0‰ (*I. fastigiata*) to 13.6‰ (*A. strobiliformis*). Among the three *Tuber* species, *T. rufum* sporophores had a significantly less negative $\delta^{13}C$ than the two others and a significantly higher $\delta^{15}N$. The immature sporophores of *T. melanosporum*, cropped in August, showed no significant differences in $\delta^{15}N$ and $\delta^{13}C$ compared with the mature sporophores cropped in January.

3.1.5. δ_{CN} index (Tab. III)

Hobbie et al. (2001) proposed a combined isotopic index $\delta_{CN} = \delta^{13}C - \delta^{15}N$ to separate ectomycorrhizal from saprotrophic fungi. In the Châteauvert stand, the two saprotrophic fungi displayed a δ_{CN} less than 20‰, while all symbiotic fungi displayed values greater than 25‰.

3.2. Greenhouse experiment

3.2.1. Total N (Tab. IV)

Total N in leaves was higher under greenhouse conditions than under field conditions (1.9% and 1.4% respectively). However, the variations between the different organs were similar under both conditions.

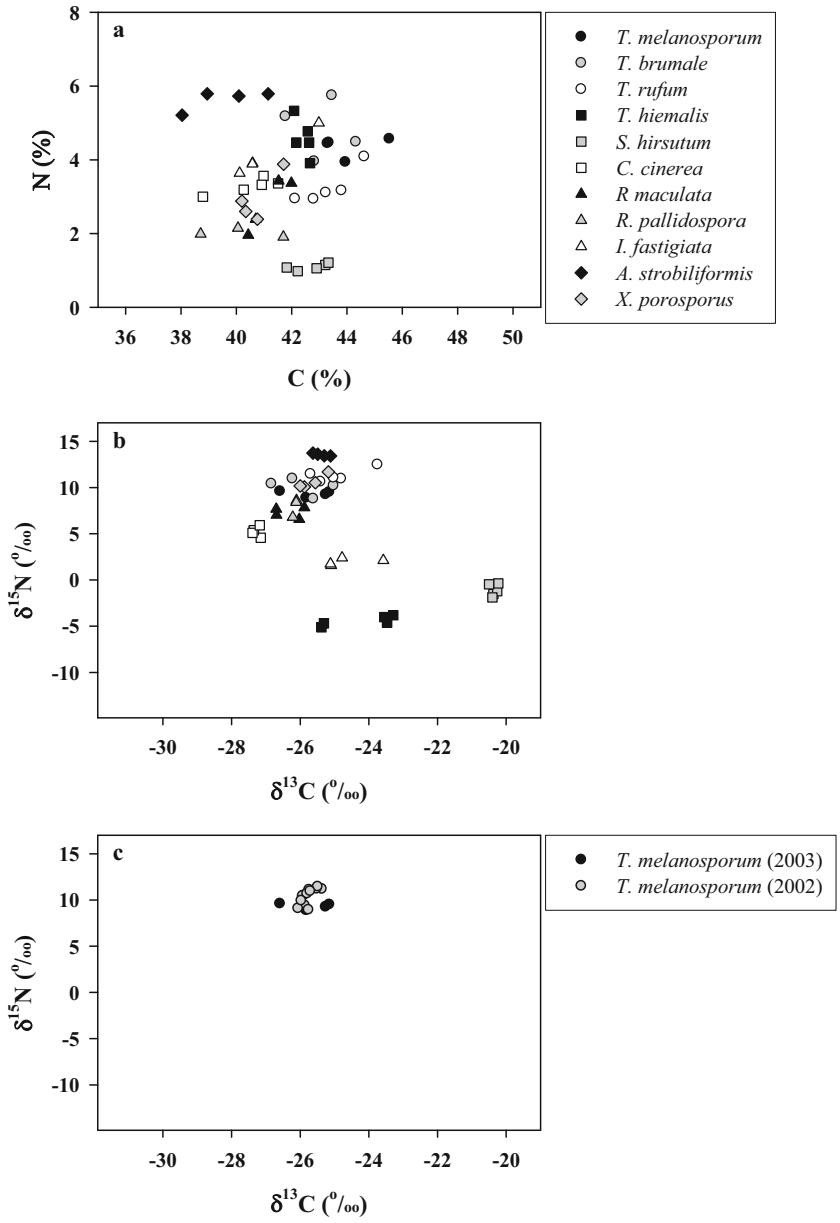

Figure 2. Discrimination among sporophores collected in the Châteauvert stand in 2003 according to: **(a)** Total C and total N (all sporophores, average and standard deviation for each species). Two species differed significantly ($P < 0.001$) from the other species for total N, *A strobiliformis* (high values) and *S. hirsutum* (low values). No significant differences were observed between saprotrophic and ectomycorrhizal fungi. **((b)** $\delta^{13}C$ and $\delta^{15}N$ (all sporophores, average and standard deviation for each species). Saprotrophic and ectomycorrhizal fungi differed significantly for $\delta^{15}N$ ($P < 0.001$). *I. fastigiata* displayed an intermediary position. *S. hirsutum* differed significantly for $\delta^{13}C$ from all of the other species ($P < 0.001$). **(c)** Discrimination among sporophores of *T. melanosporum* collected in the Châteauvert stand in 2002 (summer) and 2003 (winter) according to $\delta^{13}C$ and $\delta^{15}N$ (all sporophores, average and standard deviation for the two dates). There were no significant differences between the two dates.

3.2.2. $\delta^{13}C$ *(Tab. IV and Fig. 1b)*

When plants were grown under greenhouse conditions, $\delta^{13}C$ of oak leaves (–28.1‰) was less negative than under field conditions (–29.7%). Both under field and greenhouse conditions $\delta^{13}C$ was more negative in leaves that in fine roots, but did not statistically differ between roots and mycorrhizae.

3.2.3. $\delta^{15}N$ *(Tab. IV and Fig. 1b)*

Under greenhouse conditions, $\delta^{15}N$ did not statistically differ between leaves and fine roots, while it was largely and significantly positive in *T. melanosporum* mycorrhizae (average 4.6‰) compared to leaves (–0.2‰) and fine roots (–1.9‰).

Table III. Total C, total N, $\delta^{13}C$, $\delta^{15}N$ and isotopic index δ_{CN} ($\delta^{13}C - \delta^{15}N$) of fungal sporophores collected in the Châteauvert stand (average and standard deviation, $n = 5$ or 4).

Genus	Species	Authors	Date	C (%)	N (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta_{CN} = \delta^{13}C - \delta^{15}N$
Tuber	*melanosporum*	Vittad.	08-2003	43.0 (3.81)	4.0 (0.47)	–25.8 (0.21)	10.4 (0.87)	–36.16 (0.73)
Tuber	*melanosporum*	Vittad.	01-2003	44.0 (1.05)	4.4 (0.28)	–25.7 (0.66)	9.3 (0.32)	–35.0 (0.78)
Tuber	*brumale*	Vittad.	01-2003	43.1 (1.07)	4.9 (0.78)	–25.9 (0.78)	10.1 (0.92)	–36.0 (1.42)
Tuber	*rufum*	Pico.	01-2003	43.3 (0.96)	3.3 (0.48)	–24.9 (0.75)	11.3 (0.71)	–36.2 (0.54)
Tubaria	*hiemalis* var. *hie.*	Romagn. ex Bon	01-2003	42.4 (0.27)	4.6 (0.52)	–24.2 (1.05)	–4.4 (0.53)	–19.7 (0.69)
Stereum	*hirsutum*	(Willd.) Pers.	01-2003	42.7 (0.65)	1.1 (0.09)	–20.3 (0.11)	–1.1 (0.66)	–19.3 (0.66)
Clavulina	*cinerea*	(Bull.) J. Schröt.	01-2003	40.5 (1.05)	3.3 (0.21)	–27.3 (0.11)	5.2 (0.49)	–32.5 (0.51)
Russula	*maculata*	Quél. & Roze	01-2003	41.2 (0.72)	2.8 (0.73)	–26.3 (0.43)	7.3 (0.58)	–33.6 (0.73)
Russula	*pallidospora*	J. Blum ex Romagn.	01-2003	40.2 (1.50)	2.0 (0.12)	–26.1 (0.06)	8.2 (0.98)	–34.1 (0.93)
Inocybe	*fastigiata*	(Schaeff.) Quél.	01-2003	41.1 (1.29)	4.1 (0.61)	–24.6 (0.73)	1.9 (0.37)	–26.6 (0.63)
Amanita	*strobiliformis*	(Paulet ex Vittad.) Bertill.	01-2003	39.6 (1.35)	5.6 (0.28)	–25.4 (0.23)	13.5 (0.15)	–38.9 (0.37)
Xerocomus	*porosporus*	Imler	01-2003	40.8 (0.68)	2.9 (0.66)	–25.7 (0.37)	10.6 (0.74)	–36.3 (0.41)

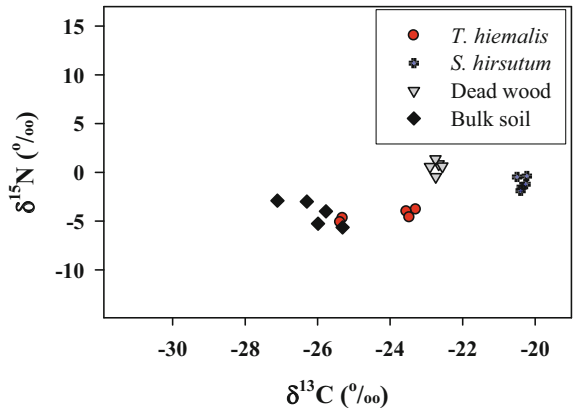

Figure 3. Discrimination among the two saprotrophic sporophores collected in the Châteauvert stand in 2003 according to $\delta^{13}C$ and $\delta^{15}N$ (all sporophores, average and standard deviation). Comparison with soil and wood $\delta^{13}C$ and $\delta^{15}N$. *Tubaria hiemalis* lives on soil and *Stereum hirsutum* on wood. There is a statistically significant $\delta^{13}C$ shift between soil and *Tubaria hiemalis* and between wood and *Stereum hirsutum*. There is no statistically significant $\delta^{15}N$ shift between the two fungi and the two corresponding substrates.

4. DISCUSSION

In the Châteauvert stand, holm oak (*Q. ilex*) leaves displayed different $\delta^{13}C$ and $\delta^{15}N$ values from those of sessile oak leaves (*Quercus petraea*) as obtained by Zeller et al. (2007) in the Breuil forest (–29.7‰ compared to –28.8‰ and –1.6‰ compared to –4.1‰). The difference was higher still for dead wood ($\delta^{13}C$ of –22.7‰ compared to –26.4‰). Similarly $\delta^{13}C$ and $\delta^{15}N$ were different in the A_1 horizon of the soils in the two sites (–26.1‰ and –4.6‰ compared to –28.3‰ and 1.4‰ respectively). These differences are probably partly due to soil and climatic differences between the two ecosystems. The Châteauvert stand is characterised by a Mediterranean climate and a calcareous soil, while the Breuil forest is characterised by a temperate climate and an acidic soil. Consequently, the C/N ratio of the A1 horizon is less than 10 in the Châteauvert stand while it is around 20 in the same horizon of the Breuil forest.

Despite these differences in ecological conditions, $\delta^{13}C$ and $\delta^{15}N$ of fungal sporophores displayed the same tendencies in the two sites. As reported by several authors (Gebauer and Taylor, 1999; Henn and Chapela, 2001; Hobbie et al., 1999, 2001; Högberg, 1997; Kohzu et al., 1999; Taylor et al., 2003, Trudell et al., 2004; Zeller et al., 2007), $\delta^{13}C$ values differed between sporophores of saprotrophic and ectomycorrhizal fungi. Sporophores of the two saprotrophic fungi from the Châteauvert site showed a ^{13}C enrichment compared to their substrates. *T. hiemalis* displayed a $\delta^{13}C$ of –24.2‰ compared to –26.1‰ for soil organic matter. Similarly, *S. hirsutum* displayed a $\delta^{13}C$ of –20.3‰ compared to –22.7‰ for dead wood (Fig. 3).

Sporophores of the two saprotrophic fungi displayed no or little ^{15}N fractionation compared to their substrate (Fig. 3). *T. hiemalis* displayed a $\delta^{15}N$ of –4.4‰, very close to the $\delta^{15}N$ of soil organic matter (–4.6‰). Similarly, *S. hirsutum* displayed a low $\delta^{15}N$ fractionation (–1.1‰ compared to 0.6‰ for dead wood). These results are very close to those obtained by Zeller et al. (2007) in the Breuil forest, where most saprotrophic fungi also had no or little effect on fractionation of stable N isotopes relative to their substrates (leaves, twigs, wood or soil).

In the Châteauvert stand,^{13}C natural abundance of ectomycorrhizal fungi sporophores varied from –24.6‰ to –27.2‰. A similar amplitude was observed in the Breuil forest (–22.2‰ to –28.1‰). According to the ^{13}C natural abundance in their sporophores, we can assume whether ECM fungi acquire more carbon from their host than from dead organic matter, or the contrary. *I. fastigiata* displayed a $\delta^{13}C$ of –24.6‰, which is close to the value found in sporophores of the saprotrophic fungus *T. hiemalis*. On the other hand, *C. cinerea* displayed a $\delta^{13}C$ of –27.3‰. It could be hypothesised that *C. cinerea* acquires more carbon from its host than *I. fastigiata*. Nevertheless, according to the isotopic index $\Delta_{CN} = \delta^{13}C - \delta^{15}N$, *I. fastigiata* is an ectomycorrhizal fungus, the limit between saprotrophic and symbiotic strategies being 25.0‰ (Hobbie et al., 2001). Indeed, *I. fastigiata* displayed a Δ_{CN} of –26.6‰.

Table IV. Nursery experiment (2003). Total C, total N, $\delta^{13}C$ and $\delta^{15}N$ in leaves, fine roots and mycorrhizae of one-year-old seedlings of *Quercus ilex* L. artificially inoculated with *T. melanosporum* (average and standard deviation, $n = 5$).

Sampling date	Material	C (%)	N (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
August 2002	Leaves	48.4 (0.77)	1. 9 (0.24)	–28.1 (1.19)	–0.2 (0.47)
	Fine roots	46.5 (0.72)	1.4 (0.27)	–26.9 (0.77)	–1.9 (0.89)
	Mycorrhizae	47.8 (0.3)	3.4 (0.20)	–27.1 (0.42)	4.6 (0.46)

$\delta^{15}N$ natural abundance of ectomycorrhizal sporophores was much more variable than $\delta^{13}C$ natural abundance. For example, *I. fastigiata* displayed a low nitrogen natural abundance ($\delta^{15}N$ of 2.0‰), while *A. strobiliformis* displayed a high natural abundance ($\delta^{15}N$ of 13.5‰). These results are congruent with those of several research teams (Gebauer and Dietrich, 1993, Henn and Chapela, 2001, Hobbie et al., 2004; Kohzu et al., 1999; Taylor et al., 2003; Trudell et al., 2004; Zeller et al., 2007) who all observed high ^{15}N abundance in ectomycorrhizal fungi sporophores. The three *Tuber* species displayed a high $\delta^{15}N$ value (9.3 to 10.4‰). Hobbie et al., (2001) also found for *T. gibbosum*, a North American truffle, high $\delta^{15}N$ values (12.0 to 16.6‰). In the Châteauvert site, the amplitude of $\delta^{15}N$ variation among ectomycorrhizal sporophores was similar to the amplitude observed in the Breuil forest (–1.0‰ to 10.2‰). From the Châteauvert results, congruent with those of Hobbie et al. (2001) for *T. gibbosum* in Oregon, it seems improbable that *Tuber* ascocarps could use, through protease excretion, nitrogen incorporated in soil organic matter which display on average a $\delta^{15}N$ of –4.6‰, while *T. melanosporum* ascocarps display $\delta^{15}N$ values ranging on average from 9.3‰ in the winter to 10.4‰ in the summer. A transfer of nitrogen via mycorrhizae, which displayed a positive $\delta^{15}N$ (4.6‰ in nursery conditions), seems much more probable. On the other hand, the saprotrophic fungus *T. hiemalis*, which displays a negative $\delta^{15}N$ similar to the $\delta^{15}N$ of the soil, probably utilises complex forms of soil organic matter for its nitrogen supply (Fig. 3).

According to Hobbie et al. (1999) and Kohzu et al. (2000), the transfer of nitrogen to trees by ectomycorrhizal fungi is a fractionating process, which could occur through amino acid biosynthesis or amino acid transfer to the host. Nevertheless, according to the Breuil results (Zeller et al., 2007), some ectomycorrhizal fungi (i.e., *Lactarius*) seem to have a different way of operating and either supply N to the host without N isotope fractionation or do not supply N to the host. Moreover, this fractioning process does not occur in all symbiotic associations. In mycoheterotrophic orchids, the host plant gain most of its nitrogen from their associated ectomycorrhizal fungi and display the same isotope signature as their fungal partner (Bidartondo et al., 2004; Gebauer and Meyer, 2003). In our nursery experiment with trees and ectomycorrhizal fungi, it is clear that the fractioning process of nitrogen started through *T. melanosporum* mycorrhizae. From –1.9‰ in fine roots, $\delta^{15}N$ shifted to 4.6‰ in *T. melanosporum* mycorrhizae. A similar shift was found in the Breuil forest for *Cortinarius* sp. and *Tricholoma sciodes* mycorrhizae (Zeller et al., 2007).

From the isotopic index $\Delta_{CN} = \delta^{13}C - \delta^{15}N$, which allows assignment of a mycorrhizal or a saprotrophic strategy for sporophore differentiation, it is obvious that *Tuber* ascocarps do not display a saprotrophic strategy. If we consider the $\delta^{13}C$ values alone, the conclusions are the same as Hobbie et al. (2001) when considering that the limit between saprotrophic and symbiotic strategies is 24‰ (Tab. III). From the Châteauvert results, it seems that, during ascocarp differentiation, *T. melanosporum, T. brumale and T. rufum* behave like ectomycorrhizal fungi and not like saprotrophic fungi, despite the fact that sporophore initiation and development are rapid (some days) in ectomycorrhizal Basidiomycetes and very slow in *Tuber* species (six to nine months). Moreover, there were no statistically significant differences between newly formed *T. melanosporum* ascocarps collected in the summer (August) and mature ascocarps collected in the winter (January of the following year). This implies that the processes of carbon allocation remained identical during the entire period of *T. melanosporum* ascocarp development.

It then becomes a challenge to know to which category of ectomycorrhizal fungi the species of genus *Tuber* belongs. What proportion of carbon do they derive from their host when forming ectomycorrhizae? What is the importance of their saprotroph ability during ascocarp differentiation? Although we do not know enough on the relationships between isotope signatures, metabolic processes and ecosystem functions, from the results of this study, we can hypothesise that like other ectomycorrhizal fungi, which have saprotrophic capacities, *Tuber* species depend on their host at least partly for ascocarp development and maturation. It is difficult to believe that at any step of their development *Tuber* ascocarps are completely independent from their host. Prior work has shown that young ascocarps of *T. melanosporum* never develop if separated from their host (Rouquerolle and Payre, 1975). Rommel (1938) obtained similar results with other ectomycorrhizal fungi in Sweden. Trenching completely prevented fruit body formation of ectomycorrhizal fungi. However, some ectomycorrhizal fungi may mobilise organic polymers of nitrogen, increasing their availability to host plants (Perez-Moreno and Read, 2000; Read and Perez-Moreno, 2003). Similarly, ectomycorrhizal fungi, even if they depend on their host for carbon allocation, can also express extracellular enzyme activities in the soil. Courty et al. (2005) showed that several ectomycorrhizal fungi expressed high activities of cellobiohydrolase, β-glucosidase, chitinase, laccase, glucuronidase and xylosidase, suggesting their contribution to the degradation of soil humic polymers so as to acquire extra carbon and other nutrients from polyaromatic complexes. Callot et al. (1999) found that in mycorrhizae, *T. melanosporum* hyphae are able to degrade polyphenols inside dead host cells. Similarly, *Tuber* hyphae seem able to develop in the dead cells of root bark (Callot

et al., 1999). From these different results or observations, it seems obvious that during *Tuber* sporophore differentiation, a direct transfer of hexose exists between the host and the fungus via ectomycorrhizal structures, and an indirect transfer of carbon from dead host cells or dead mycorrhizae or dead roots or exfoliating root bark via free hyphae displaying saprotrophic abilities. Nevertheless, according to Lindahl et al. (1999) and Leake (2001), ectomycorrhizal fungi do not need to produce costly ligninases to acquire nutrients from dead organic matter. Rather than direct nutrient capture, ectomycorrhizal fungi may obtain nutrients via absorption of small organic molecules exogenously decomposed by saprotrophic fungi. In two forests of southern Sweden, Staaf (1988) showed that trenching had no significant effect on total organic matter over the following six years. These results, as well as results from other similar studies, suggest that ectomycorrhizal fungi through mycorrhizae or sporophores do not play an important role in the use of soil organic matter, despite their saprotrophic abilities.

According to France and Reid (1983) and Straatsma and Bruinsma (1986), ectomycorrhizal fungi can assimilate CO_2. Lapeyrie (2002) demonstrated that *Paxillus involutus* could utilise bicarbonate ions from solution for oxalate synthesis. Barry (1993) showed that *T. melanosporum* ascocarps were able to fix CO_2(10 µg of C per g of dry matter and per hour). Lacourt et al. (2002) demonstrated the existence of an anaplerotic pathway in *T. borchii* mycelium and ascocarp. Ceccaroli et al. (2003) confirmed the existence of anaplerosis in *T. borchii* mycelium. Nevertheless, anaplerotic uptake of carbon is unlikely to provide a significant part of the carbon necessary for *Tuber* ascocarp development. Based on complete pathways analyses, Marx et al. (2001) showed that in *Corynebacterium glutamicum* anaplerotic reactions cannot contribute to biomass at a level greater than 3–5% of carbon.

Our main conclusion is that, when required climatic and edaphic conditions are met, primordial initiation of *T. melanosporum* is strictly dependent on the host as in almost all ectomycorrhizal fungi. Soil humidity is crucial for the development of young primordia into ascocarps during the summer (Le Tacon et al., 1982). During ascocarp development, carbohydrate demand could be supplied by the host as simple carbohydrates through mycorrhizae, or by an indirect transfer of carbon from dead host tissues, or by the fruit body itself through the tufts of hyphae sprouting from the tops of the scales of the peridium (Barry et al., 1994; Callot and Guyon, 1990). These hyphae colonise the surrounding soil and can probably use complex forms of organic matter (Barry et al., 1993). There is a huge difference between epigeous ectomycorrhizal fungi which produce mature sporophores in some days and truffles which produce mature ascocarps in six or nine months. Are the mycorrhizae, which have given birth to the young *Tuber* primordium in May or June, still functioning in January or February of the following year when the ascocarp is mature? Does the ascocarp get its carbon from host photosynthates through mycorrhizae during all its development or only during primordium formation? Does it obtain its organic compounds both from the host and from current decomposition activities or does it completely get its carbon from dead organic matter in the final phase of maturation? From our results, it seems improbable that carbon allocation for ascocarp development could be mainly supplied via saprotrophic pathways from surrounding soil organic matter or dead host tissues, and that sporophores could be completely independent at any time during their development, even during late maturation. Our results bring new insights on truffle ascocarp development and clearly show from $\delta^{13}C$ and $\delta^{15}N$ abundance that *Tuber* ascocarps do not exhibit a saprotrophic strategy during their development. These results contradict the statements of well recognized truffle handbooks (Callot et al., 1999; Olivier et al., 1996) and could be of some importance for the improvement of truffle cultivation methods.

However, in situ ^{13}C and ^{15}N labelling experiments are the only way to solve the question of carbon and nitrogen allocation during *Tuber* sporophore differentiation. However, the technical difficulties are great, which is likely why no convincing experiments have yet been conducted. We have started some preliminary labelling experiments and we hope to obtain new results in the coming years.

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