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Ergosterol and microbial biomass relationship in soil

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Abstract Ergosterol and microbial biomass C were measured in 26 arable, 16 grassland and 30 forest soils. The ergosterol content ranged from 0.75 to 12.94 μg g^{-1} soil. The geometric mean ergosterol content of grassland and forest soils was around 5.5 μg g^{-1}, that of the arable soils 2.14 μg g^{-1}. The ergosterol was significantly correlated with biomass C in the entire group of soils, but not in the subgroups of grassland and forest soils. The geometric mean of the ergosterol:microbial biomass C ratio was 6.0 mg g^{-1}, increasing in the order grassland (5.1), arable land (5.4) and woodland (7.2). The ergosterol:microbial biomass C ratio had a strong negative relationship with the decreasing cation exchange capacity and soil pH, indicating that the fungal part of the total microbial biomass in soils increased when the buffer capacity decreased. The average ergosterol concentration calculated from literature data was 5.1 mg g^{-1} fungal dry weight. Assuming that fungi contain 46% C, the conversion factor from micrograms ergosterol to micrograms fungal biomass C is 90. For soil samples, neither saponification of the extract nor the more effective direct saponification during extraction seems to be really necessary.

Key words Microbial biomass · Fungal biomass · Ergosterol · Fumigation extraction

Introduction

As strictly heterotrophic organisms, fungi are very effective decomposers of complex organic material and usually dominate the soil microbial biomass in most soils (Anderson and Domsch 1975; Parkinson et al. 1978), one exception being the rhizosphere (Vancura and Kunc 1977). Several methods exist for quantifying fungal biomass. Direct microscopic quantification in solid substrates suffers from difficulties in differentiating fungus from mineral soil particles and in separating live and dead fungal tissue. As a consequence, microscopic counts have been reported to overestimate (Schnürer et al. 1985) or underestimate fungal biomass (Ingham and Horton 1987). Important indirect methods are the selective inhibition technique (Anderson and Domsch 1973) and the measurement of fungus-specific components such as ergosterol. This predominant fungal sterol is endogenous only to fungi and certain green microalgae (Newell et al. 1987); negligible amounts have been found in higher plants and procaryote organisms (Weete and Weber 1980). Ergosterol is an important constituent of cell membranes, controlling their permeability, microviscosity and the activity of membrane-bound enzymes (Peacock and Goosey 1989). Ergosterol has been used to detect fungal invasion and spoilage of food and cereal grains (Seitz et al. 1977; Schnürer 1993) and fungal infection of plants (Osswald et al. 1986). The development of ectomycorrhizae (Martin et al. 1990) and vesicular-arbuscular mycorrhizae (Frey et al. 1992) was monitored by measuring their ergosterol content and ergosterol was also measured as a biomarker for fungi in coastal marine sediments (Newell et al. 1989).

Rapid ergosterol losses of more than 95% were observed by Davis and Lamar (1992) within 2 weeks after fumigation with methyl bromide. These data imply that ergosterol decays rapidly after fungal death and will not be accumulated to any great extent in humic material (Nylund and Wallander 1992). Consequently, it can be used to estimate fungal biomass in soils (West et al. 1987; Zelles et al. 1987; Davis and Lamar 1992). However, more detailed studies of the relationships between ergosterol and microbial biomass are lacking. The major aim in our work was to examine the relationship between ergosterol and microbial biomass C in soils and to study the effects of soil properties and land use on this relationship. A subsidiary aim was to see if direct saponification could be omitted for the analysis of ergosterol in soil samples.

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Materials and methods

Soils

A series of 72 soils from Germany was analysed. Thirty deciduous forest soils were collected in the areas around Braunschweig and Göttingen, Lower Saxony. They were taken from the A horizon with a spade to a depth of about 10 cm after careful removal of overlying organic layers. Properties and storage procedure of the forest soils were described in detail by Joergensen et al. (1995). Twenty six arable and 16 grassland soils were mainly collected around Göttingen and were taken with a corer also to a depth of about 10 cm as described by Joergensen et al. (1993). All field moist soils were passed through a sieve (<2 mm), given a conditioning incubation of 25°C for 10 days and stored at 4°C for at least 6 months before analysis of microbial biomass C and ergosterol content (Joergensen et al. 1995). This procedure ensured that actively growing microorganisms were not present in the soil.

Analytical procedures

Soil microbial biomass C was measured by fumigation extraction (Vance et al. 1987). Moist soil (50 g, on an oven-dry basis) was split into two portions, 25 g for the fumigated and 25 g for the non-fumigated treatment and extracted with 100 ml 0.5 *M* K_2SO_4. Organic C in the extracts were measured using a Dohrman DC 80 automatic analyser. Soil microbial biomass C was estimated from the relationship: biomass C=2.22 E_C (Wu et al. 1990), where E_C is [(organic C extracted from fumigated soil) minus (organic C extracted from non-fumigated soil)]. Analysis of soils was performed as described by Joergensen et al. (1995). The result are the means of quadruplicate analysis (ergosterol) or triplicate analysis (all other data) and are expressed on an oven-dry basis (about 24 h at 105°C). Statistical analyses were performed with the SAS program. The χ^2-test and the Kolmogorov-Smirnov test were used to assess the distribution fit. In contrast to soil pH, cation exchange capacity (CEC), soil organic C, microbial biomass C, ergosterol and the ergosterol:microbial biomass C ratio were log-normally distributed in the entire group of 72 soils, but also in the three subgroups. Pearson's correlation coefficients were calculated with these parameters after log-transformation. One-way analysis of variance was performed after log-transformation of the data to assess the effects of land use. Significance of differences was examined using the Scheffé test.

Ergosterol

Moist soil (1 g, on an oven-dry basis, between 25% and 75% of the water-holding capacity) was extracted with 80 ml bidistilled ethanol (96%, denaturized) for 30 min by oscillating shaking (250 rpm). A ratio soil:extractant smaller than 1:80 gave lower ergosterol contents in some soils. To reduce re-adsorption to soil colloids, the soil suspension was filtered (Whatman GF/A) immediately after extraction, the extract being evaporated in a vacuum rotary evaporator at 40°C in the dark. The residue was collected in 10 ml ethanol and filtered again (cellulose acetate membrane 0.45 µm). Quantitative determination of ergosterol was performed by reserved-phase HPLC analysis: main column 12.5-cm Spherisorb ODS II S5 (Knauer Vertex 12-cm main column, 0.5-cm pre-column), mobile phase 97% methanol/3% water (v/v), flow rate 1.0 ml min^{-1} and detection at 282 nm. A constant room temperature of 25°C was maintained. Retention of ergosterol with this system is 7.25±0.25 min. The detection limit for ergosterol for our system was about 0.01 µg ml^{-1}. A standard solution of ergosterol (Sigma E-6510) was prepared in bidistilled ethanol (96%, denaturized). The recovery was slightly lower in non-sterile soils, at between 92% and 101%. The extraction time (15 min to 2 h) had only a minor effect on the extracted ergosterol content (Djajakirana et al. 1993).

Saponification

According to Zelles et al. (1987), moist soil (1 g, on an oven-dry basis) was weighed into a 100-ml round-bottomed flask, 20 ml methanol, 5 ml ethanol and 2 g KOH pellets were added and then refluxed for 90 min. After cooling, 5 ml H_2O was added and then extracted with 3×20 ml petroleum benzene in a separator funnel. The petroleum benzene fraction was collected and then evaporated in a vacuum rotary evaporator at 40°C. Further steps were carried out as described above.

Results and discussion

Ergosterol

The ergosterol content ranged from 0.75 to 12.94 µg g^{-1} soil (Table 1, Fig. 1). The geometric mean ergosterol content of grassland and forest soils was nearly identical at around 5.5, that of the arable soils was 2.14, less than half that of the other groups (Table 1). The ergosterol content was highly significantly correlated with microbial biomass C in the entire group of soils (Table 2). This correlation coefficient was smaller than that between ergosterol and soil organic C. The ergosterol content showed a significant negative correlation with soil pH, but not with the CEC (Table 2). These relationships differed markedly in the three subgroups. A significant relationship between ergosterol and microbial biomass C was only found in the group of arable soils. The ergosterol content was not significantly correlated with any of the soil variables in the small group of grassland soils and only significantly correlated with the soil organic C content in the group of deciduous forest soils.

Table 1 Geometric means, minima and maxima of soil variables in the A-horizon (0–10 cm) in a series of 72 soils from Germany; geometric means of arable (n=26), grassland (n=16) and forest soils (n=30). Means with the same column followed by the same letter are not significantly different (P=0.05; Scheffé)

	CEC (µeq g^{-1})	Soil organic C (mg g^{-1})	Ergosterol (µg g^{-1} soil)	Microbial biomass C (µg g^{-1} soil)	Ergosterol microbial biomass C (mg g^{-1})
All soils	200	33.0	3.58	597	6.0
Minimum	55	5.5	0.75	151	1.4
Maximum	892	214.5	12.94	2970	7.7
Arable	191 ab	15.8 a	2.14 a	352 a	5.4 a
Grassland	281 b	38.6 b	5.52 b	900 b	5.1 a
Forest	174 a	57.3 b	5.45 b	759 b	7.2 a

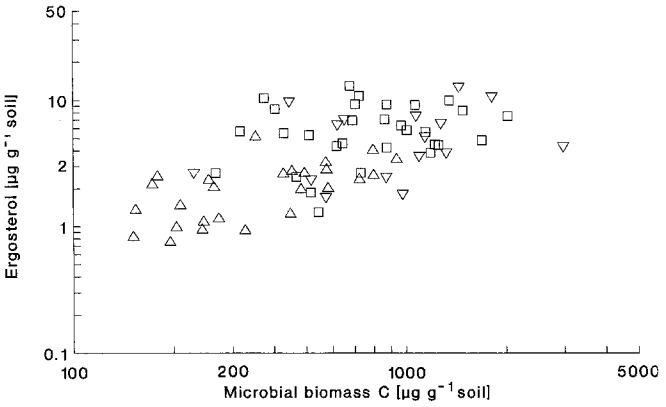

Fig. 1 Linear relationship between ergosterol and biomass C in a series of soils from Germany: arable (△); grassland (▽); forest (□); pooled standard error of replicate ergosterol measurements was 0.10 µg g^{-1} for arable soils, 0.21 µg g^{-1} for grassland soils and 0.30 µg g^{-1} for forest soils

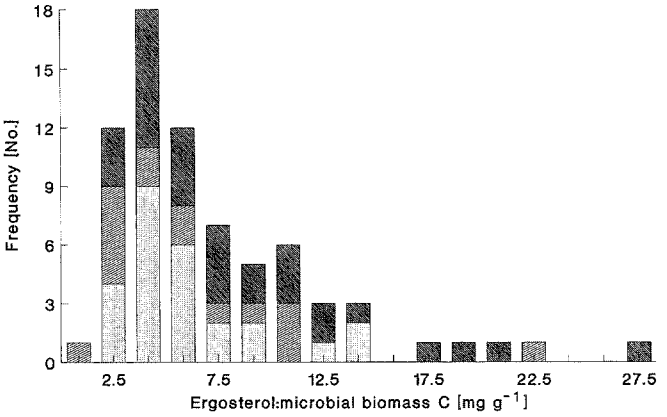

Fig. 2 Frequency distribution of the ergosterol:microbial biomass C ratio in a series of soils from Germany: arable (▨), grassland (■), forest (■)

Table 2 Correlation coefficients of ergosterol content and the ergosterol:microbial biomass C ratio and soil variables in the A-horizon (0–10 cm) in a set of arable, grassland and deciduous forest soils from Germany. All data were log-transformed except soil pH

	Microbial biomass C	Soil organic C	CEC	pH
All soils (n=72)				
Ergosterol	0.61****	0.71****	0.13	–0.32**
Ergosterol:microbial biomass C	–0.36**	–0.03	–0.55****	–0.48****
Arable soils (n=26)				
Ergosterol	0.61***	0.45*	0.43*	0.13
Ergosterol:microbial biomass C	–0.48*	–0.47*	–0.50**	–0.28
Grassland soils (n=16)				
Ergosterol	0.31	0.22	0.21	0.49
Ergosterol:microbiol biomass C	–0.57*	–0.53*	–0.57*	–0.15
Forest soils (n=30)				
Ergosterol	0.24	0.69****	0.01	–0.14
Ergosterol:microbial biomass C	–0.57**	0.21	–0.55****	–0.60***

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

Ergosterol:microbial biomass C ratio

The geometric mean of the ergosterol:microbial biomass C ratio was 6.0 mg g^{-1} (Table 1) and increased in the order grassland (5.1), arable land (5.4) and woodland (7.2), suggesting that the fungal part of the microbial biomass increased in the same order. This is consistent with the generally held opinion that fungi are more dominant in forest than in agricultural soils (Alexander 1977). However, the differences in ergosterol:microbial biomass C ratio were not significant for the different forms of land use due to large variations within each group (Fig. 2). The minimum ergosterol:microbial biomass C ratio (1.4 mg g^{-1}) was measured in a fen peat soil used as grassland, while the maximum ratio (27.7 mg g^{-1}) in an acid forest soil originated from miocenic sand. The ergosterol:microbial biomass C ratio had a strong negative relationship with CEC and pH in the entiry group of 72 soils (Table 2). The relationship of this ratio with CEC was on a similar level of significance in all three subgroups, although this applied to pH only in the group of forest soils, which indicates an increased fungal part of the total microbial biomass in soils with a smaller buffering capacity, i.e. in more acid soils. This explains why large ergosterol:microbial biomass C ratios between 9 and 28 mg g^{-1} were measured in the organic layer of an acid beech forest soil (Scholle et al. 1993a).

The ergosterol:microbial biomass C ratio in soil, representing fungal versus the whole microbial biomass, showed larger variations than the ATP:microbial biomass C ratio, representing the whole microbial biomass estimated in two ways (Jenkinson 1988). Also the fungal percentage of the total microbial biomass, estimated by selective inhibition, varied in a much smaller range (Anderson and Domsch 1975; Parkinson et al. 1978). The possibility cannot be excluded that the large variation in the ergosterol:microbial biomass C ratio is partly caused by differences in true k_{EC} values (i.e. the fraction of microbial biomass C extracted after fumigation) between the soils. Fungi with thick cell walls might be less affected by $CHCl_3$ fumigation (Ingham et al. 1991), leading to underestimation of microbial biomass C and thus an overestimation of the ergosterol:biomass C ratio. The consistent negative correlation of the ergosterol:microbial biomass C ratio in all soil groups points in this direction (Table 2). However, k_{EC} values were found to be unaffected by pH (Vance et al. 1987), clay and soil organic C content (Kaiser et al. 1992).

Ergosterol:fungal biomass ratio

A range of ergosterol concentrations is shown in Table 3 as obtained from the literature for the biomass of different fungal species. The average ergosterol concentration calculated from these data was 5.1 mg g^{-1} fungal dry weight. This mean content derived from actively growing species

Table 3 Ergosterol content of different fungal species

Fungal species	Ergosterol (mg g^{-1} dry weight)
Agaricus bisporus[be]	2.2–4.0
Alatospora acuminata[hj]	4.5–11.2
Alternaria alternata[d]	3.8–4.4
Amanita muscaria[i]	3.1–4.0
Amanita rubescens[m]	13.5–17.6
Anguillosporum longissima[j]	3.7–4.2
Articulospora tetracladia[hj]	4.2–5.0
Aspergillus amstelodami[d]	4.9–5.9
Aspergillus flavus[d]	2.3–3.3
Boletus griseus[m]	10.7–11.9
Cenococcum geophilum[m]	3.0–3.5
Clavariopsis aquatica[j]	4.2–8.0
Clavatospora longibrachiata[j]	8.3
Crucella subtilis[j]	3.3
Dendryphiella salina[l]	2.7
Entomoloma sericeum[m]	5.6–6.6
Flagellospora curvula[hj]	3.9–11.5
Fusarium solani[a]	1.7–2.4
Hebeloma crustuliniforme[im]	2.8–6.8
Hymenoscyphus sp.[l]	3.7
Lactarius pubescens[m]	5.0
Laccaria laccata[i]	3.1–3.3
Lemonniera aquatica[j]	2.3–6.8
Mycosphaerella sp.[l]	2.3
Passeriniella obiones[l]	4.6–6.1
Paxillus involutus[m]	8.1–9.2
Phaeosphaeria halima[l]	3.7
Phaeosphaeria spartinae[l]	2.6
Phaeosphaeria spartinicola[l]	8.1
Phaeosphaeria typharum[f]	1.9–2.5
Phanerochaete chrysosporium[g]	5.1
Pleospora spartinae[l]	4.2
Rhizopogon sp.[m]	5.1–7.8
Rhizopus arrhizus[c]	1.0–3.0
Rhizopus oligoporus NRRL 5905[h]	2.0–24.0
Scleroderma citrinum[m]	2.9–6.9
Suillus bovinus[i]	2.8–2.9
Tetrachaetum elegans[jk]	2.9–5.0
Tetracladium marchalianum[k]	2.6–5.0
Tricladium splendens[hk]	3.4–3.8
Variosporina ramulosa[l]	4.1
Wettsteinia marina[l]	3.2
Mean (n=42)	5.1

[a] Kok et al. (1970), [b] Holtz and Schisler (1972), [c] Weete et al. (1973), [d] Seitz et al. (1979), [e] Matcham et al. (1985), [f] Newell et al. (1987), [g] Davis and Lamar (1992), [h] Nout et al. (1987), [i] Salmanowicz and Nylund (1988), [j] Gessner and Schwoerbel (1991), [k] Gessner and Chauvet (1993), [l] Padgett and Posey (1993), [m] Antibus and Sinsabaugh (1993)

may differ from a dormant soil fungal population. Another basic problem is the inter- and intraspecies variation. An unknown part of the variation shown in Table 3 can be explained by different extraction and detection techniques used by the different authors. The major part of the variation is presumably species specific, but may also be caused by the developmental stage, age, physiological state and growth conditions such as nutrient supply or the amount and quality of the C input (Arnezeder and Hampel 1991).

However, Gessner and Chauvet (1993) found that mycelium in the stationary phase was only 10–12% lower in ergosterol content than mycelium in the growth phase. Newell et al. (1987) found more pronounced peaks of ergosterol concentrations where the phase of logarithmic growth was supposed to culminate. They observed that the ergosterol concentration of some species, but not of all species, was considerably affected by the composition of the growth medium. Consistent with this, Gessner and Chauvet (1993) found no systematic effects on the ergosterol:fungal biomass C ratio by changes in growth conditions and in availability of plant sterols. Nout et al. (1987) observed drastically reduced ergosterol contents in *Rhizopus oligoporus* when grown under low oxygen tensions. In accordance with this, we found the smallest ergosterol:microbial biomass C ratio in a fen peat soil. In contrast to soil (Fig. 2) and species (Table 3) specific variation, the ergosterol:microbial biomass C concentration was found to be very stable within one soil for a 6-week incubation period (Wolters and Joergensen 1992). This is consistent with the findings of Seitz et al. (1979) and Salmanowicz and Nylund (1988), who found a relatively constant ergosterol concentration in fungi cultures for several weeks.

Assuming that fungal biomass contains 46% C, the ergosterol:fungal biomass C ratio is 11.1 mg g^{-1}, i.e., the conversion factor from micrograms ergosterol to micrograms fungal biomass C is 90. The fungal part of the total microbial biomass is on average 54%, converting the geometric mean of the ergosterol:microbial biomass C ratio using this conversion factor. The average ergosterol concentrations of fungal and total microbial biomass fit well together considering the data of Anderson and Domsch (1973, 1975), who used the selective inhibition technique. A fungal concentration between 0.7% and 11.3% of total biomass measured by the agar film technique (Hassink et al. 1991) seems extremely small and points to the fact that direct microscopy may underestimate the fungal biomass substantially. Similarly, Newell et al. (1987) and Nout et al. (1987) found extraordinarily high ergosterol to biomass ratios in fungi grown in their natural substrate when the biomass estimates were derived from microscopic measurements of hyphal length.

Saponification

The amounts of ergosterol found after solvent extraction without saponification as performed by us are called "free" ergosterol. Saponification by addition of KOH to the filtered extract often increased the yield of ergosterol. This additional amount is the fraction called "ergosteryl esters". The addition of KOH to the solvent before extracting the sample often gave the highest yields of ergosterol. The additional fraction is called "membrane-bound" ergosterol. The fraction of "free" ergosterol comprised between 85% and 87% of total ergosterol when analysing cultured fungal species (Weete and Weber 1980; Nout et al. 1987). Saponification releases ergosterol from ergosteryl ester but at the same time causes a decay of already existing free ergosterol. So the net ergosterol yield of saponification depends on the initial ergosteryl ester concentration. A

Table 4 Comparison of our method for extracting ergosterol without saponification with the method of Zelles et al. (1987) with saponification (*CV* coefficient of variation)

Soil	Ergosterol in mg g^{-1} soil (±CV)	
	Without saponification	With saponification
Arable	1.98 (3.5)	1.01 (16.8)
Grassland	2.31 (10.4)	2.40 (12.5)
Forest	12.09 (7.6)	12.20 (22.8)

further problem is that the true chemical nature of the different ergosterol fractions is not exactly known. Originally all ergosterol must be ester bonded to cell membranes. Grant and West (1986) and West et al. (1987) used a soil:solvent ratio of less than 1:4, Zelles et al. (1987) 1:25 and we used 1:80. In preliminary experiments, we obtained an increased ergosterol yield by increasing the soil:solvent ratio from 1:20 to 1:80 similar to that of Zelles et al. (1987) by direct saponification during the extraction. In this method, KOH was added to the solvent before the extraction to give the highest yields of ergosterol.

We compared our method with that proposed by Zelles et al. (1987). However, the ergosterol content did not increase in any of the three very heterogeneous soils analysed (Table 4). Similar results were observed by Martin et al. (1990) in their study on ectomycorrhizae fungi. They found saponification to give non-significantly lower ergosterol contents. However, saponification is an indispensable step in obtaining the complete ergosterol yield in samples containing a high percentage of fatty acids and other solvent soluble material in combination with thick cell walls. In soils, neither saponification of the extract nor the more effective direct saponification during extraction seems to be really necessary.

Conclusions

The use of ergosterol as a biomarker for fungal biomass in soil has the main disadvantage that the ergosterol:biomass C ratio of soil microorganisms is more variable than the expected variations of the ratios of fungal to bacterial biomass. However, ergosterol measurements have some advantages in comparison with other methods: (1) Ergosterol is specific for fungi, in contrast to ATP and phospholipids. (2) Only negligible amounts of ergosterol are accumulated in miocrobial necromass (Davis and Lamar 1992) in contrast to chitin (Scholle et al. 1993b). (3) An exactly defined substance is determined chromatographically in contrast to the fumigation extraction method. (4) The extractability is extremely high as indicated by high percentages in recovery of added ergosterol (Davis and Lamar 1992; Djajakirana et al. 1993). (5) The extraction and measurement of ergosterol is relatively simple and quick in contrast to the selective inhibition technique. (6) The extraction and measurement of ergosterol show good reproducibility in contrast to direct microscopic measurements.

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