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Determinination of soil fungal biomass from soil ergosterol analyses

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Abstract

Determination of fungal biomass (FB) and FB-carbon (FB-C) from soil ergosterol concentration is difficult because of unknown ergosterol-to-fungal biomass (E-to-FB) conversion factors and inefficient ergosterol extraction methods. We applied a microwave-assisted extraction (MAE) and high performance liquid chromatographic (HPLC) procedure to measure ergosterol in soil samples. The E-to-FB conversion factors were determined in six species of fungi grown in vitro. The MAE method was fast and extracted up to nine times more soil ergosterol than a classical refluxing saponification method. Soil ergosterol was separated and quantified rapidly (< 10 min) by HPLC. *Alternaria alternata*, *Chaetomium globosum*, *Fusarium oxysporum*, *Penicillum chrysogenum*, *Rhizopus stolonifer* and *Trichoderma harzianum* isolated from soil and plant matrices were grown in batch. Ergosterol and biomass content were determined in mycelia harvested during the stationary and exponential phases of growth. Total mycelial ergosterol ranged from 180 to 2178 μg, and total dry biomass ranged from 17 to 595 mg. Total ergosterol and fungal dry biomass were strongly associated ($r^2 = 0.95$). The C content in mycelial mats averaged 43% (±1.1, SD), and was similar among fungal species and growth phases. The analyses of variance showed that the E-to-FB ratio was similar among fungal species or growth phase. An average ergosterol concentration of 4 μg mg^{-1} dry biomass was determined for the six species of fungi, which gave a conversion factor of 250 μg dry biomass μg^{-1} ergosterol. The MAE method recovered an average of 62% (±11%, SD) of the ergosterol added in mycelial mats to soils prior to extraction, and its recovery was independent of soil properties. The E-to-FB ratio and percent recovery of mycelial ergosterol helped establish for the first time relationships determining soil FB and FB-C from soil ergosterol concentration. The amount of FB ranged from 155 to 4745 μg g^{-1} and that for FB-C ranged from 67 to 2040 μg g^{-1} for different soils, and was higher in samples taken from native undisturbed land than in samples taken from adjacent cultivated fields. Measurement of soil ergosterol concentration is a useful estimate content of the living soil FB.

Keywords: Microwaving; HPLC; Soil; Fungal biomass; Ergosterol; Soil ergosterol-to-fungal biomass

1. Introduction

Common methods used to extract soil ergosterol are slow, use large sample sizes (g), reagent volumes, and include steps of refluxing, saponification or sonication (Grant and West, 1986; Stahl et al., 1995; Ruzicka et al., 1995). Young (1995) developed an efficient microwave-assisted method (MAE) to extract ergosterol from a variety of matrices. The MAE method which

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uses small sample size (mg) and reagent volumes (ml), is about six-times faster than the routine methods (Seitz et al., 1977; Scheu and Parkinson, 1994), but has not been thoroughly tested in soil heterogeneous matrices. The MAE method has not been applied to soil samples. An efficient extraction method for extracting and quantifying soil ergosterol would facilitate determinations of soil fungal biomass (FB), since ergosterol is used as a marker for living fungi (Grant and West, 1986).

Ergosterol plays an important role in the growth of fungi (Lees et al., 1995), and is found in the phospholipid bilayer of cell membranes mostly in its free state and to a lesser extent esterified to fatty acids (Martin et al., 1990). Ergosterol is synthesized mainly by fungi and also by some microalgae (Grant and West, 1986; Newell et al., 1987). Ergosterol measurements are used to study fungi for economical and ecological reasons (Johnson and McGill, 1990; Gessner and Chauvet, 1993). Ergosterol is used as a marker of living FB because of its rapid turnover in dead cells (Grant and West, 1986); and because soils, plant residues and grains do not favour the growth of microalgae (Matcham et al., 1985; Grant and West, 1986; Tunlid and White, 1992).

The amount of FB is determined using an ergosterol-to-fungal biomass (E-to-FB) conversion factor from cells growing in pure culture (Antibus and Sinsabaugh, 1993; Gessner and Chauvet, 1993). E-to-FB conversion factors have been reported for different aquatic and ectomycorrhizal fungi (Antibus and Sinsabaugh, 1993; Newell, 1994), and range between 2.2 and 5.5 μg ergosterol mg^{-1} dry weight of mycelia (Matcham et al., 1985; Gessner and Chauvet, 1993). Factors for estimating FB in soils are not yet defined. Heterogeneous soil matrices, the growth environment and analytical recovery of ergosterol, may however, influence the factors to estimate FB. Thus, the estimation of FB in terrestrial ecosystems may benefit from establishing efficient ergosterol extraction methods and E-to-FB conversion factors. Our objectives were to (a) adopt an MAE method to measure ergosterol in soil, and (b) establish E-to-FB conversion factors for soils.

2. Materials and methods

2.1. Soils

To test the MAE method, we selected seven soil series having widely different pedogenesis, properties and management histories (Table 1). Soil samples were collected from the 0–15 cm of the A horizon in cultivated and undisturbed Brunisolic, Gleysolic, Chernozemic and Gray Luvisolic soils. The cultivated Brunisolic soils corresponded to the Rideau and Grenville series, and were collected at the Central Experimental Farm

Table 1
Some soil and climate characteristics for samples taken from the 0–15 cm depth of the A horizon in seven different soil series from Canada

Soil/land use[a]	pH[b]	Content (g kg^{-1})				Mean annual	
		OC[c]	Clay	Silt	Sand	T (°C)[d]	P (mm)[d]
Brunisolic							
Grenville, cul, LS	6.7	19.2	29	119	852	6.0	870
Rideau, cul, SiC	5.9	29.8	537	423	40		
Gleysolic							
North Gower, for, SiCL	6.4	100.0	353	503	144	6.0	870
North Gower, cul, SiCL	6.4	19.2	372	488	140		
Brandon, cul, L	5.4	20.5	262	364	374		
Brown Chernozemic							
Swinton, ngra, SiL	6.8	22.1	215	510	275	3.5	359
Dark Brown Chernozemic							
Lethbridge, ngra, L	7.1	25.1	170	420	410	5.0	414
Lethbridge, cul, L[e]	7.2	13.5	180	440	380		
Gray Luvisolic							
Breton, bgra, L	n.d.	41.8	210	400	390	2.1	547
Breton, cul, L[f]	6.9	11.2	190	400	410		

[a] for = forested, cul = cultivated; ngra = native grassland, bgra = brome; LS = loamy sand, SiC = silty clay, SiCL = silty clay loam, L = loam, SiL = silty loam.
[b] pH measured in 10 mM $CaCl_2$.
[c] OC = organic carbon.
[d] T = temperature, P = total precipitation.
[e] Series B (wheat-fallow) in long-term crop rotation plots at Lethbridge, Alta.
[f] Series E, plot 1, and east half in long-term crop rotation plots at Breton, Alta.

(CEF), Ottawa, Ontario in the spring of 1994. Samples were also taken from Humic Gleysolic soils in a cultivated Brandon series at the CEF, and in a forested North Gower series near Winchester, Ontario in the spring of 1994. Soil samples from the Chernozemic and Gray Luvisolic soils were taken in 1992. Samples from the native Brown Chernozemic soil corresponded to the Swinton series and were taken near Swift Current, Saskatchewan. Samples of the Lethbridge series of the Brown Chernozem were taken at Lethbridge, Alberta; and the Breton series from the Gray Luvisol was sampled at the Breton plots of the University of Alberta, near Breton, Alta. Moist soil samples were air-dried and subsequently sieved to 841 μm (20 mesh) and stored until chemical analysis. Any visible plant and animal debris was removed from soil samples before and after sieving.

2.2. Selection of fungal species for batch culture

We obtained six fungal species from the Canadian Collection of Fungal Cultures (DAOM). Species were selected according to their relative growth rate in pure liquid culture media, and the milieu from which they were originally isolated. During exponential growth, species yielding a biomass < 2 mg h^{-1} were considered slow growers, those yielding between 2 and 5 mg h^{-1} had medium rates of growth, and those yielding > 5 mg h^{-1} were defined as fast growers. *Rhizopus stolonifer* DAOM 216617 (a slow grower) had been isolated from decaying roots of *Amorphophallus* sp. in Indonesia. Fungi with medium growth rates were obtained from British Columbia, Canada and included *Penicillium chrysogenum* DAOM 215337, found in western hemlock (*Tsuga heterophylla*) lumber, and *Chaetomium globosum* DAOM 221179, isolated from a Podzolic soil. Fast growers included *Fusarium oxysporum* DAOM 145356, isolated from a Brunisolic soil of Ont., Canada; *Trichoderma harzianum* DAOM 216191, found in maize (*Zea mays*), and *Alternaria alternata* DAOM 216367, isolated from a shrub (*Staphylea trifolia*) found in the United States.

Preserved fungal species were reconstituted by mixing freeze-dried inoculum with sterile, deionized water. The solutions containing *P. chrysogenum*, *R. stolonifer* or *T. harzianum* were plated out and grown on malt extract agar (MEA) plates. Solution containing *F. oxysporum* was plated out on synthetic nutrient agar (SNA) with filter paper, *C. globosum* on oatmeal agar (OA) plates (Samson et al., 1996), *A. alternata* on potato dextrose agar (PDA, Difco). These cultures were grown under light for 10 days at 25°C and determined to be free of contaminants. Two plugs (4 mm diameter) from each of the agar plates were used to replate each species into the same type of media and under the same conditions. Spores produced in the latter plates were used to inoculate broth media.

2.3. Growth media and inoculation

Each fungal species was grown in batch under optimal nutrient conditions using three different broth media: *R. stolonifer*, *P. chrysogenum* and *C. globosum* were grown in malt extract broth (MEB) (Samson et al., 1996). The species *T. harzianum and A. alternata* were grown using Czapek Dox broth (CDB) (Ahmad and Baker, 1988; Ozeelik and Ozeelik, 1990). *Fusarium* broth (FB) was used to grow *F. oxysporum* (Miller et al., 1991).

Spores from each species were collected from the agar plates by swirling 1.5 ml of the appropriate broth on the agar plate and using a sterile inoculation loop to lift the spores off the plates. The spore suspension was then used to inoculate 100 ml of the appropriate broth to make inoculum cultures of each fungus. Inoculum cultures were grown on an oscillator shaker (108 rev min^{-1}) at 24 ± 0.6°C for 2–7 days depending upon the species. Once specific inoculum cultures had produced enough biomass, sterile distilled water (100 ml) was added and the whole solution was homogenized in a sterile blender.

Homogenized aliquots (2 ml) were then used to inoculate 50 ml of the appropriate broth in sterile 250 ml Erlenmeyer flasks. To minimize the variability of inoculum size the whole solution was blended for 30 s before taking an aliquot. The inoculated flasks were placed randomly in the oscillating shaker (108 rpm) and incubated at 24 ± 0.6°C to define growth curves. Observations made under the light microscope revealed that mycelia of *P. chrysogenum* and *F. oxysporum* contained few spores (less than 1% of the mycelia's volume). The mycelial mats of the four other cultured species did not contain spores.

2.4. Experimental design and statistical analisis

Batch growth studies were designed as factorial experiments with fungal species and time as the main factors. For each time of sampling, every fungal species was replicated four times in separate flasks. Batch cultures in flasks were sampled hourly during 3–6 days (*T. harzianum*, *F. oxysporum*, *A. alternata*, *C. globosum*), or 8–10 days (*P. chrysogenum*, *R. stolonifer*).

For each fungal species, replicated flasks were randomly removed from the shaker at each sampling time. The contents of each flask were vacuum filtered using Buchner funnels with tared oven dried (100°C, 3 h) Whatman #1 filter paper (55 mm dia). Fifty milliliters of Aqueous NaCl (0.1%, w/v) was used to wash the fungal mycelia. Immediately after washing, the fun-

gal mycelia and filter paper were placed in liquid N_2 and freeze-dried for 12 h. Freeze-dried pelletized mycelia samples clean from filter paper fibers were weighed and stored in plastic vials at −20°C until chemically analyzed.

Statistical analysis were made using Systat (Wilkinson, 1996; Wilkinson and Coward, 1996). A two factor analysis of variance (ANOVA) was used to examine the influence of species and growth stage on total FB, biomass-C, total ergosterol content and concentration. A *t*-test was used for determining differences in total ergosterol content between cells harvested during the stationary and exponential growth phases. Box plots of Systat were used to represent the variability of total ergosterol and the E-to-FB ratio determined for the six fungal species. Pearson correlation and Spearman rank correlation were used to compare the relative strength of relations between the measured soil properties.

2.5. Microwave-assisted extraction (MAE)

All reagents and solvents used for extraction and chromatography were of analytical-reagent grade. Enough sample for detection of ergosterol (250 mg of soil or 50 mg of fungal mycelium) were placed into a 27 ml culture tube, treated with 2 ml of methanol and 0.5 ml of 2 M NaOH, and tightly sealed with a teflon-lined screw cap. Each culture tube was then placed within screw capped 1 l plastic bottle and tightly sealed. This combination was then placed at the centre of a domestic microwave oven (Panasonic NN-7553) operating at 2450 MHz and 900 W maximum output and irradiated at medium power (55% of maximum output power, manufacturer's setting) for 18 s. After cooling, ca. 15 min, the culture tubes were irradiated for an additional 17 s at medium power. After cooling, the tubes were removed from the plastic outer bottle. The contents were neutralized with 1 M HCl, treated with 2 ml of methanol, vortexed and then extracted with pentane (3× ca. 2 ml), all within the culture tube. The combined pentane extracts were passed through a 0.45 μm Acrodisc filter (Gelman), and then evaporated and taken to dryness under a stream of N_2 gas. The residues were then made up to 200 μl in methanol prior to HPLC or gas chromatography–mass spectrometric (GC–MS) analysis.

2.6. Routine extraction method

The routine procedure consisting of extraction and saponification was based on that of Seitz et al. (1977) as modified by Miller et al. (1983), and Scheu and Parkinson (1994). Samples (8 g) were extracted by blending for 2 min in methanol (2 × 30 ml), the combined extract treated with ethanol (50 ml) and KOH (1.6 g) and refluxed for 30 min, cooled, diluted with water and extracted with pentane (3 × 50 ml). The combined pentane extracts were evaporated to dryness and then made to 1 ml in methanol prior to analysis.

2.7. High performance liquid chromatographic analysis

Ergosterol was separated from soil extracts on a 150 × 4.6 mm stainless steel column packed with ODS 3 μm (CSC, Montrèal, Que., Canada) and eluted with acetonitrile–methanol (80:20, v/v) at a flow rate of 1.3 ml min^{-1}. Ergosterol was detected with a Varian 2550 variable wavelength detector (Varian Analytical Instruments, San Fernando, CA) set at 282 nm.

Ergosterol from fungal and soil extracts was also separated on a 30 × 5 mm Nova Pak C18 (WAT052834) reverse-phase column packed with ODS 4 μm preceded by a Nova Pak C18 15220 guard column (Waters) and eluted with acetonitrile–methanol (98:2, v/v) at a flow rate of 2 ml min^{-1}. Ergosterol was detected with a Waters 486 variable wavelength detector (Waters) set at 282 nm.

Ergosterol content was determined by comparing sample peak areas with those of external standards. Ergosterol was confirmed by comparing retention times with the external standard or by coinjection with the standard. Ergosterol was purchased from Sigma and recrystallized from methanol.

2.8. Gas chromatography–mass spectrometric (GC–MS) and elemental analysis

The presence of ergosterol in fractions collected during the HPLC analyses was confirmed using a Finnigan 4500 quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) operating in the electron ionization (EI) mode at 70 eV. Ergosterol was quantified by multiple ion monitoring (*m*/*z* 337, 363, 396) with pure ergosterol as an external standard. A J & W Scientific DB5-MS 15 m × 0.25 mm I.D. column with 0.26 μm film (J & W Scientific, Folsom, CA) was used with on-column injection and the column was temperature programmed from 150 to 300°C at 20°C min^{-1}. The concentration of C and H in freeze-dried fungal mycelia was determined by dry combustion using a LECO 600 C-H-N analyzer.

2.9. Recovery of ergosterol from soil treated with mycelia

To determine the efficiency of the MAE method to extract ergosterol from added cell tissue, freeze-dried mycelia from *C. globosum*, *F. oxysporum* and *R. stolonifer* was added to three soils with differing pedogenetic histories, soil organic C and clay content. Before addition of mycelia, samples from the Gleysolic, Gray Luvisolic and Dark Brown Chernozemic soil were con-

ditioned at room temperature and a moisture content of 75% field capacity for 3 days. Freeze-dried mycelia of the three species were dissolved in 2 ml of methanol and added to 250 mg of conditioned soil and mixed thoroughly with a vortex. Ergosterol was extracted using the MAE method and its concentration determined by HPLC.

3. Results and discussion

3.1. Extraction and analyses of soil ergosterol

Since the MAE procedure had shown promise in a variety of matrices, including soil (Young, 1995), a more rigorous application to soils was investigated. A soil sample size of 250 mg was found to provide sufficient ergosterol for ready detection by HPLC-UV. Rupturing of the culture tubes under the high temperature and pressure conditions was occasionally a problem, which was overcome by switching to larger (27 ml) culture tubes and by irradiation in two stages. Formation of emulsions and frothing was encountered in some soils, and this was overcome by addition of 2 ml methanol immediately before pentane extraction. The small amount of soil taken for analysis requires samples to be thoroughly homogenized. On the whole, the method showed a relative standard deviation of less than 10% for soil ergosterol.

Ergosterol eluted quickly at about 4 min. With the absorbance set at 282 nm, other non- or lower-wavelength UV absorbing compounds that might co-elute do not interfere with the analysis. This reduces the need for prior sample extract cleanup. Some of these compounds include fatty acids and cholesterol, which were detected in the mass spectrometric analysis (data not shown). Ergosterol elution time using acetonitrile:methanol (80:20, v/v) at a flow rate of 1.3 ml min^{-1} with the longer column was about 2 min slower.

Relative to the routine method, the MAE method presents important advantages for the analyses of soil ergosterol. The MAE method extracted from 2.5 to 9 times more ergosterol than the routine method in 4 soils (Table 2). The MAE method measures total ergosterol, requires small (mg) amounts of soil, small solvent volumes and includes two short (35 s total) microwaving steps. In comparison, the routine method requires large sample size (g) and solvent volumes, and includes a time consuming (30 min) step of sample refluxing and saponification with alkali.

Validation of the development of the microwaving extraction step in relation to the routine and CO_2 supercritical fluid extractions was reported by Young (1995). The MAE method permits extracting ergosterol from about 60 soil samples daily, depending on the operator. The MAE method enabled almost complete recovery of ergosterol added in solution (99% from a blank, no soil), and $90 \pm 6\%$ (relative SD) when added at a concentration of 1 μg g^{-1} to three soils prior to extraction. Other studies using different methods for extracting and analyzing ergosterol obtained similar relative recoveries (Martin et al., 1990; Bermingham et al., 1995).

Table 2
Comparison of MAE and routine[a] extraction methods for the analysis of ergosterol in soil samples[b]

	Ergosterol (μg g^{-1})	
Soil series/texture[c]	MAE	Routine
Grenville, LS	1.15 (0.04)[d]	0.13 (0.01)
Rideau, SiC	0.85 (0.06)	0.24 (0.01)
Brandon, L	0.69 (0.05)	0.24 (0.03)
Swinton, SiL	3.10 (0.18)	1.21 (0.21)

[a] Routine = extraction, refluxing and saponification.
[b] Sample size was 250 mg for the MAE and 8 g for the routine method.
[c] LS = loamy sand, SiC = silty clay, L = loam, SiL = silty loam.
[d] Number in brackets represent relative standard deviation values of four replicates.

3.2. Fungal biomass and ergosterol synthesis in vitro

Fungal species isolated from different milieus grew at different rates, and synthesized different amounts of dry biomass and ergosterol (Fig. 1, Table 3). On average, *R. stolonifer* grew the slowest (0.5 mg h^{-1}) and *F. oxysporum* grew the fastest (8.4 mg h^{-1}) during the exponential phase. The fast growth of *F. oxysporum* is consistent with observations reported by Seitz et al., (1977) and Martin et al., (1990). Total dry biomass production for all species ranged from 53 to 595 mg, and ergosterol content ranged from 87 to 2178 μg (Table 3). In general, total dry biomass and ergosterol values in all fungal species showed low variability during the exponential and stationary phases of growth. The average standard deviation was $<10\%$ for total dry biomass (Fig. 1), and total ergosterol (data not shown).

These results are in contrast to the large variability (coefficient of variation of up to 122%) in biomass and ergosterol content reported for several aquatic fungi (Bermingham et al., 1995). In our study, an important factor to reduce experimental variability was to ensure a homogeneous inoculum size. This was achieved by blending mycelia mats for 30 s just before transferring the mycelial inoculum into the culture flasks. Tests had indicated that the latter step reduced the variability of dry biomass considerably (up to 50%) during incubation. The large variabilities observed by Berming-

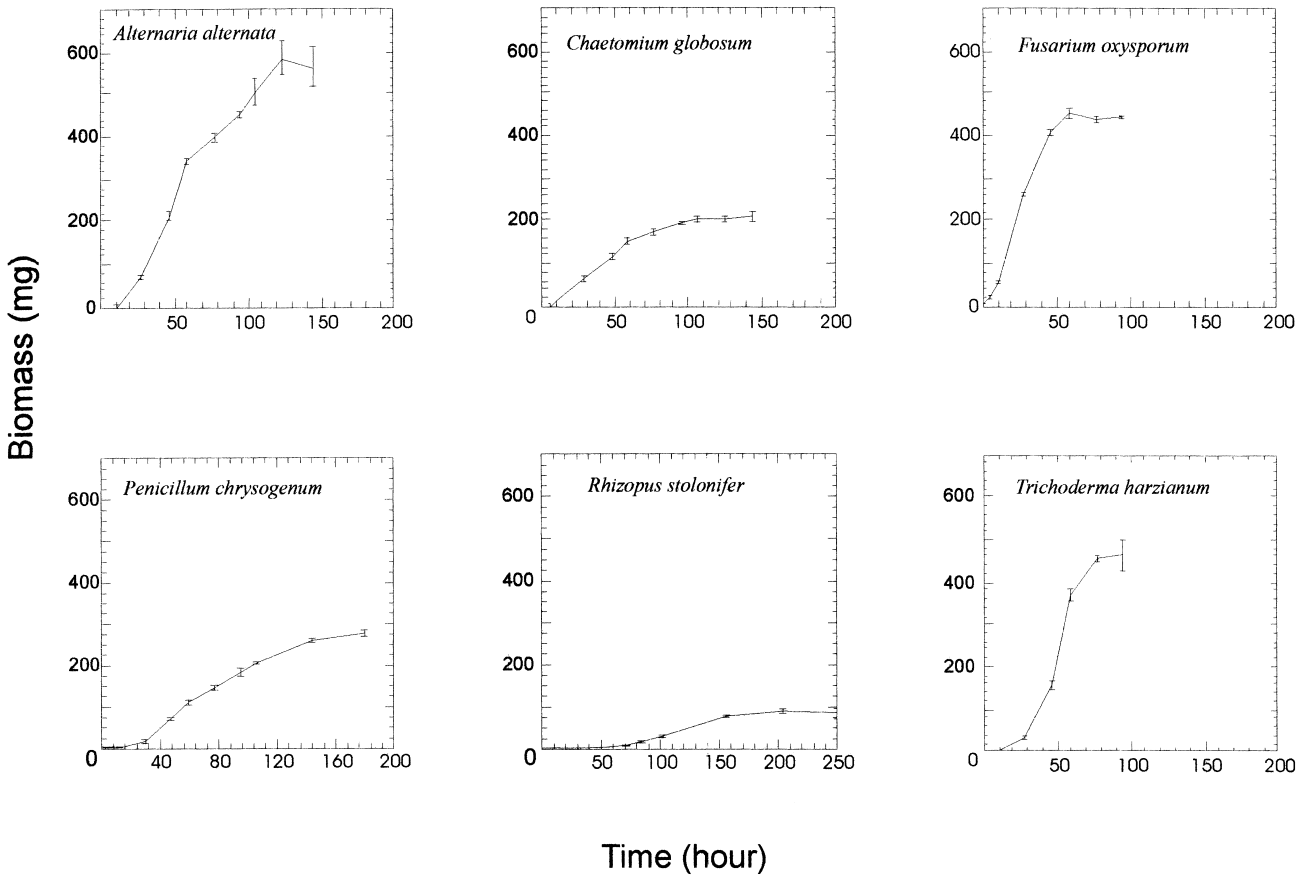

Fig. 1. Kinetics of total dry biomass in six fungal species originating from soil and plant materials. Vertical bars represent standard error of the mean ($n = 4$).

ham et al., (1995) appear to be related to subsampling of heterogeneous wet mycelia and other factors, as discussed by Newell (1996). In our study, the relative content of C and H in mycelial mats remained relatively constant during growth and was not affected by species or growth phase (Table 3).

The effect of fungal species and growth stage on total ergosterol content during the incubation was examined. The ANOVA showed a significant effect of species and growth stage on total ergosterol content ($F = 12.2$, $P < 0.01$; and $F = 23.0$, $P < 0.01$, respectively); and the two factors did not interact to influence ergosterol content ($F = 1.6$, $P = 0.21$). The total ergosterol content in mycelial mats harvested during the stationary phase was significantly higher than the content measured during the exponential phase, indicating a low rate of ergosterol synthesis by mycelia during the first 48 h ($t = -2.94$, $P = 0.005$, $n = 39$;

Table 3
Mean growth rate and range for total dry biomass and ergosterol synthesized by mycelial mats of fungal species harvested during the exponential and stationary growth phases

Species	Growth[a](mg h^{-1})	Biomass (mg)	Ergosterol (μg)	C (%)	H (%)
F. oxysporium	8.4	53–445	180–1648	43.8 (0.4)[b]	7.1 (0.1)
T. harzianum	7.3	140–486	420–2104	44.7 (0.7)	7.2 (0.0)
A. alternata	5.0	199–595	663–2178	42.0 (0.1)	7.0 (0.1)
P. chrysogenum	2.1	106–284	394–1024	42.0 (0.1)	6.7 (0.2)
C. globosum	2.0	61–209	229–675	42.6 (0.2)	7.0 (0.0)
R. stolonifer	0.5	17–118	87–345	43.2 (0.6)	7.2 (0.1)

[a] Average growth rate during exponential phase.
[b] Number within parentheses represent standard deviation values ($n = 4$).

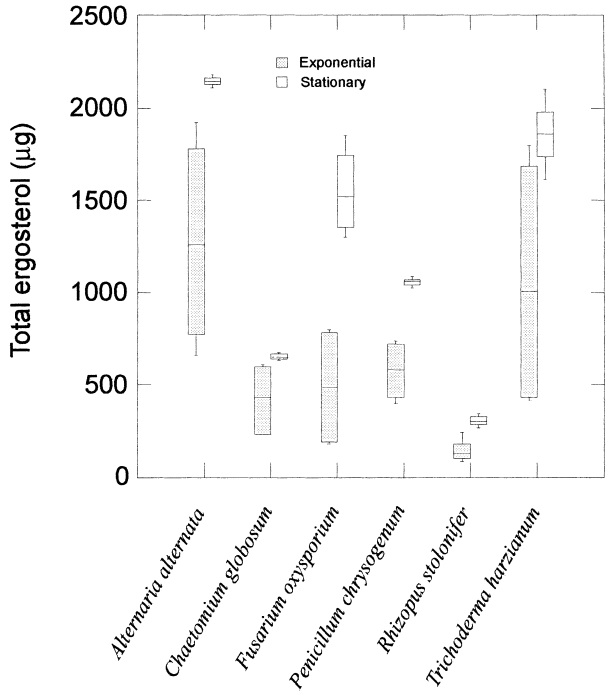

Fig. 2. Box plot of the total ergosterol content during the exponential and stationary growth phases in pure cultures of six fungal species originating from soil and plant materials.

Fig. 2). Mycelial ergosterol content harvested at the end of the exponential phase was the same as that measured during the stationary phase (data not shown). The latter results are consistent with observations made by Behalova et al., (1994).

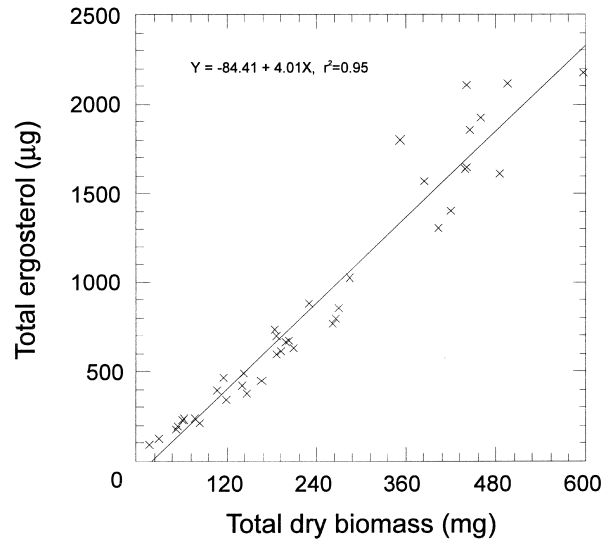

Fig. 3. Linear regression for total ergosterol content versus total dry biomass of pure cultures of six fungal species originating from soil and plant materials. Standard error of estimate = 145.

The total ergosterol content was closely correlated with total dry biomass production during the growth of each of the fungal species (Fig. 3). The slope of the regression in Fig. 3 indicates an average total ergosterol content of 4 µg mg^{-1} biomass produced. The coefficient of determination ($r^2 = 0.95$) is stronger than those reported for ergosterol and dry mycelial mass in ectomycorrhizal basidiomycete and aquatic fungi (Johnson and McGill, 1990; Newell, 1994).

3.3. Ergosterol-to-fungal biomass conversion factor

The ANOVA and Fig. 4 indicated that the E-to-FB ratio was not affected by species or growth phase ($F = 1.1$; $P = 0.38$ and $F = 0.2$, $P = 0.67$, respectively); and the two factors did not interact to influence the ratio ($F = 1.1$; $P = 0.37$). Although the E-to-FB ratio was determined in mycelia growing in vitro under unlimited nutrient conditions, it appears to be a useful parameter to estimate FB in terrestrial ecosystems. The E-to-FB was also robust under unfavourable conditions of growth such as those found during the stationary phase. The consistent response of the E-to-FB ratio among the different fungal species and growth stages may help to better represent conditions of asynchronous growth, species diversity, variable nutrient availability and environmental regimes found in soils.

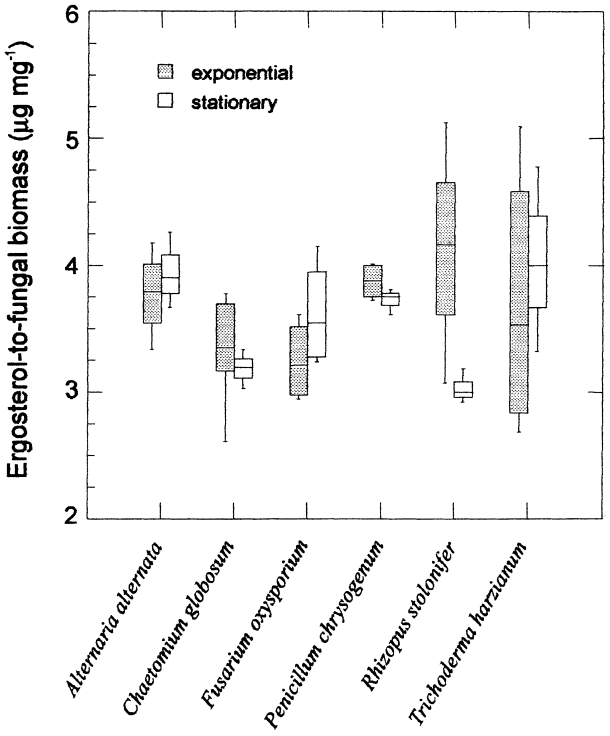

Fig. 4. Box plot of the ergosterol-to-fungal biomass ratio during the exponential and stationary growth phase of pure cultures of six fungal species originating from soil and plant materials.

The ergosterol concentration for mycelial mat samples harvested during the exponential and stationary phases ranged between 2.6 and 5.1 μg mg^{-1} biomass, and the estimated average for all samples was 3.65 μg mg^{-1} of dry FB (SD = 0.6, n = 39) (Table 4). This average value was similar to the slope (4.0 μg mg^{-1}) determined from the total ergosterol versus dry biomass regression of the same samples (Fig. 3). The range for ergosterol concentration in fungi grown in vitro under different temperatures, with limiting and excess substrate supply range from 0.35 to 12 μg mg^{-1} (Bermingham et al., 1995), although a more common range for ergosterol concentration in fungi is 4–12 μg mg^{-1} (Newell et al., 1987; Davis and Lamar, 1992). We accept the regression slope value of 4 μg mg^{-1} as representing the average value for ergosterol content mg^{-1} FB. The regression function explicitly considers the different variabilities in ergosterol content observed during the stationary and exponential growth phases.

The acceptance of a mean value of 4 μg mg^{-1}biomass compensates for potential differences in soil E-to-FB ratio that may be associated with the ubiquitous nature of living microhabitats and available substrates in soils (Monreal and Kodama, 1997). Not all living fungal species and strains grow in a synchronized manner in soils. Most likely, lag, exponential and stationary growth phases for soil fungi occur at different rates, microniches and periods that cannot be differentiated with the methods used to take and handle soil samples. Therefore, we consider the average ergosterol concentration of 4 μg mg^{-1} biomass as an acceptable value for measuring FB in bulk soil. This average ergosterol value compares well with the average concentration (4.53 μg mg^{-1}) estimated by other authors for several species of aquatic hyphomycetes grown in vitro with different media and temperature (Table 4).

The average conversion factor (f) for mycelial mats of all species was 274 μg FB μg^{-1} ergosterol (Table 4). This value is similar to that determined from the regression slope in Fig. 3 (250 = [1 mg FB/4 μg ergosterol] × 1000 [μg mg^{-1}]). The f value for two species of soil fungi (*F. oxysporum* and *C. globosum*) were similar to those estimated for fungi isolated from plants (Table 4). Thus, we propose that the conversion factor derived for six fungal species isolated from soil and plants can be used as a factor to determine FB in soil–plant systems. Thus, the use of f = 250 is strongly supported by our data. The proposed conversion factor needs further testing since supply of lipids (Newell, 1992, 1994) and oxygen tension (Safe, 1973; Nout et al., 1987) may influence fungal ergosterol concentration in soil systems. The latter awaits further research. The testing of f may be important when determining FB in soil catenas with variable lipid content and texture (Dinel et al., 1998). Further research on the effects of extreme soil environmental conditions on the E-to-FB ratio is warranted.

3.4. Using ergosterol concentration to determine fungal biomass in vivo soils

Table 5 shows that the average relative recovery of

Table 4
Average ergosterol concentration and E-to-FB conversion factors for pure culture of fungi originating from different milieus and grown under different temperatures and substrates

Fungal species	Ergosterol (μg mg^{-1})	f[a]	Reference
Aquatic fungi			
Number of species			
5	4.89	205[a]	Cook (1987)[b]
4	5.40	185	Gessner and Schwoerbel (1991)
14	5.50	182	Gessner and Chauvet (1993)
9	1.21	826	Bermingham et al. (1995)
7	5.64	177	Newell (1994)
Average (weighted)	4.53	221	
Soil and plant fungi (this study)			
Species names			
A. alternata	3.84	260	
C. globosum	3.29	304	
F. oxysporium	3.43	292	
P. chrysogenum	3.81	263	
R. stolonifer	3.69	271	
T. harzianum	3.82	262	
Average	3.65	274	
Slope in Fig. 3	4.01	250	

[a] f = conversion factor (i.e., [1 mg FB/4.89 μg^{-1} ergosterol] × 1000 [μg mg^{-1}] = 205).
[b] P.E. Cook, unpublished Ph.D. thesis, City of London Polytechnic, 1987.

ergosterol added in solution to soils prior to extraction was 90%, and the variability of percent recovery was low. Davis and Lamar (1992) noted that incomplete recovery of solution ergosterol added to soil samples was associated with adsorption or oxidation of the sterol by soil components. Physico-chemical adsorption of ergosterol onto soil inorganic colloids is less likely because the molecule is not electrically charged. We carried out complementary steps of sonicating soil samples as described by Ruzicka et al., (1995), and found no increased recovery of ergosterol added in solution or of that found in situ (data not shown).

Table 5 shows that the average relative recovery for added ergosterol in the mycelia of three species was 62% and ranged from 50 to 88%. The average relative recovery was not associated with species of fungi, soil series, organic matter content, pH or texture (data not shown). The relative recovery may be related to protection of fungal cell walls by soil particles. Scheu and Parkinson (1994) indicated that the susceptibility of soil fungi and bacteria to air drying depends on soil material. The amount of microbial biomass and the number of bacteria, fungi and actinomycetes were closely associated with the equivalent spherical diameter of soil particles (Monreal and Kodama, 1997). The average relative recovery of mycelial ergosterol also provides indirect evidence that the physical protection of fungal tissue is an important mechanism stabilizing soil organic matter in soils. The recovery factor value of 1.61 (1/0.62) in the present study is higher than the recovery factor (3.0 = 1/0.33) proposed for estimating soil microbial biomass-C from chloroform-fumigation extractable soil C studies (Tate et al., 1988). The MAE method appears to extract a greater proportion of the fungal tissue C than the chloroform fumigation–extraction technique normally used to extract C pools from the soil microbial biomass.

From these results, we concluded that determination of soil FB on the basis of ergosterol analysis requires correcting ergosterol concentration by the proportion of unextracted mycelial ergosterol. Thus, FB is calculated as follows:

$$\text{FB}\ \left(\mu\text{g g}^{-1}\ \text{soil}\right) = \text{Ergosterol}\ \left(\mu\text{g g}^{-1}\ \text{soil}\right) \times f \times R_f \quad (1)$$

where f = 250 (1/4 × 1000, mg biomass μg^{-1} ergosterol), and R_f = 1.61 (correction factor for average percent recovery, 1/0.62). FB may also be expressed in terms of the C content of the mycelial mats:

$$\text{FB-C} = \text{FB} \times \text{C} \quad (2)$$

where FB-C = fungal biomass-carbon, and C = 0.43, average C content in fungal species (Table 3). Scheu and Parkinson (1994) used substrate-induced respiration and ergosterol measurements to estimate a mean ratio of 91 μg FB-C μg^{-1} ergosterol in forest soils of Canada. The latter E-to-FB-C ratio is 20% lower than ours (i.e., 108 μg FB-C μg^{-1} ergosterol, assuming a FB-C content of 43%), and did not consider losses of soil ergosterol during extraction and analyses. Relative to our method of calculation, their published values for FB-C may have been underestimated. Our calculation explicitely considers the recovery of ergosterol in mycelia added to different soil matrices.

Eqs. (1) and (2) were used to determine the content

Table 5
The recovery of ergosterol from fungal mycelia and pure solution added to soils prior to extraction

Soil[b]	Recovery of mycelial and solution ergosterol (as % of added)[a]				
	C. globosum	*F. oxysporum*	*R. stolonifer*	Mean	Solution
N.G., for, SiCL	nd[c]	77 (10)	55 (2)	66	88 (7)
N.G., cul, SiCL	73 (20)[d]	40 (9)	78 (14)	64	90 (8)
Breton, gra, L	80 (16)	40 (20)	41 (17)	54	nd
Breton, cul, L[e]	82 (30)	47 (14)	70 (14)	69	90 (6)
Lethbridge, gra, L	67 (19)	65 (4)	64 (14)	73	nd
Lethbridge, cul, L[f]	42 (7)	45 (5)	nd	44	93 (5)
Brandon, cul, L	nd	nd	nd		87 (9)
Average				62 (11)	90 (2.3)

[a] Recovery = $[(\text{Erg}_{\text{spiked}} - \text{Erg}_{\text{soil}})/\text{Erg}_{\text{added}}] \times 100$, where $\text{Erg}_{\text{spiked}}$ is the concentration of ergosterol measured in the soil sample spiked with mycelia or pure solution, Erg_{soil} is the ergosterol concentration measured in the soil sample, and $\text{Erg}_{\text{added}}$ is the ergosterol concentration in the added mycelia or solution.

[b] N.G. = North Gower, Ontario; for = forested, cul = cultivated, gra = grassland; SiCL = silty clay loam, L = loam.

[c] nd = not determined.

[a] Number in brackets represent standard deviation for n = 3.

[e] Series E, plot 1, east half, in long-term crop rotation plots at Breton, Alta.

[f] Series B (wheat-fallow), long-term crop rotation plots at Lethbridge, Alta.

Table 6
The amount of soil fungal biomass determined by using Eqs. (1) and (2) and ergosterol concentration measured in the 0–15 cm depth of A horizons

Soil[a]	Ergosterol (μg g^{-1})	Fungal biomass (μg g^{-1})	Fungal biomass-C (μg g^{-1})
North Gower, for, SiCL	2.25 (0.15)[b]	906	390
North Gower, cul, SiCL	1.29 (0.19)	519	223
Breton, bgra, L	11.79 (0.94)	4745	2040
Breton, cul, L	0.91 (0.18)	366	157
Lethbridge, ngra, L	1.79 (0.21)	720	310
Lethbridge, cul, L	0.62 (0.11)	155	67
Brandon, cul, L	0.69 (0.03)	278	120

[a] for = forested, cul = cultivated, bgra = brome, ngra = native grassland; SiCL = silty clay loam, L = loam.
[b] Number within brackets represent standard deviation values of four replicates.

of FB and FB-C on the basis of soil ergosterol concentration in soils with different pedogenesis, properties and climate (Table 6). Samples from undisturbed soils under grass and forest had from 2- to 12-fold greater content of living FB and FB-C than samples taken from adjacent cultivated soils. Eash et al., (1996) reported similar range in soil ergosterol content, and indicated greater ergosterol content in a prairie soil than in a close by soil cultivated to a corn–soybean rotation. Relative to agricultural soils, ergosterol content in forested and peaty soils are much higher and range between 2 and 200 μg g^{-1} (Anderson et al., 1994; Scheu and Parkinson, 1994). According to Table 6, the living FB-C observed in the cultivated Gray Luvisol represents 50% of the average microbial biomass-C reported earlier for samples taken from the same crop rotation plot (McGill et al., 1986). Interestingly, ergosterol content, and thus FB and FB-C were not linearly correlated with the total content of soil organic C or texture as indicated by the correlation matrix of Spearman (Table 7) and Pearson (data not shown). The latter strongly suggests that the growth and activity of soil FB may be more closely associated with specific chemical pools of soil organic matter (i.e., substrate quality) or type of crop production system. This awaits further research.

Table 7
Spearman correlation matrix examining relationships between soil ergosterol and other properties in samples taken from the 0–8 cm depth of seven soil series shown in Table 1

	Ergosterol	pH	OC	Clay	Silt	Sand
Ergosterol	1.00					
pH[a]	−0.02	1.00				
OC[b]	0.71	−0.35	1.00			
Clay	0.11	−0.73	0.40	1.00		
Silt	0.33	0.08	0.26	0.47	1.00	
Sand	−0.06	0.59	−0.33	−0.95	−0.66	1.00

[a] Soil pH in 10 mM $CaCl_2$.
[b] OC = soil organic carbon.

4. Conclusions

The MAE is an effective and rapid method to extract soil ergosterol. Soil FB can be determined from measurements of soil ergosterol concentration using an ergosterol-to-biomass conversion factor of 250 μg biomass μg^{-1} ergosterol, and a recovery factor of 1.61 for mycelial ergosterol added to soil. These factors were consistent for mycelial mats from six fungal species and soils with different pedogenesis, management, and properties. Living FB content can be determined from measurements of soil ergosterol content.

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