In Vivo Natural-Abundance $^{13}$C Nuclear Magnetic Resonance Studies of Living Ectomycorrhizal Fungi

OBSERVATION OF FATTY ACIDS IN CENOCOCCUM GRANIFORME AND HEBELOMA CRUSTULINIFORME

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FRANCIS MARTIN*, DANIEL CANET, JEAN-PIERRE MARCHAL, AND JEAN BRONDEAU
Laboratoire de Microbiologie Forestière, Centre de Recherches Forestières, Institut National de la Recherche Agronomique, Champenoux, 54280 Seichamps, France (F. M.); and Laboratoire de Méthodologie RMN, E.R.A. CNRS No. 22, Université de Nancy I, B.P. 239—54506 Vandoeuvre les Nancy Cedex, France (D. C., J-P. M., J. B.)

ABSTRACT

Natural-abundance $^{13}$C nuclear magnetic resonance spectroscopy has been used to study intact mycelia of the ectomycorrhizal fungi Cenococcus graniforme (Ascomycetes) and Hebeloma crustuliforme (Basidio-

mycetes). A number of sharp resonances are observed in living fungi. These signals primarily arise from fatty acyl chains and carbohydrate nuclei. The spectra are interpreted in terms of relative concentrations of the major fatty acids present in the fungal triglycerides. The small line widths of fatty acids (mainly oleic, linoleic, and palmitic acids) resonances and spin-lattice relaxation time are indicative of fast rotational reorientations and are consequently thought to arise from fatty acyl chains in fat droplets. We were able to locate the site of lipid accumulation within mycelia using light microscopy and histological staining. Many lipid droplets were observed in mycelia of both species.

These results suggest that fatty acids droplets could be involved in carbon storage and metabolism from ectomycorrhizal fungi.

We report here the results of natural-abundance $^{13}$C NMR studies of triacylglycerides from the Ascomycete Cenococcus graniforme and the Basidiomycete Hebeloma crustuliforme. $^{13}$C resonances allow measurement of the fatty acyl chains composition and provide intrinsic and nonperturbing probes for the molecular dynamics of constituent lipids.

MATERIALS AND METHODS

Growth and Harvesting of Mycelia. Cenococcus graniforme Ferd. and Wing, strain Kiffer 1973, and Hebeloma crustuliforme, strain S 166, were grown in medium containing 110 mM glucose, 28 mM maltose, 7 mM KH$_2$PO$_4$, 2.7 mM MgSO$_4$, 2.5 mM diammonium tartrate and micronutrients. Mycelia were cultured in 1-L Erlenmeyer flasks containing 500 ml of medium, with aeration provided by a gyratory shaking. Mycelia were grown at 25°C during the night and at 28°C to 29°C during the day. Samples were harvested by filtration and washed with 3 volumes of distilled H$_2$O to remove traces of growth medium. The packed mycelium was used immediately for $^{13}$C NMR.

$^{13}$C Nuclear Magnetic Resonance. Packed living mycelia (about 2 g) were studied in 10 mm o.d. spinning sample tubes at 30°C. $^{13}$C NMR spectra, in the Fourier transform mode with proton broad band decoupling (1 w power, phase modulation), were measured at 50.3 MHz with a modified Bruker WP200 spectrometer interfaced to a Nicolet 1180 computer. The following conditions were used: spectral window, 15,150 Hz; quadrature phase detection; 8K data storage array; observing pulse of 10 μs (corresponding to a 30° flip angle); 0.77 s repetition rate. Ten thousand transients were accumulated for normal spectra aiming at chemical shift determinations or at quantitative analysis. Spectra with short accumulation times (15 min) were taken before and after a long term accumulation. At the exception of a lower signal to noise ratio, they did not show any significant difference. This indicates that the experimental conditions do not cause any alteration of the metabolites observed by NMR. Chemical shifts are reported relative to substituted TMS sample. Resonance intensities were estimated by measuring peak area.

Longitudinal relaxation time ($T_1$) measurements were performed according to the Fast Inversion Recovery Sequence (3): (180° − τ − 90° − T), with a waiting time T of 1.27 s, n = 4500 transients, and 11 τ values ranging from 0.01 to 0.40 s.

RESULTS AND DISCUSSION

$^{13}$C NMR Spectra. The proton-decoupled natural-abundance $^{13}$C NMR spectra of intact mycelia from the ectomycorrhizal fungi...
Acids in *C. graniforme* and *H. crustuliniforme*: oleic (C18:1) and linoleic (C18:2) acids. Resonances arising from these two fatty acids are readily assigned in the olefinic region (peaks 2 and 3) (Fig. 1, A and B). Oleic acid has two vinyl carbons, both of which contribute to peak 2. Linoleic acid has four vinyl carbons, two of which contribute to peak 2 and the other two to peak 3. The intensities lead to the estimation of the relative contribution of oleic and linoleic acids of 0.45 and 0.55, respectively, in *C. graniforme* and 0.07 and 0.93, respectively, in *H. crustuliniforme*. Thus, the basidiomycete is much richer in di-unsaturated fatty acyl chains than the ectomycorrhizal ascomycete.

From the assignments in Table I, the relative concentrations of saturated fatty acids triglycerides observed by NMR can be determined by using the intensity ratio of peaks 6 and 7 from the methylene region (Fig. 1, A and B), together with the relative concentrations of unsaturated fatty acids, as determined by analysis of the olefinic carbons from the same spectra (2, 10). The only major saturated fatty acid in both ectomycorrhizal fungi is palmitic acid (C16:0) (9). Palmitic, oleic, and linoleic acids have 10, 8, and 5 carbons which contribute to peak 6 (Fig. 1, A and B) and they have 0, 2, and 2 carbons, respectively, which contribute to peak 7 (10). Analysis of the intensities indicates the occurrence of about 5% of oleyl-type fatty acyl chains, 54% of linoleyl-type chains, and 40% of palmitoyl-type species in 10-week-old *H. crustuliniforme*. In 10-week-old *C. graniforme*, the analysis leads to an estimation of the relative concentrations of oleic, linoleic, and palmitic acids of 37%, 31%, and 32%, respectively. The two ectomycorrhizal fungi could be distinguished by their fatty acids pattern, although C18 unsaturated fatty acids constitute the main component in each fungus. The values obtained are not in agreement with the chromatographically determined (9) values of 17%, 74%, and 9% for oleic, linoleic, and palmitic acids measured in *H. crustuliniforme* and 22%, 58%, 20%, respectively, found in *C. graniforme*. However, the discrepancy could result from difference in strains used and age of the fungi. Also, the chromatographic analysis is based upon extracted lipids and is therefore a destructive analysis which may modify the relative proportions of fatty acids.

From spectra similar to those of Figure 1, relative proportions of saturated and unsaturated fatty acids, i.e., palmitic, oleic, and linoleic acids, were monitored in mycelium of *C. graniforme* from day 23 to day 68 (Table II). The predominant NMR-observable fatty acids are always oleic, linoleic, and palmitic acids. Their relative proportions showed no significant change during the life span of the mycelium.

![Fig. 1](image_url) Natural abundance $^{13}$C NMR spectra at 50.4 MHz of intact mycelia from (A) *Cenococcum graniforme* and (B) *Hebeloma crustuliniforme*. Assignments are given in Table I. Chemical shifts are expressed in ppm downfield from TMS. Each spectrum represents the time average of 10,000 scans of 30° flip angle pulses with a 0.77-s repetition rate.

Fungi *Cenococcum graniforme* and *Hebeloma crustuliniforme* are shown in Figures 1, A and B. Spectra revealed high internal amounts of mobile metabolites in both fungi. Comparison of these spectra with previously reported $^{13}$C NMR spectra of plant tissues (6, 10, 11), animal tissues (12, 14, 15), and lipids (1, 2) suggest most of the assignments. Lines appearing at 173 ppm from TMS (Fig. 1, peak 1) are due to carboxyl carbons of various types, mainly those of fatty acyl chains of lipids (triacylglycerols) (10). Peaks 2 and 3, located in the vinyl region (130 ppm) are attributed to olefinic carbons of fatty acids. Resonances between 60 and 100 ppm arise from the carbons in a not yet identified carbohydrate or polyol in the case of *C. graniforme* (Fig. 1A) and likely from glycogen in the case of *H. crustuliniforme* (Fig. 1B) (12). Lines ranging from 20 to 40 ppm (peaks 4–9) can be assigned to the methylene of the fatty acids; finally, the line appearing at 14 ppm (peak 10) is due to their methyl carbon. Assignments were also checked against solutions of standard fatty acids.

The present report concerns the composition and physical state of fatty acyl chains observed in intact mycelia.

As demonstrated by GLC and TLC (9; Martin and Mourot, unpublished results), there are only two major unsaturated fatty acids in *C. graniforme* and *H. crustuliniforme*: oleic (C18:1) and linoleic (C18:2) acids. Resonances arising from these two fatty acids are readily assigned in the olefinic region (peaks 2 and 3) (Fig. 1, A and B). Oleic acid has two vinyl carbons, both of which contribute to peak 2. Linoleic acid has four vinyl carbons, two of which contribute to peak 2 and the other two to peak 3. The intensities lead to the estimation of the relative concentration of oleic and linoleic acids of 0.45 and 0.55, respectively, in *C. graniforme* and 0.07 and 0.93, respectively, in *H. crustuliniforme*. Thus, the basidiomycete is much richer in di-unsaturated fatty acyl chains than the ectomycorrhizal ascomycete.

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<table>
<thead>
<tr>
<th>Peak in Fig. I</th>
<th>Chemical Shift (ppm)</th>
<th>Palmitic acid</th>
<th>Oleic acid</th>
<th>Linoleic acid</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>171.78</td>
<td>C-1</td>
<td>C-1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>129.77</td>
<td>C-9, C-10</td>
<td>C-9, C-13</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>128.10</td>
<td>C-10, C-12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>33.83</td>
<td>C-2</td>
<td>C-2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>32.13</td>
<td>C-14</td>
<td>C-16</td>
<td>C-16</td>
</tr>
<tr>
<td>6</td>
<td>29.89</td>
<td>C-4 to C-13</td>
<td>C-4 to C-7</td>
<td>C-4 to C-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C-12 to C-15</td>
<td>C-15</td>
</tr>
<tr>
<td>7</td>
<td>27.33</td>
<td></td>
<td>C-8, C-11</td>
<td>C-8, C-14</td>
</tr>
<tr>
<td>8</td>
<td>24.94</td>
<td>C-3</td>
<td>C-3</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>22.83</td>
<td>C-15</td>
<td>C-17</td>
<td>C-17</td>
</tr>
<tr>
<td>10</td>
<td>14.10</td>
<td>C-16</td>
<td>C-18</td>
<td>C-18</td>
</tr>
</tbody>
</table>

Table I. $^{13}$C Chemical Shifts and Assignments of Palmitic, Oleic, and Linoleic Acid Carbon Nuclei in the Intact Ectomycorrhizal Fungi *Cenococcum graniforme* and *Hebeloma crustuliniforme*

Chemical shifts are expressed in ppm downfield from TMS.
Table II. Relative Concentrations of Palmitic, Oleic, and Linoleic Acids as a Function of Age in Mycelium of Cenococcum graniforme

Mycelium was grown as described in “Materials and Methods” and was sampled at intervals from 23 to 68 d after inoculation.

<table>
<thead>
<tr>
<th>Time after Inoculation (d)</th>
<th>Relative-Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Palmitic acid</td>
</tr>
<tr>
<td>23</td>
<td>0.30</td>
</tr>
<tr>
<td>36</td>
<td>0.36</td>
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<tr>
<td>38</td>
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<tr>
<td>57</td>
<td>0.34</td>
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<tr>
<td>68</td>
<td>0.32</td>
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13C Nuclear Magnetic Relaxation in Fatty Acids. The small line widths of fatty acid resonances are indicative of high chain flexibility and fast chain segmental motion. The high resolution of the mycelium 13C spectra as well as their suitably high signal to noise ratios permit the determination of the spin-lattice relaxation time \( T_1 \) pertaining to some well resolved lines or to some group of resonance. All the carbons of fatty acids (peaks 4-10) had \( T_1 \)'s of 0.23 s. Such relatively important \( T_1 \) values indicate at least the presence of a fast motion in the fatty acid chains. Indeed, an effective correlation time of 100 ps, indicating rapid motion, can be estimated assuming isotropic reorientation and a single type of motion (4). However, this procedure is very oversimplified since one expects at least two motions: a fast one due to internal rotations and a slower one corresponding to the reorientation of the molecule as a whole. In order to proceed further, \( T_1 \) and nuclear Overhauser enhancements should be measured at several frequencies.

Presently, the structural organization in the fatty acids which emerges from the nuclear relaxation parameters derived from the 13C NMR spectra of intact mycelia is the one of a system with lipid hydrocarbon chains able to move in a fluid matrix. This is consistent with a droplet organization rather than a membrane location.

We were able to locate the site of fatty acids deposit within the mycelium of both fungi using light microscopy and histological staining. Many refringent droplets were seen in the mycelia. These droplets were stained by Red Suddan, a lipid-specific stain (data not shown). This further supports the view that the observed 13C NMR resonances of fatty acids arise from lipids in fat droplets.

Fatty acid droplets have been observed in a number of fungi including endomycorrhizal fungi (5) but to our knowledge the present report is the first devoted to observation and dynamics of these compounds in living microorganisms. It is believed that fat droplets can serve as a reserve of carbon skeletons and energy, and, in view of the high amount of carbon contained in the fatty acids, these compounds are probably a major store in the studied ectomycorrhizal fungi. Thus, 13C NMR spectra should be valuable in characterizing nutritional state and fatty acids pathway of these symbiotic microorganisms. The use of 13C-labeled precursors should permit the study of metabolic pathways in vivo and thus the way is open to extend the present investigations to other aspects of ectomycorrhizal fungi physiology.

13C NMR is a potentially valuable analytical tool in the investigation of lipid composition of plant materials since it offers a method for performing such analysis without the need for chromatographic separation or chemical alteration of the various lipid components.

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LITERATURE CITED