Symbiotic versus saprotrophic strategy during Tuber melanosporum ascocarp development. Preliminary results based on 13C and 15N natural abundance and on in situ 13CO2 pulse-labelling

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SYMBIOTIC VERSUS SAPROTROPHIC STRATEGY DURING *TUBER MELANOSPORUM* ASCOCARP DEVELOPMENT. PRELIMINARY RESULTS BASED ON ^{13}C AND ^{15}N NATURAL ABUNDANCE AND ON *IN SITU* $^{13}CO_2$ PULSE-LABELLING.

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ABSTRACT

Despite their renown, the life cycle of the true truffles belonging to the genus *Tuber* is not well known. The growth of the ascocarp is poorly understood. It is not known if a direct transfer of carbohydrates takes place between the host tree and the developing ascocarps through the ascogonial filament or whether ascocarps become independent from their hosts after several weeks or months and are able to use dead host tissues or soil organic matter as carbon and nitrogen sources. From a first work based on ^{13}C and ^{15}N natural abundance, we found that *Tuber* ascocarps do not exhibit a saprotrophic strategy during their development [1]. 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* ascocarp differentiation. A first *in situ* $^{13}CO_2$ pulse-labelling experiment was carried out in 2010 on a 20-year-old hazel tree mycorrhized with *Tuber melanosporum*. The preliminary results showed that the transfer of carbon from the leaves to the fine roots is slow but continuous during several months even during winter at low temperature. The fine roots act as a pipe to transfer the carbon to the mycorrhizas. From the mycorrhizas, ^{13}C accumulates into the ascocarps, which constitutes a carbon sink. These results contradict the statements of recognized truffle handbooks and could be of some importance for the improvement of truffle cultivation methods.

Keywords: *Tuber*; Ascocarp; Development; Carbon; Nitrogen.

INTRODUCTION

The development of *Tuber* ascocarps is poorly understood. It is not known if a direct transfer of carbohydrates takes place between the host tree and the developing ascocarps through the ascogonial filament or whether ascocarps become independent from their hosts after several weeks or months and are able to use dead host tissues or soil organic matter as carbon and nitrogen sources. According to several studies, the ascocarp becomes independent from the host very early [2, 3, 4]. It would be able to use soil carbon sources through external mycelium independently from the host. During its development, the truffle ascocarp develops from its

peridium external mycelium which could colonize dead cells from living roots, dead roots, other dead organic tissues or mineral structures [5]. These external hyphae could absorb, water, orthophosphates and simple sugars, which would be then transferred inside the ascocarp [6]. Similarly, in pure culture, the mycelium of *T. melanosporum* could use cellulose, cellobiose, lignin, chitin and tannins as sources of carbon [7, 5]. Nevertheless, the sequencing of *T. melanosporum* genome has shown that this fungus has much fewer GH-encoding genes than saprotrophs [8]. The cellulases from families GH6 and GH7 are absent, whereas family GH45 cellulase and hemicellulases from families GH10 and GH43 are present. *T. melanosprum* has an invertase gene allowing the hydrolysis of the plant-derived sucrose.

The aim of our work was to determine the way of carbon allocation to the ascocarps: saprotrophic pathways from surrounding soil organic matter or dead host tissues or symbiotic pathways through carbon allocated by the host.

The first part of our work was based on the determination of ^{13}C and ^{15}N natural abundance in different compartments of a truffle orchard of holm oaks in the South East of France. The second part of this work was based on an *in situ* $^{13}CO_2$ pulse-labelling experiment carried out on a 20-year-old hazel nut tree mycorrhized with *Tuber melanosporum* in a truffle orchard of hazel nut trees in East of France.

MATERIAL AND METHODS

^{13}C and ^{15}N natural abundance. Samples were collected at Châteauvert, Visan, Vaucluse, France, in a holm oak truffle orchard (Longitude: 4.916° - Latitude: 44.366°; altitude: 200 m, annual winter-dominant rainfall 759 mm; mean annual temperature 13°C. The bedrock consists of calcareous alluvial deposits. The soil is a deep brown calcarisol with a sandy-silty texture, high alkaline $pH_{(H2O)}$ (8.5) and a limestone content of 41%. It is very poor 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 and irrigated (100 mm in summer).

The holm oak (*Quercus ilex* L.) stand (10000 m^2) was planted in 1976 on a vineyard site. The seedlings were mycorrhized with undetermined naturally occurring fungi and were naturally mycorrhized with *T. melanosporum* and other ectomycorrhizal fungi.
In 2002, five trees, having produced *T. melanosporum* ascocarps over many years, were selected for sampling. Leaves, fine roots, branches, mycorrhizas, soil samples and *Tuber* ascocarps were collected in August 2002 and January 2003 underneath these five trees. In January 2003, sporophores of epigeous fungi were also collected throughout the site.

Percentages of C and N plus isotopic composition were determined at 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 $^{heavy}X/^{light}X$.

$^{13}CO_2$ pulse-labelling experiment. The experiment was carried out in Rollainville situated in the West part of the Vosges, France, on a limestone plateau of the Jurassic era (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 calcarisol with a silty clay texture, a high alkaline pH (water pH 7.97), a moderate content of organic matter (9.4%) and a limestone content of 8.8%. It is poor in available phosphorus and moderate in available K and Mg. It is a free draining soil highly granular and aerated.

Established in 1990 and 1991 on a previously cultivated site with *Quercus robur* (L.) and

hazel nuts trees previously inoculated with *Tuber aestivum* (Vittad.) (summer truffle) and *T. melanosporum* (Vittad.) (black Perigord truffle). The first truffle crop has started in November 1995 for *T. melanosporum* and in October 1996 for *T. aestivum*.

In 2010, we have started a labelling experiment with $^{13}CO_2$ on a hazel nut tree selected for having previously produced *T. melanosporum* ascocarps. On the day of labelling, the whole tree was enclosed in a 28 m^3 cylindrical polyethane chamber into which $^{13}CO_2$ gas was injected. The content of $^{13}CO_2/^{12}CO_2$ was followed by a Mahiac (SICK 710). A first introduction of $^{13}CO_2$ was done the 6th of July 2010 and a second one the 1st of September 2010. During the two labelling periods, the tree has assimilated 16.7 g de ^{13}C.

The isotopic composition of leaves, fine roots, mycorrhizas and ascocarps was determined as previously described for natural abundance.

RESULTS AND DISCUSSION

^{13}C and ^{15}N natural abundance. As reported by several authors [9, 10, 11, 12, 13, 14, 15, 16, 17], in the Châteauvert site $\delta^{13}C$ values differed between sporophores of saprotrophic and ectomycorrhizal fungi (Figure 1b), while total C did not differed among the two groups (Figure 1 a).

Sporophores of the two saprotrophic fungi from the Châteauvert site showed $\delta^{13}C$ enrichment compared to their substrates. *T. hiemalis* displayed a $\delta^{13}C$ of -24.2‰ against -26.1‰ for soil organic matter (Figure 2). Similarly, *S. hirsutum* displayed a $\delta^{13}C$ of -20.3‰ against -22.7‰ for dead wood (Figure 2). Cellulose and lignin degradation could be involved in ^{13}C enrichment of sporophores of saprotrophic fungi, although few fungal culture studies on known ^{13}C complex substrates have yet been conducted.

Sporophores of the two saprotrophic fungi displayed no or little ^{15}N fractionation compared to their substrate (Figure 2). *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‰ against 0.6‰ for dead wood).

^{13}C natural abundance of ectomycorrhizal fungi sporophores varied from -24.6‰ to -27.2‰ (-25.7 to -25.8‰ for *T. melanosporum*) (Figure 1 b).

$\delta^{15}N$ natural abundance of ectomycorrhizal sporophores was much more variable than $\delta^{13}C$ natural abundance. For example, *I. fastigiata* displayed a weak nitrogen natural abundance ($\delta^{15}N$ of 2‰), while *A. strobiliformis* displayed a huge natural abundance ($\delta^{15}N$ of 13.5‰). These results are congruent with those of several research teams [10, 12, 13, 18, 15, 17, 19, 20] 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. [21] also found for *T. gibbosum*, a North American truffle, high $\delta^{15}N$ values (12 to 16.6‰). From the Châteauvert results, congruent with those of Hobbie et al. [21] 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 winter to 10.4‰ in summer. A transfer of nitrogen *via* mycorrhizas, which displayed a positive $\delta^{15}N$ (4.6‰ in nursery conditions), seems much more probable. 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 (Table 1). If we consider the $\delta\ ^{13}C$ values alone, the conclusions are the same as Hobbie et al. [21] when considering that the limit between saprotrophic and symbiotic strategies is 24‰. 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 months ore more). Moreover, there were no statistically significant differences between newly formed *T. melanosporum* ascocarps collected in summer (August) and mature ascocarps collected in winter (January of the following year) (Figure 1c). This implies that the processes of carbon allocation remained identical during the entire period of *T. melanosporum* ascocarp development.

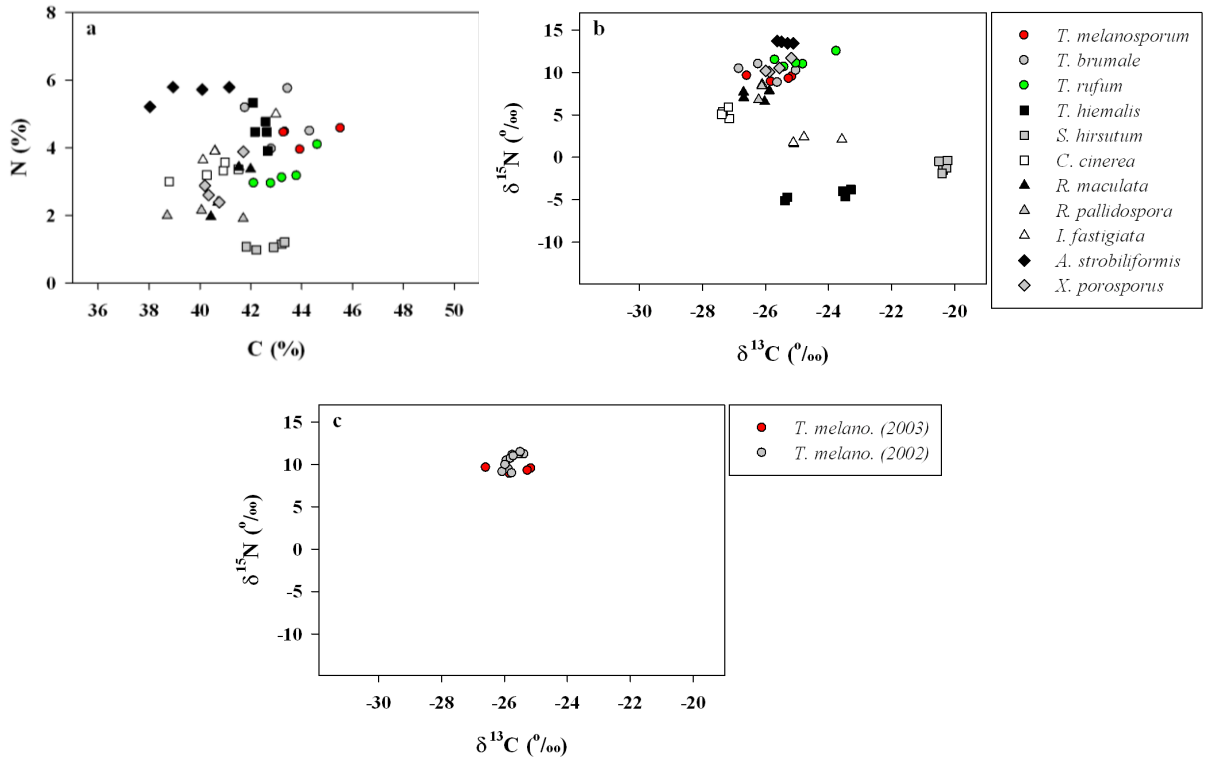

Figure 1: Discrimination among *Tuber* ascocarps (*Tuber melanosporum* Vittad., *Tuber brumale* Vittad. and *Tuber rufum* Pico) and sporophores of epigeous fungi (two saprotrophic species, *Stereum hirsutum* (Willd.) Pers. and *Tubaria hiemalis*, var. *hiemalis* Romagn. ex. Bon, six ectomycorrhizal species, *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) 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.

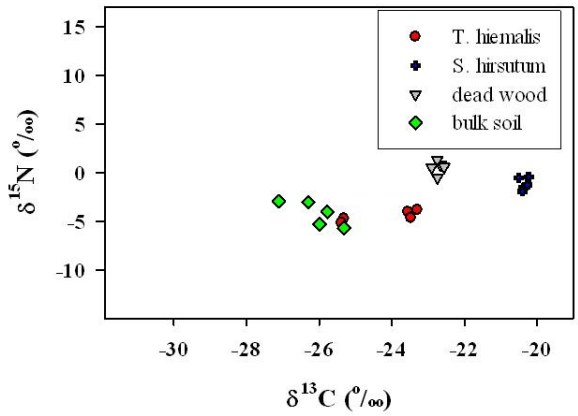

Figure 2: Discrimination among the two saprotrophic sporophores collected in the Châteauvert stand in 2003 according to $\delta^{13}C$ and $\delta^{15}N$ (all sporophores, average and standard deviation). Comparison with soil and wood $\delta^{13}C$ and $\delta^{15}N$. *Tubaria hiemalis* lives on soil 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.

Table 1: 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 (%)**	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**	**Δ_{CN} = $\delta^{13}C$ - $\delta^{15}N$**
Tuber	*melanosporum*	Vittad.	08-2003	43.0 (3.81)	4.0 (0.47)	-25.76 (0.21)	10.40 (0.87)	-36.16 (0.73)
Tuber	*melanosporum*	Vittad.	01-2003	44.0 (1.05)	4.4 (0.28)	-25.71 (0.66)	9.32 (0.32)	-35.02 (0.78)
Tuber	*brumale*	Vittad.	01-2003	43.1 (1.07)	4.9 (0.78)	-25.93 (0.78)	10.10 (0.92)	-36.03 (1.42)
Tuber	*rufum*	Pico.	01-2003	43.3 (0.96)	3.3 (0.48)	-24.94 (0.75)	11.32 (0.71)	-36.26 (0.54)
Tubaria	*hiemalis var. hie.*	Romagn. ex Bon	01-2003	42.4 (0.27)	4.6 (0.52)	-24.20 (1.05)	-4.45 (0.53)	-19.75 (0.69)
Stereum	*hirsutum*	(Willd.) Pers.	01-2003	42.7 (0.65)	1.1 (0.09)	-20.35 (0.11)	-1.09 (0.66)	-19.26 (0.66)
Clavulina	*cinerea*	(Bull.) J. Schröt.	01-2003	40.5 (1.05)	3.3 (0.21)	-27.28 (0.11)	5.25 (0.49)	-32.54 (0.51)
Russula	*maculata*	Quél. & Roze	01-2003	41.2 (0.72)	2.8 (0.73)	-26.33 (0.43)	7.27 (0.58)	-33.60 (0.73)
Russula	*pallidospora*	J. Blum ex Romagn.	01-2003	40.2 (1.50)	2.0 (0.12)	-26.15 (0.06)	8.21 (0.98)	-34.08 (0.93)
Inocybe	*fastigiata*	(Schaeff.) Quél.	01-2003	41.1 (1.29)	4.1 (0.61)	-24.65 (0.73)	1.96 (0.37)	-26.60 (0.63)
Amanita	*strobiliformis*	(Paulet ex Vittad.) Bertill.	01-2003	39.6 (1.35)	5.6 (0.28)	-25.39 (0.23)	13.54 (0.15)	-38.93 (0.37)
Xerocomus	*porosporus*	Imler	01-2003	40.8 (0.68)	2.9 (0.66)	-25.66 (0.37)	10.62 (0.74)	-36.27 (0.41)

$^{13}CO_2$ pulse-labelling experiment. The flux of pulse-derived ^{13}C from the tree to the fine roots, the mycorrhizas, and the ascocarps was traced and quantified over a seven-month post-labelling period. The preliminary results of this $^{13}CO_2$ pulse-labelling experiment showed that a significant transfer of ^{13}C towards the roots was detectable 5 days after labelling. However, the $\delta^{13}C$ of fine roots always remained low during all the post-labelling period, indicating that fine roots acted mainly as a pipe (Table 2). The transfer of ^{13}C from the fine roots to the *T. melanosporum* mycorrhizas occurred between 5 and 20 days after labelling (Table 2). The mycorrhiza $\delta\ ^{13}C$ reached a maximum of +22.75 ‰ 22 days after the first labelling. Then it decreased when the young truffles started to grow. It increased again after the second labelling to reach a maximum of 55.35 ‰ before decreasing again. The mycorrhizas formed a carbon sink and accumulated ^{13}C for all the tree growing period and for all the period of the ascocarp development. Then the mycorrhizas transferred their ^{13}C to the truffles, which accumulated carbon from the host until their complete maturity, 200 days after the first labelling. At the end of September, the gleba of the ascocarps, which were in full growth, was six times more enriched in ^{13}C than the mycorrhizas and ten times than the fine roots (Table 3). This role of carbon sink of the *Tuber* ascocarps occurred several months after the end of carbon assimilation by the host and at low temperature.

Table 2: Rollainville: Evolution of the $\delta^{13}C$ of leaves, fine roots and *T. melanosporum* mycorrhizas in ‰ according to the time (average of 4 samples at each date)

Days after labelling	1	4	5	22	55	83	133	264
Leaves	-27.66	290.71	35.26	29.65	469.07	11.45	-4.45	76.70
Fine roots	-27.62	-26.62	- 9.73	-13.15	-19.34	- 6.73	9.87	3.82
Tuber mycorrhizas	-27.50	-24.09	-24.60	22.75	-5.31	26.3	52.35	18.85

Table 3: Rollainville: Evolution of the $\delta^{13}C$ of *T. melanosporum* ascocarps (peridium and gleba) in ‰ according to the time (average of 3 to 7 samples at each date; the $\delta^{13}C$ of *T. melanosporum* ascocarps cropped under non labelled trees were in average -25.7‰ for the gleba and -26.25‰ for the peridium).

Days after labelling	83	101	133	168	204
Peridium	87.01	60.36	69.56	59.43	77.36
Gleba	125.38	78.94	79.13	67.17	82.12

CONCLUSIONS

^{13}C and ^{15}N natural abundance measurements and a $^{13}CO_2$ pulse-labelling experiment gave results going in the same direction. *Tuber* ascocarps do not exhibit a saprotrophic strategy during their development. The results demonstrate for the first time under field conditions that *Tuber* mycorrhizas provide a slow but important pathway of carbon flux from tree to the ascocarps. Almost the whole of carbon used by the truffle ascocarps seems to be allocated by the host. It becomes now evident that *Tuber* ascocarps are dependent from their hosts during their whole development. These preliminary results contradict the statements of well-recognized truffle handbooks and could be of some importance for the improvement of truffle cultivation methods.

A replication of the $^{13}CO_2$ labelling experiment is in course. We have also carried out two new experiments of soil labelling (^{15}N and ^{13}C) in order to determine if truffle ascocarps could partly use dead host tissues or soil organic matter as carbon and nitrogen sources.

Nevertheless some questions are remaining. It is not known how the transfer of carbohydrates takes place between the host tree and the developing ascocarps. The most probable is a transfer through the ascogonial filament, which could provide a direct connection between mycorrhizas and ascocarps.

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