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解　説

Methods to study the role of ectomycorrhizal fungi in forest carbon cycling 2 : Ergosterol analysis method to quantify the fungal content in ectomycorrhizal fine roots

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Abstract: At the first report of this series, we reconfirmed the importance of the study to quantify the role of mycorrhizal fungi in forest carbon cycling. In this report, we focused on the ergosterol analysis method, a general method to quantify the biomass of fungi in ectomycorrhizal fine roots, and its procedure was detail described. Concentration of the ergosterol in ectomycorrhizal fungi is used as a conversion factor and this value has the great influence on the estimation of fungal biomass in ectomycorrhizal fine roots. We reviewed the data about conversion factor and recognized that the necessity of further data accumulations.
Keywords: biochemical indicator, ergosterol, fine root, fungal biomass, phospholipid fatty acid (PLFA)

森林の炭素循環における外生菌根菌の役割を研究する手法2: 外生菌根の菌類含量を定量化するエルゴステロール分析手法:里村 多香美（広島大学大学院生物圏科学研究科），橋本 靖（帯広畜産大学環境総合科学講座），木下 晃彦（広島大学大学院生物圏科学研究科），堀越 孝雄（広島大学大学院総合科学研究科）

要　旨：このシリーズの第一弾では，森林の炭素循環において菌根菌の定量が重要であることを再確認した。この第二弾となる報告では，外生菌根の形成された植物細根の中の菌類バイオマスの定量の際に用いられる一般的な手法であるエルゴステロール分析法に焦点をあて，その手順を詳述する。外生菌根菌のエルゴステロール濃度は変換係数として用いられ，その値が根の中の外生菌根菌のバイオマスを推定値に与える影響は大きい。我々は変換係数の値をレビューし，更なるデータの蓄積が必要であることを認識した。
キーワード：エルゴステロール，菌類のバイオマス，細根，生化学指標，リン脂質脂肪酸（PLFA）

Introduction

In the study of forest carbon cycling, it is important to assess the fungal biomass in ectomycorrhizal fine roots (reviewed by Satomura et al., 2006). Ergosterol is a fungal specific sterol and has been used as an indicator of fungi in mycorrhizal roots (e.g. Nylund and Wallander, 1992; Satomura et al., 2003) and ergosterol analysis method is a major method to quantify the biomass of fungi in ectomycorrhizal fine roots. In this article, we review the historical aspect of ergosterol analysis and the procedure of ergosterol method is detail described. We affirm the importance to inquire a suitable conversion factor (ergosterol concentration in ectomycorrhizal fungi) that translates ergosterol content in ectomycorrhizal fine roots into fungal biomass in them.

Historical aspects of ergosterol analysis

Ergosterol is a sterol that is specific to fungal taxonomical groups, such as Zygomycetes and higher fungi (Ascomycetes, Basidiomycetes and their anamorphs) (Weete and Gandhi, 1996). All of the known taxa of mycorrhizal fungi are fall into these taxonomical groups (VA mycorrhizal fungi had been categorized into Zygomycota, which now

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belong to the Glomeromycota; Walker and Trappe, 1993). The ergosterol method has an advantage over other estimation methods, such as the dissection method and/or the image analysis, in that it can be easily applied to roots of any type of mycorrhizal plants, i.e., not only ectomycorrhizal plants but also VA and ericoid mycorrhizal plants. However, the wide taxonomic range of the ergosterol is also weak point.

The fungal ergosterol concentrations were reported about the dozens of ectomycorrhizal fungi (Table 1), but there are few data on fungi of other mycorrhizal types, such as VA mycorrhizal fungi (e.g. Frey et al., 1992; Fujiyoshi et al., 2000). In these reports about ectomycorrhizal fungi (Table 1), ergosterol has been detected in all kinds of ectomycorrhizal fungi. The median (minimum-maximum) value was 4.10 (0.86-17.55) mg ergosterol g^{-1} fungal DW (Table 1). On the contrary, available data suggest that VA

Table 1. Ergosterol concentrations of the mycelium of various ectomycorrhizal fungi. Data are sorted according to the order of fungal species and average ergosterol concentration of mycorrhizal fungi.

Fungal genus / Species	Isolate	Ergosterol concentration (mg g^{-1} fungal dw)	Growth condition: Incuvation time	Growth condition: Temperature (°C)	Reference*
Amanita					
A. muscaria		3.11	4 months	-	1
		4.03	7 months	-	1
		4.68	40 days	25	2
A. rubescens		13.52	21 days	25	3
		17.55	21 days	25	3
Boletus					
B. griseus		10.65	21 days	15	3
		11.88	21 days	25	3
Cenococcum					
C. geophilum		1.54	40 days	25	2
	(CLU-011)	3.00	21 days	15	3
		3.28	21 days	25	3
	(CLU-029)	3.41	21 days	25	3
		3.49	21 days	25	3
Entoloma					
E. sericeum		5.59	21 days	25	3
		6.62	21 days	25	3
Hebeloma					
H. crustuliniforme		2.84	3 weeks	-	1
		3.04	4 months	-	1
		3.13	40 days	25	2
		3.36	2 weeks	-	1
		3.59	4 weeks	-	1
		6.43	21 days	25	3
		6.84	21 days	25	3
H. cylindrosporum	(D16)	3.00	6 weeks	-	4
Laccaria					
L. bicolor	(CRBF 569)	4.90	4 weeks	-	4
	(CRBF 581)	3.90	4 weeks	-	4
L. laccata		3.06	2 weeks	-	1
		3.07	3 weeks	-	1
		3.31	4 weeks	-	1
		6.55	40 days	25	2
	(Bruchet)	0.86	5 weeks	-	4
		1.04	5 weeks	-	4
		1.14	5 weeks	-	4
		15.10	6 weeks	-	4
	(CNRF)	2.00	6 weeks	-	4
	(S238)	6.50	6 weeks	-	4
Lactarius					
L. pubescens		4.96	21 days	25	3
		4.98	21 days	25	3
Paxillus					
P. involtus		6.28	40 days	25	2
		8.12	21 days	25	3
		9.22	21 days	15	3
Pisolithus					
P. tinctorius		2.24	40 days	25	2
	(441)	1.19	5 weeks	-	4
		1.58	5 weeks	-	4
		1.76	5 weeks	-	4
		8.40	4 weeks	-	4
Rhizopogon					
R. luteus		7.98	40 days	25	2
Rhizopogon	371	10.20	20 days	-	5
	378	10.40	20 days	-	5
	2272	10.50	20 days	-	5
Rhizopogon sp.		4.54	21 days	25	3
		5.08	21 days	5	3
		7.75	21 days	15	3
Scleroderma					
S. citrinum	(CLU-012)	2.93	21 days	25	3
		3.59	21 days	25	3
	(CLU-013)	6.93	21 days	15	3
		6.94	21 days	25	3
Suillus					
S. bovinus		2.84	3 weeks	-	1
		2.88	4 weeks	-	1
S. luteus		3.90	40 days	25	2
S. pungens		12.50	20 days	-	5
Thelophora					
T. terrestris		4.17	40 days	25	2
Maximum		17.55			
Minimum		0.86			
Average		5.46			
Median		4.10			

* 1) Salmanowicz and Nylund (1988), 2) Sung et al. (1995), 3) Antibus and Sinsabaugh (1993), 4) Martin et al. (1990), 5) Bidartondo et al. (2001).

mycorrhizal fungi contain a small quantity of ergosterol (at most 0.63 mg ergosterol g^{-1} fungal DW) (Frey et al., 1992; Weete and Gandhi, 1996; Gramdomougin-Ferjani et al., 1999; Fujiyoshi et al., 2000). Recently, some researchers questioned whether ergosterol is a constituent of the arbuscular mycorrhizal fungi (Olsson et al., 2003), even though several studies showed positive correlations between mycorrhizal colonization intensity of mycorrhizal fine roots and ergosterol content of mycorrhizal fine roots (Hart and Reader, 2002; Fujiyoshi et al., 2000). The ergosterol method for detecting arbuscular mycorrhizal fungi is now controversial (Olsson et al., 2003; Hert and Reader, 2003). For ectomycorrhizal fungi, however, there is no doubt that ergosterol is a constituent and thus ergosterol is considered to be a good chemical indicator for quantifying the active fungal mass (Nylund and Wallander, 1992).

Like other sterols, ergosterol is an important component of the fungal plasma membrane (Weete et al., 1974). The biochemistry and function of ergosterol are not mentioned in the present paper as they have already been reviewed by Weete and Gandhi (1996). Unlike chitin (another fungal specific biochemical indicator that constitutes the cell wall), ergosterol has been used as a 'metabolically active' fungal indicator to quantify the biomass of fungi growing in various habitats since its first use by Seitz et al. (1977). One of the PLFA (phospholipid fatty acid), linoleic acid (18:2 ω 6,9) have been also used as a 'metabolically active' fungal indicator to quantify the biomass of fungi in soil (e.g. Olsson, 1999; Olsson et al., 2003). However, plant also has linoleic acid as a major lipid compound (Hitchcock, 1975; Zells, 1997). Consequently, linoleic acid can be a good indicator for fungi when plant cells are not present, and this indicator is not suitable to quantify the fungi in ectomycorrhizal fine roots (plant-fungal interface). Ergosterol has been used widely to quantify the biomass of fungi in ectomycorrhizal fine roots since the study of Nylund and Wallander (1992), in which the procedure of ergosterol method was also mentioned in detail. The ergosterol content and the content of active fungi in ectomycorrhizal fine roots were considered to show a positive correlation (Nylund and Wallander, 1992). Some reports even found a correlation between the ergosterol content of ectomycorrhizal fine roots and ectomycorrhizal colonization intensity (Ekblad et al., 1995; Manninen et al., 1998). The suitability of this method has been affirmed by a large number of reports in which the ergosterol content of ectomycorrhizal fine roots was analyzed. So far, the ectomycorrhizal fine root ergosterol content was reported in dozens of papers on various ectomycorrhizal plant species.

The procedure of ergosterol analysis

A flowchart of the ergosterol analysis procedure is shown in Fig. 1. This method is based on the procedures used by Kasai and Horikoshi (1997) and Satomura et al. (2003). Root samples were washed, freeze-dried, weighed and put into a test tube (61 ml) with a Teflon-lined screw-cap. Twenty-five ml of methanol, 5 ml of ethanol, and 2 g KOH (pellets) were added. After the KOH dissolved, boiling chips were added and the mixture was saponified at about 75°C for 30 min. The capped test tube was cooled to room temperature with running water, and 10 ml purified water was added. The saponified samples were extracted once with 10 ml and two times with 5 ml portions of petroleum ether and vigorous shaking. The etheric phases were collected and combined and evaporated under a stream of nitrogen. The dried samples were redissolved in 2 ml of dichloromethane:methanol (1:1/v:v), sonicated for 1 min, filtered through a 0.2 μm pore-size polytetrafluoroethylene (PTFE) membrane filter (DISMIC-13HP, Toyo Roshi, Tokyo) and analyzed by reversed phase HPLC. The HPLC system (Shimadzu, Kyoto) consisted of a Shim-pack HRC-ODS column (250-mm long, 4.6-mm i.d.) with 5 μm particle size, a guard column (Shim-pack GHRC-ODS), a LC-10AD pump, and a SPD-10A uv-vis detector. Injection volume was usually 10 μl. Methanol was used as the mobile phase at a flow rate of 1.0 ml min^{-1}. The effluent was monitored at A_{282} at which ergosterol has a strong absorption. Under these conditions, ergosterol was detected at approximately 16 min after injection. The peak of ergosterol was identified by comparing its retention time with that of authentic ergosterol, purchased from Sigma Chemical (St. Louis) (Fig.2).

Ergosterol content translate into fungal content of fine root

The biomass of fungi in ectomycorrhizal fine roots (B_{mf}) (kg ha^{-1} ground area) and the fine root fungal content (FC) (% fungi per root DW) are considered to be a function of both the fine root ergosterol content (E_r) (μg ergosterol g^{-1} root DW) and the ergosterol concentration of fungi (E_{mf}) (mg ergosterol g^{-1} fungal DW). The latter value (E_{mf}), i.e. the amount of ergosterol per unit mass (weight) of fungi, has been used as a conversion factor in previous studies (Salmanowicz and Nylund, 1988; Antibus and Sinsabaugh, 1993; Ekblad et al., 1995). Biomass of fungi comprised in ectomycorrhizal fine roots (B_{mf}) is expressed with the following proportion:

$$B_r \times E_r : B_{mf} = E_{mf} \times 10^3 : 1 \quad \cdots \text{(Eq.1)}$$

where B_r (kg ha^{-1} ground area) is the measured ectomycorrhizal fine root biomass. The product ($B_r \times E_r$) gives the amount of ergosterol in the

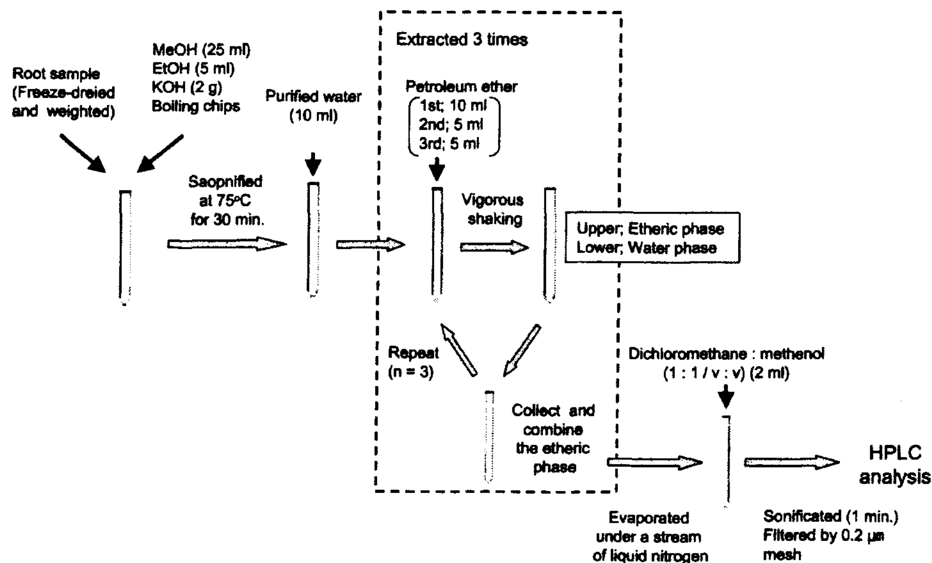

Fig. 1 Protocol of ergosterol analysis. MeOH, methanol; EtOH, ethanol; KOH, potassium hydroxide. The detailed procedure of the ergosterol analysis is described in the text

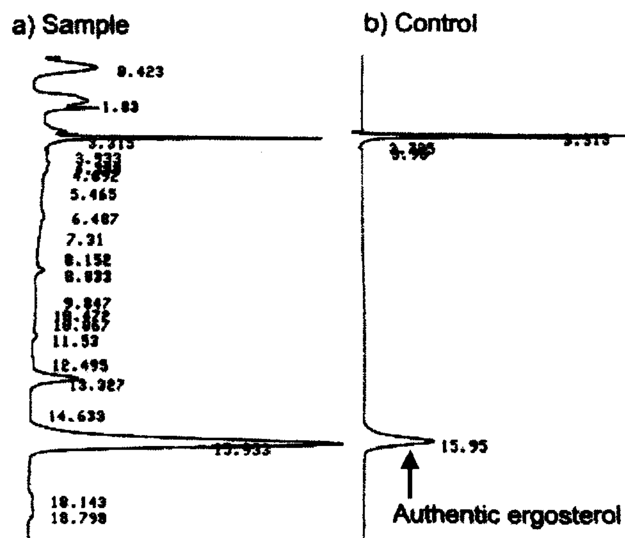

Fig. 2 HPLC profile of an an ectract from tree roots. Ergosterol in the ecxtract (a) is identified by comparison of their retention time on HPLC with authentic ergosterol (Sigma Chem., St. Louis) (b).

ectomycorrhizal fine roots in a unit ground area. Rewrite the equation as follows:

$$B_{mf} = B_r \times E_r \times E_{mf}^{-1} \times 10^{-3} \quad \cdots \text{(Eq.2)}$$

Based on these values, the fungal content of ectomycorrhizal fine roots (FC) (% fungi per root DW) is also calculated with the following equation:

$$FC = B_{mf} \times B_r^{-1} \times 10^2 \quad \cdots \text{(Eq.3)}$$

$$= (B_r \times E_r \times E_{mf}^{-1} \times 10^{-3}) \times B_r^{-1} \times 10^2 \quad \cdots \text{(Eq.4)}$$

$$= E_r \times E_{mf}^{-1} \times 10^{-1} \quad \cdots \text{(Eq.5)}$$

The biomass of the host tissues in ectomycorrhizal fine roots is obtained by subtracting the biomass of fungi comprised in ectomycorrhizal fine roots (B_{mf}) from the ectomycorrhizal fine root biomass (B_r).

Further considerations

The conversion factor (E_{mf}), the ergosterol concentration of mycorrhizal fungi, obviously plays an important role in the calculation (Eq.2-5). The ergosterol concentrations of the ectomycorrhizal fungi varied widely (Table 1), which implies that the estimated values of the fungal content in ectomycorrhizal fine roots and biomass of fungi in ectomycorrhizal fine roots would vary widely depending on the conversion factor that was used. For example, on the supposition that the ergosterol content of ectomycorrhizal fine root samples as 100 μg ergosterol g^{-1} root DW, 11.6%, 2.4% and 0.6% fungal contents of ectomycorrhizal fine roots were obtained from the minimum, median and maximum E_{mf} values (0.86, 4.10 and 17.55 mg ergosterol g^{-1} fungal DW, respectively), respectively. For ectomycorrhizal plants, the conversion factors used in the previous studies were 2.06 (Ekblad et al., 1995), 3.15 (Salmanowicz and Nylund, 1988), 4.10 (Satomura et al. 2003) and 5.7 mg ergosterol g^{-1} fungal DW (Antibus and Sinsabaugh, 1993) and 1.9 mg ergosterol g^{-1} fungal FW (Martin et al. 1990). These values are within a narrow range and are not so different from the median value (4.10 mg ergosterol g^{-1} fungal DW) and the average value (5.46 mg ergosterol g^{-1} fungal DW) of the literature data (Table 1).

The estimated values of the fungal biomass in ectomycorrhizal fine root and the fungal content in ectomycorrhizal fine roots are apparently altered by the conversion factor (E_{mf}). In Table 1, in order to recognize the conversion factor effect, the estimated values of the ectomycorrhizal fine root fungal content in the literature are given with each conversion factor value that was used in the original papers. The conversion factor, i.e. the value of ergosterol concentration of mycorrhizal fungi, is in inverse proportion to the biomass of fungi in ectomycorrhizal fine roots and the fungal content in ectomycorrhizal fine roots (Eq. 2-5). If we use higher value for the ergosterol concentration of ectomycorrhizal fungi than were used in the other reports, the biomass of fungi in ectomycorrhizal fine roots and the fungal content of ectomycorrhizal fine roots would be lower. If the fungal content of ectomycorrhizal fine roots is evaluated by the ergosterol analysis method, it is unknown whether we can apply a single value as a conversion factor for all types of forests without having data taken in various types of forests under various environmental conditions. Having a suitable conversion factor in each forest would help to give a more precise estimation of carbon dynamics, since the ergosterol concentrations of a single strain of fungi could be altered by growth conditions (cf. Antibus and Sisabaugh, 1993) and the ergosterol contents vary among fungal species (Salmanowicz and Nylund, 1988; Martin et al., 1990; Antibus and Sisabaugh, 1993; Sung et al., 1995; Bidartondo et al., 2001) (Table 1). Further information about the ergosterol concentrations of various kinds of ectomycorrhizal fungi and mechanisms that alter the fungal ergosterol concentration are needed to better understand the ecosystem carbon dynamics and quantitative evaluation of the fungal role in carbon dynamics.

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