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# The extraction and quantification of ergosterol from ectomycorrhizal fungi and roots

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**Abstract.** Recently, ergosterol analysis has been used to quantify viable fungal biomass in resynthesized ectomycorrhizae. An objective of our study was to quantify ergosterol in a range of ectomycorrhizal isolates under differing growth conditions. In addition, we tested the applicability of the method on field-collected roots of ectomycorrhizal and vesicular-arbuscular (VA) mycorrhizal plants. Quantification of sitosterol as a biomass indicator of plant roots was also undertaken. Ergosterol was not detected in roots of uninoculated *Betula populifolia* seedlings, and sitosterol was not detected in an ectomycorrhizal fungal isolate but was present in birch roots. Ergosterol was produced in all isolates examined, which represented the major orders of ectomycorrhizal fungi. The range of values obtained, from 3 to nearly 18 μg ergosterol $mg^{-1}$ dry mass, agrees well with reported values for other mycorrhizal and decomposer fungi. Hyphal ergosterol was the same during growth on phytic acid and $KH_2PO_4$. Reduction of growth temperature from 25° C to 15° C had little effect on ergosterol content of cultures harvested at similar growth stages. Ergosterol and sitosterol were detected in field-collected ectomycorrhizae of *B. populifolia* and *Pinus sylvestris* and VA mycorrhizae of *Acer rubrum* and *Plantago major*. Both ergosterol content and ergosterol to sitosterol ratios were significantly lower in VA mycorrhizae than ectomycorrhizae. Calculations of viable fungal biomass associated with field-collected roots were in agreement with those reported by others using the method on resynthesized ectomycorrhizae. Estimates of total mass could be obtained for field-collected *B. populifolia* roots by a simultaneously using ergosterol to estimate fungal biomass and sitosterol to estimate root mass. Some potential applications and limitations of sterol quantification in studies of mycorrhizal physiology and ecology are discussed.

**Key words:** Biomass – Ectomycorrhizae – Ergosterol – Sitosterol – Vesicular-arbuscular mycorrhizae

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## Introduction

Mycorrhizal fungi are found upon and within roots of the majority of vascular plant species (Harley 1989). Experiments with individual plants show that the presence of mycorrhizal fungi can increase phytobiont growth and survival by enhancing the capacity to acquire essential resources (Harley and Smith 1983). Improved resource acquisition, however, entails a carbon cost to the host proportional to utilization of photosynthetic carbon by the mycobiont (Paul and Kucey 1981). Complete estimates of mycorrhizal costs involve measuring the effect of colonization on root respiration and exudation, as well as accurate assessment of fungal biomass and turnover (Jakobsen and Rosendahl 1990). Measurement of mycorrhizal fungal biomass is also important from an ecosystem perspective. Mycorrhizae represent a poorly understood component of the net primary production in forest soils (Fogel 1985). Mycorrhizae, extramatrical hyphae and fruiting bodies serve as food for soil fauna (Ponge 1988) and small mammals (Maser et al. 1978), and contribute both to soil aggregate formation (Miller and Jastrow 1990) and soil enzyme production (Linkins and Antibus 1982). However, despite their importance in soil, estimation of mycorrhizal fungal biomass has received little attention to date.

Progress in the study of mycorrhizal biomass and fungal production has been hampered in part by methodological limitations. The intimate association between plant and fungus has limited attempts to physically separate and quantify biomass (Fogel 1980). Most workers have indirectly quantified fungal biomass using visual estimates of root colonization. Quantification of $^{14}C$ movement into the fungal component (Jakobsen and Rosendahl 1990) or measurement of fungus-specific chemical components such as chitin (Hepper 1977; Plassard et al. 1983) represent advances in our ability to quantify root-associated fungal biomass. However, most current methods do not allow separation of carbon invested in living versus nonliving mycelium.

Recently, the technique of ergosterol analysis has gained favor in ecological studies concerned with quantification of viable fungal biomass (Osswald et al. 1986; Newell et al. 1987; West and Grant 1987). Ergosterol is the dominant sterol in the membranes of higher fungi (Weete 1980), and is not found in significant quantities in bacteria or higher plants. Unlike chitin, ergosterol is rapidly degraded upon cell death and hence is a potential marker of viable biomass. Several workers have measured the ergosterol contents of ectomycorrhizal (ECM) fungi grown in pure culture, and these values have subsequently been applied to measure viable biomass associated with resynthesized ECM (Salmanowicz and Nylund 1988; Johnson and McGill 1990a, b; Martin et al. 1990). The method appears to have great potential but has not been widely tested. The present study was undertaken to further test sterol analysis with cultures of ECM fungi and field-collected mycorrhizae. Our specific objectives for the pure culture studies were: 1) to determine ergosterol content in a range of isolates representing the major orders of ECM-forming species, and 2) to examine whether culture conditions affected the ergosterol contents of common ECM fungi. The objectives of field studies were: 1) to use ergosterol analysis to estimate the potential contribution of ECM fungi to root biomass, and 2) to determine whether ergosterol could be detected in vesicular-arbuscular mycorrhizal (VAM) plants. Finally, we developed a method of dual sterol analysis using ergosterol to measure fungal biomass and sitosterol to simultaneously measure root biomass. Sitosterol was selected because this is an important sterol in higher plants (Bergmann 1953). This method can be applied as a way of testing the validity of viable fungal biomass estimates in mycorrhizal studies.

## Materials and methods

### *Fungal isolates and culture conditions*

The isolates used in this study, their putative host plants and dates of isolation are listed in Table 1. With the exception of *Cenococcum geophilum* Fr. (CLU 011 and 029), all isolates were obtained from sporophore tissue.

Stocks of isolates were maintained on solid MMN medium (Molina and Palmer 1982) at 25°C. Starters were established by transferring four 5-mm diameter plugs cut from the mycelial margin into 250-ml Erlenmeyer flasks containing 50 ml liquid MMN. These were grown for 14 days at 25°C. Mats of starter mycelium were homogenized in a sterile Eberbach semimicro-homogenizer and centrifuged for 10 min on a tabletop centrifuge. The mycelial pellets were rinsed in sterile water, recentrifuged and resuspended in 50 ml of sterile water. Flasks containing 50 ml of the appropriate growth medium were inoculated with 1 ml of suspension dispensed with 5-ml wide-opening pipettes. The growth medium used in all experiments contained: 0.5 g $NH_4Cl$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.28 g KCl, 0.05 g $CaCl_2$, 0.01 g $FeCl_3$, 5.0 μg biotin, 1.0 mg thiamine HCl, 0.172 g citric acid, 0.929 g sodium citrate and 10 g glucose per 1000 ml of distilled $H_2O$. In routine experiments, phosphorus (50 μM P) was supplied as $KH_2PO_4$, the media was adjusted to pH 5.0 and isolates were grown in complete darkness for 21–22 days at 25°C.

To determine whether P source affects ergosterol content, a subset of six ECM isolates was grown with P added at a final concentration of 50 μM as either $KH_2PO_4$ (Pi medium) or phytic acid (Po medium). A subset of five isolates was grown at 5, 15 and 25°C to examine the effects of growth temperature on ergosterol content. Mycelia in temperature studies were harvested when growth appeared (by visual assessments) to match that obtained after 21 days at 25°C. This was done to assure that mycelia at different temperatures were at similar developmental stages when harvested.

At the appropriate harvest dates, the contents of five to ten replicate flasks per fungus were transferred to separate screw-top centrifuge tubes. Mycelia were pelleted at $2000 \times g$ for 10 min, and the medium was decanted and allowed to drain from pellets by inverting the tubes for 2 min. Pellets were divided in half and weighed. One portion of the pellet, dried at 105°C overnight, was used for fresh weight to dry weight conversions. The remaining mycelium was placed in 15 ml of HPLC-grade methanol in glass scintillation vials and stored in the dark at 4°C. To determine whether lyophilization would affect ergosterol content, we harvested *Suillus tomentosus* (Kauff.) Sing., Snell & Dick as described above, except that one portion was frozen at −20°C, lyophilized, weighed and stored dry at −20°C prior to extraction.

### *Field sampling*

Fruiting bodies of *Paxillus involutus* (Fr.) Fr. and *Lactarius pubescens* Smith & Hesler were collected beneath *Betula papyifera* Marsh. on the Clarkson University campus, a site from which cultures of these fungi had been previously obtained. Slices of cap and stipe tissue were taken from each of three fruiting bodies of both species. Approximately 500 mg fresh wt. of tissue from each

**Table 1.** Ectomycorrhizal isolates used in ergosterol analyses

| Species | Isolate | Associated host | County | State | Isolation date |
|---|---|---|---|---|---|
| *Amanita rubescens* | CLU-018 | *Pinus sylvestris* | St. Lawrence | N.Y. | 1986 |
| *Boletus griseus* | CLU-023 | *Fagus grandifolia* | St. Lawrence | N.Y. | 1986 |
| *Cenococcum geophilum* | CLU-011 | *Pinus resinosa* | Worcester | Mass. | 1988 |
| | CLU-029 | *Salix* sp. | Yukon/Kuskokwim | Alaska | 1979 |
| *Entoloma sericeum* | VT-1003 | *Salix rotundifolia* | Barrow/Kobuk | Alaska | 1977 |
| *Hebeloma crustuliniforme* | CLU-026 | *Pseudotsuga menziesii* | Missoula | Mont. | 1982 |
| *Lactarius pubescens* | CLU-052 | *Betula papyifera* | St. Lawrence | N.Y. | 1987 |
| *Paxillus involutus* | CLU-050 | *Betula papyifera* | St. Lawrence | N.Y. | 1987 |
| *Rhizopogon* sp. | CLU-042 | *Pseudotsuga menziesii* | Missoula | Mont. | 1983 |
| *Scleroderma citrinum* | CLU-012 | *Tsuga canadensis* | St. Lawrence | N.Y. | 1986 |
| | CLU-013 | *Fagus grandifolia* | St. Lawrence | N.Y. | 1987 |
| *Suillus tomentosus* | CLU-077 | *Pinus banksiana* | Clinton | N.Y. | 1990 |

fruitbody was used to determine fresh weight to dry weight conversions, whereas a second set was fixed in methanol for sterol extraction.

Mycorrhizal roots of *B. populifolia* Marsh., *Acer rubrum* L. and *Pinus sylvestris* L. were collected in October 1989 and June 1990 from local native and plantation forests, respectively. These dates represent late and early growing season for our area. All roots were excavated by hand, placed in plastic bags with damp leaf mulch and stored in an insulated cooler until processed. Roots were washed thoroughly over a fine screen and placed in 0.5 mM $CaCl_2$. Roots were subjected to further cleaning with fine forceps under a stereomicroscope. ECM short roots judged to be viable (Harvey et al. 1976) were excised from suberized long roots and placed in vials by morphotype. Segments of suberized *P. sylvestris* roots from which ECM tips had been removed were also saved for analysis. Roots were blotted dry, weighed and stored in HPLC-grade methanol at 4°C until analyses could be performed. Parallel sets of samples were weighed, dried at 105°C overnight and reweighed to obtain fresh weight to dry weight conversions.

To see whether we could detect ergosterol in a putative VAM herbaceous plant, *Plantago major* L. was collected from a fertilized garden on two dates in June 1990. At each date, three plants were excavated with roots and soil intact from each of three areas within the site. Roots were washed under running water and lateral roots were excised at the root crown. The lateral roots from three adjacent plants were pooled into samples, with three samples per date. For both *A. rubrum* and *P. major*, the roots in each sample were allocated to two groups, blotted dry, and weighed. One portion of roots was fixed in formalin–acetic acid–alcohol (FAA), the other was transferred to HPLC-grade methanol and stored at 4°C for sterol analysis. Root segments fixed in FAA were subsequently cleared in KOH and stained with chlorazol black E to verify the presence of VAM structures (Brundrett et al. 1984).

Seed of *B. populifolia* were surface-sterilized and germinated on sterile moist filter paper to provide nonmycorrhizal roots for sitosterol analysis. Seedlings were transplanted into pots containing 400 ml of autoclaved, sieved forest soil and grown under artificial light (330–380 $\mu E\ cm^{-2} \cdot s^{-1}$) for 2 months. The nonmycorrhizal status of seedlings was confirmed at harvest by visual inspection and chlorazol black E staining.

### *Sterol extraction and quantification*

A review of methods used in the isolation of sterols has been provided by Heupel (1989); the procedure used to extract and quantify ergosterol was based on that used for leaf decay fungi by Newell et al. (1988). These works should be consulted for details concerning extraction and storage of samples. To test the extraction efficiency for ECM fungi, we added internal ergosterol standards (0, 10, 20, 30, 50 $\mu g\ ml^{-1}$) to samples of known fresh wt. (500–515 mg) of *Entoloma sericeum* (Bull. ex Marat) Quelet. prior to extraction.

Samples to be analyzed were brought to a volume of 50 ml in methanol and transferred to 250-ml extraction flasks. These were refluxed for 2 h in methanol and filtered to remove undigested materials. Dry weights were obtained on roots at this point. The filtrate was saponified with 5 ml of 4% KOH in ethanol and refluxed for an additional 30 min. After cooling, the extracts were transferred to 125-ml separatory funnels containing 10 ml of water. Each sample was extracted three times with pentane (10, 5, and 5 ml). Pentane extracts were combined, evaporated in a hood and redissolved in 1.0 ml of HPLC-grade methanol prior to quantification.

Sterol concentrations were quantified using a reverse-phase HPLC system configured as follows. Solvent: methanol; flow rate: 2 ml $min^{-1}$; sample size: 100 µl; column: Altex Ultrasphere ODS, 0.46 × 15 cm; absorbance detector set at 282 nm for ergosterol with a range of 0–0.500; quantification: two-channel Waters 745B integrator with attenuation at 16; ergosterol retention time: 4.1 min; replication: three injections per sample; standard: 25 µg ergosterol. All ECM isolate data were collected on a minimum of five replicate flasks per fungus and treatment.

Roots subjected to sitosterol analysis were harvested, cleaned, stored and extracted by the procedures detailed above. Sitosterol separations were carried out using the same column, solvent, injection and running conditions described above. Under the described conditions, sitosterol demonstrated absorbance peaks at 202 nm, but absorbance was measured at 205 nm to reduce background. Retention times for sitosterol were 6.2–6.25 min. Routine calibration runs used commercially available sitosterol (Sigma, St. Louis, Mo.).

## Results

After 21 days growth, ergosterol ranged from 0.02 µg $mg^{-1}$ in *Rhizopogon* to 0.84 µg $mg^{-1}$ in *Amanita rubescens* (Pers. ex Fr.) S.F.G. on a fresh weight basis (Table 2). On a dry weight basis, values ranged from 3.06 µg $mg^{-1}$ in *S. tomentosus* to 17.55 µg $mg^{-1}$ in *A. rubescens*. The mean for ECM isolates grown on Pi medium at 25°C for 21 days was 6.64 ± 4.3 µg $mg^{-1}$ dry

**Table 2.** Ergosterol contents of selected ectomycorrhizal fungi when grown in axenic culture for 21 days on inorganic P (Pi) or phytic acid (Po) at 25°C. The data shown are means ± standard deviations ($n = 10$)

| Fungus | P source | Ergosterol | |
|---|---|---|---|
| | | (µg $mg^{-1}$ fresh wt.) | (µg $mg^{-1}$ dry wt.) |
| Basidiomycotina | | | |
| *Amanita rubescens* | Pi | 0.84 ± 0.19 | 17.55 ± 3.73 |
| | Po | 0.64 ± 0.10 | 13.52 ± 0.43 |
| *Entoloma sericeum* | Pi | 0.12 ± 0.02 | 6.62 ± 0.56 |
| | Po | 0.10 ± 0.03 | 5.59 ± 1.41 |
| *Hebeloma crustuliniforme* | Pi | 0.40 ± 0.06 | 6.43 ± 0.58 |
| | Po | 0.34 ± 0.04 | 6.84 ± 0.36 |
| *Lactarius pubescens* | Pi | 0.16 ± 0.01 | 4.98 ± 0.15 |
| | Po | 0.23 ± 0.03 | 4.96 ± 0.23 |
| *Scleroderma citrinum* | Pi | 0.10 ± 0.04 | 2.93 ± 0.99 |
| (CLU-012) | Po | 0.11 ± 0.01 | 3.59 ± 0.26 |
| Deuteromycotina | | | |
| *Cenococcum geophilum* | Pi | 0.06 ± 0.01 | 3.41 ± 0.19 |
| (CLU-029) | Po | 0.05 ± 0.01 | 3.49 ± 0.20 |

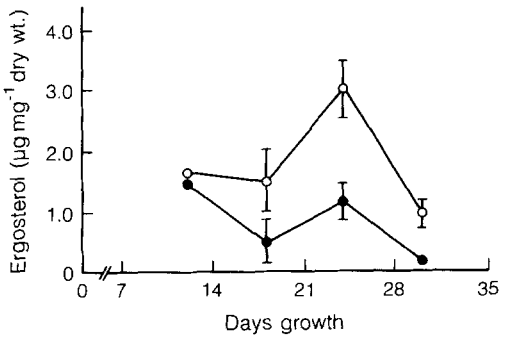


**Fig. 1.** The effects of culture age on ergosterol content of *Suillus tomentosus* mycelia extracted directly at harvest (○) or following freeze-drying (●). Values shown are means; *vertical bars* represent standard deviations ($n=5$)

**Table 3.** Ergosterol contents of selected ectomycorrhizal fungi when grown in axenic culture at different temperatures. The data shown are means ± standard deviations ($n=10$)

| Fungus | Temperature (°C) | Ergosterol (μg mg$^{-1}$ dry wt.) |
|---|---|---|
| Basidiomycotina | | |
| *Boletus griseus* | 15 | 10.65±1.40 |
| | 25 | 11.88±0.79 |
| *Paxillus involutus* | 15 | 9.22±1.48 |
| | 25 | 8.12±0.30 |
| *Rhizopogon* sp. | 5 | 5.08±0.10 |
| | 15 | 7.75±0.44 |
| | 25 | 4.54±0.74 |
| *Scleroderma citrinum* (CLU-013) | 15 | 6.93±0.21 |
| | 25 | 6.94±0.29 |
| Deuteromycotina | | |
| *Cenococcum geophilum* (CLU-011) | 15 | 3.00±0.14 |
| | 25 | 3.28±0.16 |

wt., and the median ergosterol content was 5.70 μg mg$^{-1}$ dry wt. Interspecies patterns in ergosterol content were similar whether expressed on a fresh or dry weight basis.

The correlation coefficients ($r$) between observed and expected ergosterol contents of *E. sericeum* mycelium spiked with internal standards were 0.98 and 0.97 on a fresh and dry weight basis, respectively. These results showed that the method gave excellent recovery of ergosterol across a range of concentrations.

In *S. tomentosus*, ergosterol values for lyophilized mycelium ranged from 88 to 18% of those for fresh mycelium; the fractional loss associated with lyophilization increased with mycelial age (Fig. 1). Decreased recoveries observed with older cultures may relate to difficulties associated with quickly freezing larger masses of mycelium with the method we used.

P source (Pi or Po) did not have a consistent or significant effect on the ergosterol contents of any of the fungi examined (Table 2). Only *Rhizopogon* sp. displayed sufficient growth to permit ergosterol analyses at all three temperatures utilized. For *Rhizopogon*, ergosterol content was highest at 15°C (Table 3), whereas in the other isolates examined, growth at 15°C had little effect compared to 25°C or caused a slight increase in ergosterol content on a dry weight basis.

Ergosterol was detected in fruitbodies and spore prints of two ECM basidiomycetes sampled. Ergosterol contents of 2.41±0.58 and 2.44±0.29 μg mg$^{-1}$ dry wt. were measured with stipe and cap tissue of *L. pubescens*, whereas stipe and cap tissue of *P. involutus* contained 3.55±0.87 and 2.11±0.13 μg mg$^{-1}$ dry wt., respectively. These values are lower than those obtained for pure cultures of these species (Tables 2 and 3).

Ergosterol was present at readily detectable levels in naturally occurring short lateral ECM roots of *B. populifolia* and *P. sylvestris* (Tables 4 and 5). Ergosterol was not detected in roots of *B. populifolia* seedlings raised in autoclaved soil (Table 5). Ergosterol invariably exceeded values of 1 μg mg$^{-1}$ dry wt. in field-collected short lateral ECM roots. Careful selection of viable roots and removal of woody root material allowed us to minimize variability among replicate samples of individual morphotypes. Coefficients of variation were 5–7%. Ergosterol contents varied by a factor of two among the morphotypes examined. We used the median ergosterol content (5.70 μg mg$^{-1}$ dry wt.) obtained from isolates examined in this study to estimate the proportional contribution of viable fungal tissue to short lateral root mass (Table 4). Values ranged from nearly 18% in a smooth birch morphotype to nearly 37% in a tomentose birch morphotype.

Estimates of percent viable fungal biomass for field-collected *P. sylvestris* roots using our range of conversion factors demonstrate how the factor employed and the scale at which the root system is examined affects the apparent proportional contribution of fungi to root

**Table 4.** Ergosterol contents and fungal biomass estimates for field-collected roots. All samples collected during October 1989, except *Plantago major*

| Plant | Mycorrhizal type | | Ergosterol (μg mg$^{-1}$ dry wt.)[a] | Fungal mass (%)[b] |
|---|---|---|---|---|
| *Betula populifolia* | Smooth-orange | ECM | 1.95±0.10 | 34.2 |
| | Smooth-white | ECM | 1.02±0.05 | 17.9 |
| | Smooth | ECM | 1.34±0.09 | 23.5 |
| | Tomentose-white | ECM | 2.08±0.12 | 36.5 |
| *Pinus sylvestris* | Smooth-brown | ECM | 1.16±0.02 | 20.4 |
| *Acer rubrum* | Beaded | VAM | 0.18±0.05 | 3.2 |
| *Plantago major* (harvest 1 – June 1990) | | VAM | 0.02±0.01 | 0.4 |

[a] Means ± standard deviations
[b] Fungal biomass calculated assuming a median value for ergosterol of 5.70 mg$^{-1}$ dry wt.

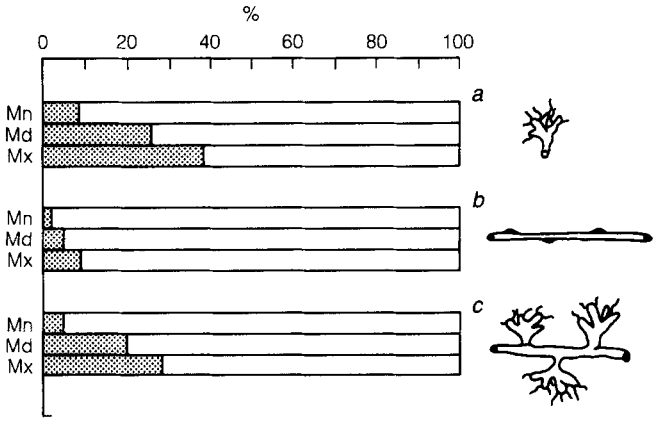


**Fig. 2.** Estimates of fungal biomass (▨) as a percentage of total root biomass. Values based on ergosterol data for field-collected *Pinus sylvestris* roots. *a* White-fuzzy ectomycorrhizae; *b* suberized long roots, and *c* combined long root and white-fuzzy ectomycorrhizal segments. Minimum (*Mn*), maximum (*Mx*) and median (*Md*) biomass estimates were calculated using the maximum (17.6), minimum (3.1), and median (5.7 μg $mg^{-1}$ dry wt.) fungal ergosterol contents measured in this study. Roots are depicted at the right

mass (Fig. 2). Depending on the conversion factor used, viable fungal tissue might constitute from 8.5% to nearly 48% of the mass of this ECM morphotype (Fig. 2a). Fungal hyphae were observed on the surface of and between cortical cells of suberized long roots of *P. sylvestris*. Long roots from which ECM fungi had been removed did contain ergosterol. Use of our ergosterol conversion factors suggested that viable mycelium would not exceed 10% of the mass of these long roots (Fig. 2b). Likewise, living fungi comprised from 5% to 28% of the total mass of "typical" long root segments with their attached clusters of one ECM morphotype (Fig. 2c).

Quantifiable levels of ergosterol were extracted (Tables 4, 5) from beaded, short lateral roots of *A. rubrum* and roots of *P. major*, both of which bore VAM fungal structures. The ergosterol contents of *A. rubrum* roots were approximately an order of magnitude lower than those of ECM short roots, whereas those of *P. major* were two orders of magnitude lower at both harvests. The observation that *P. major* roots contained less ergosterol than *A. rubrum* is largely a reflection of differences in root morphology. *A. rubrum* samples consisted of highly branched, heavily VAM-colonized fine roots, whereas *P. major* samples were a mix of larger, uncolonized storage roots and heavily colonized, fine roots.

Extracts of *E. sericeum* displayed minimal absorbance (205 nm) at a retention time of the commercial sitosterol standard (Table 5). Sitosterol, if present in this ECM fungus, is a very minor component and would not interfere with our ability to determine root biomass using this sterol as a specific biochemical marker. Likewise, ergosterol was not detected in extracts of *B. populifolia* roots raised in autoclaved soil and, therefore, would not interfere with estimates of viable fungal biomass. On a dry weight basis, sitosterol contents of plant roots forming VAM associations were low in comparison to those of ECM plants (Table 5). However, the ergosterol to sitosterol ratios support results of ergosterol measures on a root mass basis in that ergosterol:sitosterol values are lower for VAM roots (0.03–0.11) than ECM roots (0.44–0.54). Thus viable fungal biomass, assuming similar conversion factors for ECM and VAM fungi, contributes a greater proportion of root mass in ECM roots than VAM roots of *A. rubrum* or *P. major*.

## Discussion

Based on retention times of HPLC eluates, all ECM isolates examined in our study contained readily detectable quantities of ergosterol. The fungi examined represent most major orders of Basidiomycetes involved in ECM formation (Miller and Watling 1987), including species of the Agaricales, Tricholomatales, Pluteales, Russulales, Boletales, Hymenogastrales and Sclerodermatales. In addition, data were collected for the imperfect fungus *Cenococcum geophilum*. Given that the fungi examined represented a range of habitats and hosts and were taxonomically diverse, we felt the range of ergosterol contents observed was surprisingly narrow. Our data are consistent with those from other studies where ECM fungi were grown on liquid media (Salmanowicz and Nylund 1988; Martin et al. 1990) and gave a range of mycelial ergosterol contents

**Table 5.** Ergosterol and sitosterol contents for roots collected in the field during June 1990. ND, No ergosterol detected

| Sample | Mycorrhizal type | Ergosterol (μg $mg^{-1}$ dry wt.) | Sitosterol (μg $mg^{-1}$ dry wt.) | Ergosterol / Sitosterol |
|---|---|---|---|---|
| *Betula populifolia* | Smooth ECM fungus | 1.27 | 2.84 | 0.45 |
| | Smooth-white ECM fungus | 1.10 | 2.48 | 0.44 |
| | Nonmycorrhizal roots | ND | 3.73 | – |
| *Pinus sylvestris* | Smooth-brown ECM fungus | 1.17 | 2.18 | 0.54 |
| | Yellow tomentose | 1.17 | 2.49 | 0.47 |
| *Acer rubrum* | VAM fungus | 0.13 | 1.20 | 0.11 |
| *Plantago major* (harvest 2) | VAM fungus | 0.01 | 0.33 | 0.03 |
| *Entoloma sericeum* | ECM fungus | 7.23 | 0.06 | 120.5 |

of 2–15 μg $mg^{-1}$ dry wt. Combining data from these studies with our results produces mean and median ergosterol contents of 5.89 (±4.07) and 4.54 μg $mg^{-1}$ dry wt., respectively, for 23 ECM isolates studied to date. The values for ECM fungi are in close agreement with those presented for a diverse group of decomposer species by Newell et al. (1987).

The two slowest growing isolates used in our study, *A. rubescens* and *Boletus griseus* Frost in Peck, had the highest ergosterol contents. The rapid growth displayed by *S. tomentosus* was associated with low ergosterol contents. Relationships between growth rate and ergosterol content were shown for chemostat-grown yeast cells (Arnezeder and Hampel 1990). These authors found ergosterol content doubled as growth rate declined under nitrogen limitation in *Saccharomyces cerevisiae*. Growth rate and growth stage influence the ratio of active cytoplasm to cell wall material, and hence ergosterol dry weight relationships. This is supported by reports that ergosterol contents decreased from the growing margins to the center portions of ECM fungal colonies (Martin et al. 1990). Our observation that fruitbody tissues of *L. pubescens* and *P. involutus* have lower ergosterol contents than pure cultures are consistent with this suggestion. Likewise, we found, and others reported (Newell et al. 1987), that ergosterol content varied with time in cultures of fungi. However, as a measure of fungal biomass, ergosterol content varied far less with mycelial age than did chitin (Newell et al. 1987). Interactions between growth rate, mycelial age and ergosterol content warrant further study, especially in light of the profound effect that ECM formation elicits on fungal growth and morphology (Harley and Smith 1983).

Although medium composition can influence fungal ergosterol content (Newell et al. 1987), we did not observe any differences between ECM fungi grown on a defined medium with an inorganic versus an organic P source, and growth rates were very similar on the two P sources used (Antibus, unpublished work).

Temperature elicits profound effects on the lipid composition of fungi grown in culture (Weete 1980). Dexter and Cooke (1984) found that ergosterol content in *Mucor* species increased with increasing growth temperature. We knew that temperature would affect isolate growth rate, and tried to minimize the effect of this confounding factor by harvesting fungi from temperature treatments at similar developmental stages. Under these conditions, only *Rhizopogon* demonstrated a substantial change in ergosterol content in response to a 10° C shift in growth temperature. We recommend that experimenters wishing to derive ergosterol conversion factors to calculate ECM biomass in controlled inoculations grow cultures of their fungi at temperatures approximating those anticipated in the rooting medium.

A useful feature of ergosterol analysis in fungal biomass determinations is that large numbers of samples can be stored in methanol for subsequent analysis. However, there may be times when protocol necessitates drying or freeze-drying samples prior to extraction. Newell et al. (1988), in an examination of colonized *Spartina alterniflora* Loisel. leaves, found lyophilization to be superior to oven-drying but to decrease extractable ergosterol by nearly 20%. Results obtained with *S. tomentosus* support these findings. The extent to which modified freezing methods or extraction procedures can minimize such losses warrants further study.

One potential use for ergosterol measurement is estimation of viable root-associated fungal biomass. We found that *B. populifolia* roots grown in sterile soils lacked ergosterol, and Salmanowicz and Nylund (1988) reported similar findings for *P. sylvestris*. We have detected ergosterol in healthy field-collected ECM roots of both species. In these species, small quantities of short roots could be assayed (1–5 mg dry wt.) as they contained high amounts (>1 μg $mg^{-1}$ dry wt.) of ergosterol. Although we are unaware of any published ergosterol estimates of field-collected ECM, our values are in close agreement with data for ECM resynthesized under controlled conditions with known fungi (Salmanowicz and Nylund 1988; Johnson and McGill 1990a).

A difficulty encountered in ecological studies of ECM fungal biomass and production arises in determining what fraction of individual ECM root tips are attributable to fungal structures (Fogel 1980; Vogt et al. 1982). Chitin estimates have been successfully applied to quantify ECM fungal biomass on seedling root systems (Plassard et al. 1983; Rousseau and Reid 1991), but have not been applied to individual ECM tips. Most ecosystem studies rely on Harley's determination that the sheath of beech ECM accounted for nearly 40% of the short root dry weight. This figure was obtained by dissecting and weighing the mantle, and does not account for the Hartig net (Harley 1971). Salmanowicz and Nylund (1988) used an ergosterol value of 3.15 mg $g^{-1}$ for cultures of *Laccaria laccata* (Scop.: Fr.) Berk. & Broome and estimated that this fungus made up 25.5% of the biomass of resynthesized ECM root tips. When we applied our median ergosterol value (5.7 μg $mg^{-1}$ dry wt.), viable fungal biomass accounted for 18 to 37% of ECM mass in field-collected roots. Such variability as observed with *B. populifolia* is likely related to any or all of the following factors: fungal ergosterol content, mantle thickness, extent of external mycelium, and ECM fungal viability.

The selection of a conversion factor in biomass estimates is important, as is clearly illustrated by our *P. sylvestris* data. We felt that application of our median value was sound for the following reasons. First, the median ergosterol content of our isolates is similar to the mean value obtained for a wider range of ECM species grown in liquid culture. Our second justification is the result of dual sterol analyses with *B. populifolia*. We used our sitosterol conversion factor to estimate root mass and summed this with our previously determined estimate of fungal biomass. For the smooth ECM and smooth-white ECM fungi, the numbers summed to 98 and 86% of the total root biomass, respectively.

In the case of the smooth-white ECM fungi, one or both of the conversion factors could be in error, which would explain the 14% underestimate of root biomass. We could apply a lower fungal conversion factor (a value $<5.7$ $\mu g\ mg^{-1}$ dry wt.), which would increase the fungal contribution and bring the short root biomass total to 100%. However, as we do not know how ECM formation influences the sterol content of either root or fungus, an a priori rationale for doing this does not exist. Some evidence suggests that fungal growth on solid substrates influences hyphal ergosterol concentrations (Newell et al. 1987). Martin et al. (1990) detected less ergosterol in *L. laccata* and *Pisolithus tinctorius* (Pers.) Coker & Couch grown on solid compared to liquid media. The reported conversion factors, near 1 $\mu g\ mg^{-1}$ dry wt., for isolates grown on agar would not appear applicable to resynthesized or field-collected ECM fungi. As ergosterol contents of ECM fungi often exceed 1 $\mu g\ mg^{-1}$ dry wt. of root, use of these conversions would imply that fungi comprise the entire mass of ectomycorrhizae and is, therefore, unreasonable.

Our work with *A. rubrum* and *P. major* shows that ergosterol can be detected in roots colonized by VAM fungi. Ergosterol is lacking or present at extremely low levels in nonmycorrhizal *P. major* roots; we have not determined whether ergosterol is present in nonmycorrhizal roots of *A. rubrum*. By assuming similar conversion factors for ECM and VAM fungi, the viable fungal biomass was lower in VAM compared to ECM associations. These results support the widely held view (Brundrett 1991) that the standing crop of fungal hyphae is less in VAM than ECM associations; however, the assumption of similar conversion factors awaits verification on isolated VAM mycelium.

Our use of this conversion factor is realistic for several reasons. Zygomycetous fungi produce ergosterol at levels similar to those observed for ECM isolates (Dexter and Cooke 1984). Total sterol esters occur at concentrations similar to ECM mycelium in spores of *Gigaspora* and *Glomus* (Jabaji-Hare 1988). Finally, Frey et al. (1992) reported that ergosterol is the dominant fungal sterol in *Glomus intraradices*-infected *Zea mays* and *Trifolium alexandrinum* roots. The levels they detected in heavily colonized roots are similar to those we obtained.

Our findings and the work of others suggest that fungal and plant sterol analyses methods have numerous potential applications in mycorrhizal ecology and physiology. The ergosterol method gave efficient recovery and was simple to perform. Martin et al. (1990) indicated that it is possible to further simplify the process by eliminating the saponification step. The method is amenable to processing a wide range of sample sizes. The extreme sensitivity with which ergosterol can be measured should permit accurate detection of "immature" mycorrhizae not easily apparent by visual inspection (Frankland and Harrison 1985). In studies of nutrient uptake or enzyme activities, where differences among ECM morphotypes are related to fungal surface area, ergosterol could prove a more meaningful measure than dry weight. Finally, it may be possible to follow incorporation of radiolabelled carbon into ergosterol and sitosterol allowing simultaneous estimates of the production of viable root and fungal biomass. Recently, Newell and Fallon (1991) have successfully followed labelled acetate incorporation into ergosterol to measure production rates of decomposer fungi on leaves.

Clearly more research is needed before the full potential of the methods described can be realized. Presently, the method is most efficiently applied in pot studies, where known symbionts and hosts with known sterol compositions are being studied. Selection of an appropriate conversion factor will remain problematic in field studies. However, the situation is no different with other biomass measures currently in use. Ergosterol has an advantage over chitin in that it measures viable biomass, and at some future time may permit mycobiont identification.

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