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Authors
Balser, TC
Treseder, KK
Ekenler, M

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Short communication

Using lipid analysis and hyphal length to quantify AM and saprotrophic fungal abundance along a soil chronosequence

T.C. Balser[a,*], K.K. Treseder[b], M. Ekenler[a]

[a]*Department of Soil Science, University of Wisconsin, 1525 Observatory Drive, Madison, WI, 53706 USA*
[b]*Ecology and Evolutionary Biology and Earth System Science, University of California, Irvine, CA, 92697 USA*

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Abstract

We evaluate the use of signature fatty acids and direct hyphal counts as tools to detect and quantify arbuscular mycorrhizal (AM) and saprotrophic fungal (SF) biomass in three Hawaiian soils along a natural soil fertility gradient. Phospholipids16:1ω5c and 18:2ω6,9c were used as an index of AM and saprotrophic fungal biomass, respectively. Both phospholipid analysis and hyphal length indicated that the biomass of AMF was greatest at the highest fertility site, and lowest where phosphorus limits plant growth. Saprotrophic fungal biomass did not vary. Hyphal length counts appeared to under-estimate SF abundance, while the phospholipid AMF:SF ratio was in line with expectations. This study indicates that phospholipids may be a valuable and reliable tool for studying the abundance, distribution, and interactions between AM and saprotrophic fungi in soil.

Keywords: Arbuscular mycorrhizal fungi; Saprotrophic fungi; Lipid analysis; Fungal biomass; Tropical soil; Soil chronosequence

Arbuscular mycorrhizal fungi (AMF) form extensive mycelia in soil (often constituting up to 30% of total soil microbial biomass) and play a significant role in the functioning of ecosystems (Read, 1991; van der Heijden et al., 1998; Olsson and Wilhelmsson, 2000; van der Heijden et al., 2003). In particular, they are critical components of soil phosphorus and nitrogen cycling, and may directly control the above ground structure of plant communities. Because of their importance, accurate estimation of AMF biomass is necessary for a complete understanding of soil nutrient dynamics.

Microscopic measurements of hyphal length have been a commonly used method for estimating AMF biomass in soil (Sylvia, 1992; Olsson, 1999). However, methods based on microscopical analysis do not allow any systematic or functional separation of different fungal mycelia, nor reliable separation of dead and live fractions of fungal biomass (Sylvia, 1992). Moreover, they are difficult and time consuming.

The use of a specific chemical biomarker can provide a more objective quantification of AMF biomass. Chitin is a common biomarker found in SF and AMF cell walls (Bethlenfalvay and Ames, 1987). However, because chitin is also produced by other soil organisms, it may lead overestimation of fungal biomass in soil (Sylvia, 1992). Also, because chitin persists after fungal death, it may not be suitable for estimation of living biomass. Another biochemical indicator is ergosterol (found only in the cell membranes of fungi) (Klamer and Bååth, 2004). However, ergosterol is neither precise nor specific enough for use in most systems (Olsson et al., 1998). A considerable amount is lost during extraction from soil and purification. In addition, the content of this compound in mycorrhizal fungi is much lower than in other fungi.

Phospholipid fatty acid analysis is seeing increased use in soil community analysis, and has potential as a sensitive biochemical indicator capable of simultaneous estimation of, and distinction between, AMF and SF biomass (Olsson et al., 1995; Jansa et al., 1999; Olsson, 1999; Ruess et al., 2002). The phospholipid 16:1ω5c has been shown to be the dominant membrane lipid in AM fungi, while 18:2ω6,9c is

* Corresponding author. Tel.: +1 608 262 0132; fax: +1 608 265 2595.
E-mail address: tcbalser@wisc.edu (T.C. Balser).

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dominant in ectomycorrhizae and saprotrophic fungi (and is negligible in the mycelium of AM fungus) (Muller et al., 1994; Frostegard and Bååth, 1996; Larsen et al., 1998; Olsson, 1999; Ruess et al., 2002). However, studies using phospholipids to quantify and distinguish AMF and SF have primarily taken place in artificial growth systems. Little is known about the use of signature fatty acids for estimation of AM and saprotrophic fungal abundance in field soils (Larsen et al., 1998; Olsson, 1999).

In this paper, we compare the use of direct hyphal length counts with phospholipid biomarker analysis in quantifying AMF and SF biomass in three Hawaiian forest soils along a natural fertility gradient. Specifically, we assess whether the two methods yield similar patterns of abundance across sites, and relative abundance of AMF and SF within a site. Our data were obtained from two larger, independent studies at the Hawaiian field sites (see Balser, 2001; Treseder and Allen, 2002). Soil samples were obtained from three sites described in detail by Crews et al., (1995). The sites are at different stages of soil development (300-, 20,000-, and 4,100,000-yr old), and it has been shown that aboveground productivity is limited primarily by N at the youngest site (Vitousek et al., 1993), by P at the oldest site (Herbert and Fownes, 1995), and by neither N or P independently in the relatively fertile 20,000-yr old site (Vitousek and Farrington, 1997).

We collected samples from the control plots of a long-term fertilization experiment (Vitousek and Farrington, 1997). In the youngest and intermediate age sites, there are four replicate control plots, whereas at the oldest site there are three. For the phospholipid fatty acid data, soil samples were collected in June, 2000. To reduce variability and workload, four sets of replicate cores (5 cm×15 cm depth) were pooled from each plot. For extraradical hyphal data, a 5-cm diameter soil core was used to excise roots growing to 10-cm depth. Two cores were collected from each plot. Roots were not sorted by species. All cores were collected from beneath the canopy of *Metrosideros polymorpha*, the dominant evergreen canopy tree (Kitayama and Mueller-Dombois, 1995).

Phospholipid fatty acids from microbial cell membranes were extracted from 0.5 g lyophilized soil samples, purified, and identified using a modified Bligh and Dyer (1959) technique, described further in Balser (2001). We analyzed samples using a Hewlett-Packard 6890 Gas Chromatograph with an Ultra 2 (5%-phenyl)-methylpolysiloxane column (25 m×0.2 mm×0.33 μm) (Hewlett-Packard). Internal standards were used to convert fatty acids peak areas to mol% of total fatty acids extracted.

Extraradical hyphae were extracted from two soil cores per plot and their lengths were quantified (Sylvia, 1992). The method is described fully in Treseder and Allen (2002). AM hyphae were distinguished from saprotrophic hyphae by examining morphological structures (AM hyphae are non-septate, have irregular walls and display angular, unilateral branching, Bonfante-Fasolo, 1986). Results are reported as mm hyphae g^{-1} dry soil. Because hyphal length data are not normally distributed, statistical tests were conducted on ranked data.

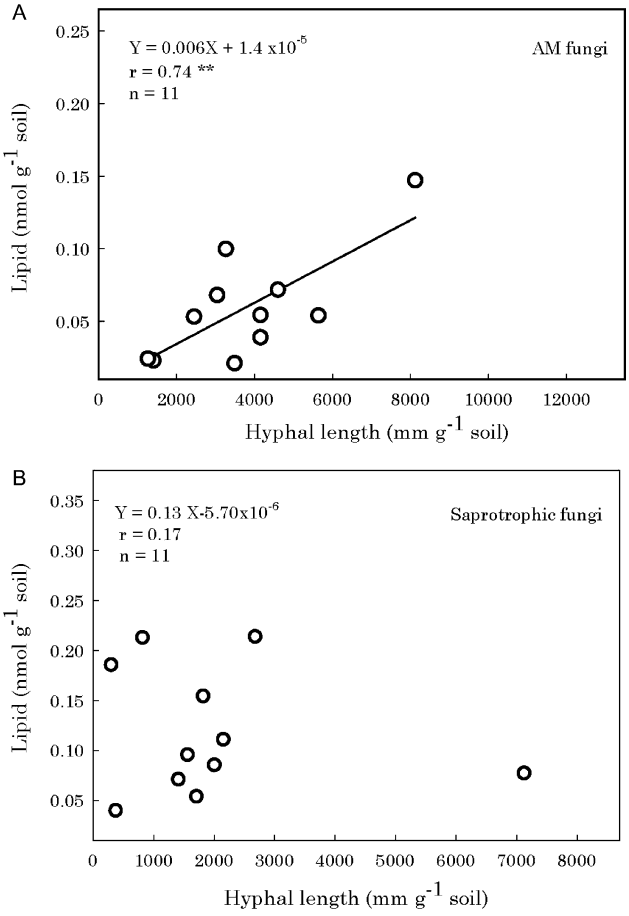

Fig. 1. Relationship between the biomass of (A) AM and (B) saprotrophic fungi calculated from extraradical hyphal length (microscopic method) and bioindicator lipids (16:1ω5c and 18:2ω6,9c).

We found that hyphal length and PLFA abundances were not highly related to measures of AM hyphal length (Fig. 1A and B), although the regression relationships appear to be driven by outliers. Possible explanations are numerous. Given the small sample size, and variability between sites, it is possible that we are simply unable to detect a relationship. Or, it may be that the high carbon content of these soils obscures the relationship via interference from humic-acid derived fatty acids (Bååth and Anderson, 2003). Finally, hyphal length determination does not account for differences in hyphal diameter among fungal species, nor does it distinguish between live and dead hyphal biomass. Thus, if there was a disproportionately high abundance of senescent SF hyphae, or fungal populations with disproportionately smaller hyphal diameters, then the relationship between hyphal length and lipid abundance could be affected. Further investigation is warranted to determine the utility of PLFA 18:2ω6,9c as an indicator of general fungal biomass in these soils (Table 1).

Table 1
Selected soil properties

Site	Parent material age (years)	pH[a]	C[b] kg m^{-2}	N[b] kg m^{-2}	P[b] kg m^{-2}
N-limited	300	4.35	15.3	0.98	0.036
Fertile	20,000	3.40	32.4	1.56	0.155
P-limited	4,100,000	3.18	24.1	1.13	0.112

[a] Soil:0.1 M $CaCl_2$ (ratio = 1:10).
[b] Soil carbon, nitrogen and phosphorus (Crews et al., 1995).

While there was no strong relationship by regression analysis, hyphal length and PLFA yielded qualitatively similar results for AMF and SF biomass across the soil chronosequence (Fig. 2A and B). Both methods indicated that AMF biomass was highest in the fertile and N limited sites, and lowest in the phosphorus limited site. Hyphal counts of SF indicated no significant difference in abundance across sites, although the 18:2ω6,9c lipid

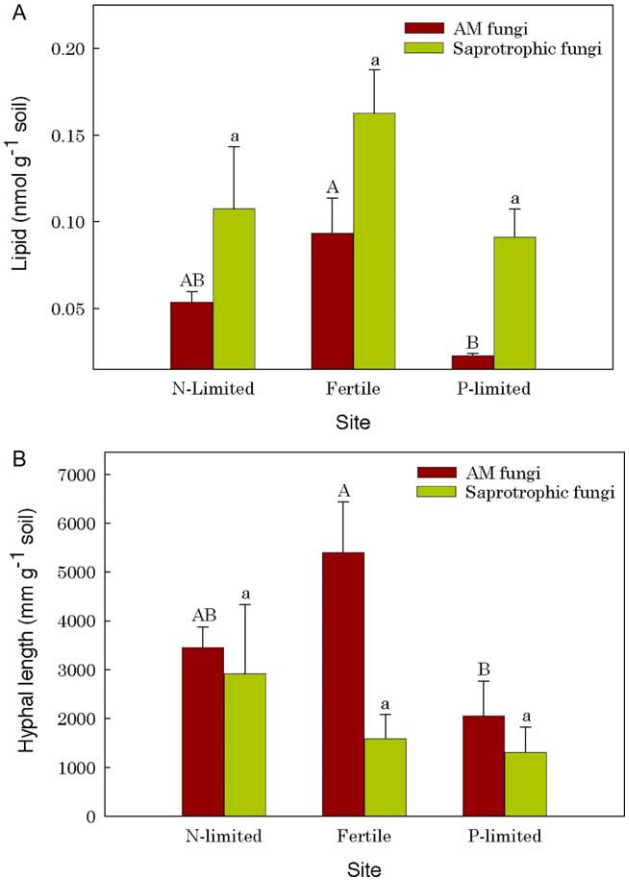

Fig. 2. Hyphal biomass of arbuscular mycorrhizal (AM) and saprotrophic fungi in soil, as determined by (A) phospholipid fatty acid analysis (PLFA), or (B) by hyphal extraction and identification by gross morphology. Bars represent the mean of three or four plots ± 1 SE. Across sites, bars with the same letter are not significantly different at $P < 0.05$ level using LSD (AM fungi upper-case letter and saprotrophic fungi lower-case letter). One-way ANOVA, and Tukey post hoc tests were conducted with site as independent variable, and PLFAs and hyphal lengths of both fungi as dependent variable. All statistical analyses were performed with statistical software by SAS Inc.

Table 2
AMF:SF ratios

Site	Parent material age (years)	AMF:SF ratio (lipids)	AMF:SF ratio (hyphal length)
N-limited	300	0.62 ± 0.12[a]	1.9 ± 0.53[a]
Fertile	20,000	0.62 ± 0.16	5.7 ± 3.3
P-limited	40,100,000	0.20 ± 0.01	2.1 ± 0.87

One way ANOVA indicates no significant differences among the soil ages for a given ratio.
[a] All values are mean of three or four plots ± 1 SE.

(SF biomass) displayed a trend consistent with that of the AMF lipid (Fig. 2A and B).

Despite internal consistence (correlation between methods, same qualitative trend across sites) between the two methods tested here, we found that hyphal counts and lipid biomarker estimates of biomass differed substantially in their absolute estimates of AMF versus SF abundance within a given site (Fig. 2). This is indicated by the ratios of AMF:SF biomass (Table 2). The ratio calculated from hyphal counts ranged from 1.9 to 5.7, whereas that from lipids was less variable, and ranged from 0.20 to 0.62. Thus, microscopic methods revealed higher AM abundance compared to saprotrophic fungi at each site (Fig. 2), yet the PLFA method indicated the opposite. Because the hyphal count method does not distinguish between live and dead hyphae, nor account for hyphal diameter (Olsson et al., 1997; Olsson, 1999); and because soil phospholipids degrade rapidly after cell death (Klamer and Bååth, 2004), the hyphal count ratio is likely the explanation for the discrepancy. It likely either underestimates SF, or overestimates AMF, abundance in each soil. Underestimation of SF biomass may be due to greater diversity of SF species and thus variation in hyphal diameter. It is likely that the diversity of SF species is higher in these sites than that of AMF (which tends to be dominated by a single genus, the Glomalean fungi), leading to greater variability in SF hyphal length-based abundance estimates. Overestimation of AMF may be due to difficulties in microscopic identification. AM fungi have similar morphologies with other groups of fungi, potentially leading to misclassification of hyphae as AMF rather than SF (Olsson et al., 1997; Olsson, 1999).

Conclusions

While microscopic measurement of hyphal length has been the most widely used method for estimating AM biomass in soil, the method does not allow systematic or functional separation of different (e.g. AMF versus SF) fungal mycelia, nor reliable separation of the dead and live fraction of fungal biomass (Sylvia, 1992; Olsson, 1999). In contrast, lipid biomarkers allow for sensitive and simultaneous estimation of live AMF and SF biomass. Overall, we found that phospholipids 16:1ω5c and 18:2ω6,9c

(indicating AMF and SF, respectively) allowed reliable discrimination between the abundance and distribution of AM and saprotrophic fungi in field soils along a fertility gradient.

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