Applied Soil Ecology 95 (2015) 86–89

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Short communication

Comparison of biochemical and microscopic methods for quantification of arbuscular mycorrhizal fungi in soil and roots

Mahaveer P. Sharma [a], Jeffrey S. Buyer [b,*]

[a] Microbiology Section, Directorate of Soybean Research, ICAR, Khandwa Road, Indore, India
[b] USDA, ARS, BARC, Sustainable Agricultural Systems Laboratory, Beltsville, MD, United States

ARTICLE INFO

Article history:
Received 16 March 2015
Received in revised form 29 May 2015
Accepted 1 June 2015

Keywords:
Arbuscular mycorrhizal fungi
PLFA
NLFA
Ester-linked FAME

ABSTRACT

Arbuscular mycorrhizal fungi (AMF) are well-known plant symbionts which provide enhanced phosphorus uptake as well as other benefits to their host plants. Quantification of mycorrhizal density and root colonization has traditionally been performed by root staining and microscopic examination methods, which are time-consuming, laborious, and difficult to reproduce between laboratories. A number of biochemical markers for estimating mycorrhizal hyphae and spores have been published. In this study we grew maize plants in three different soils in a replicated greenhouse experiment and compared the results from two microscopic methods, spore density and root colonization, to the results from three lipid biomarker methods: neutral lipid fatty acid, phospholipid fatty acid, and ester-linked fatty acid analysis. Ester-linked fatty acid analysis gave consistent results for both spore density and root colonization, but neutral lipid fatty acid analysis had the highest correlation to AMF spore counts. Phospholipid fatty acid analysis was not correlated to spore density and did not reproducibly correlate to root colonization.

Published by Elsevier B.V.

1. Introduction

Arbuscular mycorrhizal fungi (AMF) colonize the roots of more than 80% of all terrestrial plants (Smith and Read, 2008). The extended network of mycelium and hyphae developed by AMF around the colonized plant roots enhance uptake of several plant nutrients (Clark and Zeto, 2000; Li et al., 1991). Quantification of AMF populations in soil and roots has traditionally been performed by microscopic methods such as spore density (Gerdemann and Nicholson, 1963) and root colonization (Phillips and Hayman, 1970; Giovannetti and Mosse, 1980). These methods rely on the individual scientist's skills in microscopic observations (Gange et al., 1999) and often lack reproducibility amongst different observers.

As an alternative to microscopic methods, lipid biomarker analysis has been used to measure AMF population density and also to characterize soil microbial communities. Neutral lipid fatty acids (NLFA), phospholipid fatty acids (PLFA), and ester-linked fatty acids (ELFA) represent three different fractions of the total soil lipid pool. ELFA is the largest fraction and includes NLFA, glycolipid fatty acids, and PLFA. PLFA and ELFA analyses have been used in many studies to measure soil microbial biomass and community composition (for example, Allison et al., 2007; Bååth et al., 1998; Drijber et al., 2000; Frostegård et al., 1993; Moore-Kucera and Dick, 2008; Schutter and Dick, 2000; Zelles et al., 1994). The NLFA 16:1ω5cis has been shown to be a biomarker for energy storage lipids in AMF spores (Olsson, 1999), vesicles, intraradical mycelium, and arbuscules (Larsen and Bødker, 2001; Van Aarle and Olsson, 2003), and has been successfully used in field studies to estimate AMF density (Lehman et al., 2012; Vestberg et al., 2012). The PLFA 16:1ω5cis, a structural component of AMF (Olsson, 1999), has been used as a biomarker for viable AMF hyphal density (Buyer et al., 2010; Olsson, 1999) although it is also found in Gram-negative bacteria (Zelles, 1997). The ELFA 16:1ω5cis and 18:1 ω5cis were also used as biomarkers for AMF (Grigera et al., 2007).

For studies requiring both microbial community characterization and AMF population density, microscopic methods may not be necessary if lipid analysis provides the necessary data. While lipid analysis has been used to measure AMF density, there have been very few studies that directly compared the various lipid methods to each other (Grigera et al., 2007) or to the traditional microscopic methods for AMF (Lehman et al., 2012; Rasmann et al., 2009; Vestberg et al., 2012). In this report we compared the results from two microscopic methods, spore density and root colonization, to the results from three lipid biomarker analyses, NLFA, PLFA, and ELFA, in roots and rhizosphere soil of maize plants grown in three different soils. We tested the following hypotheses: (1) spore

* Corresponding author at: USDA, ARS, SASL, Bldg. 001 Room 140 BARC-W, Beltsville, MD 20705-2350, United States. Tel.: +1 301 504 8436.
E-mail address: jeffrey.buyer@ars.usda.gov (J.S. Buyer).

http://dx.doi.org/10.1016/j.apsoil.2015.06.001
0929-1393/Published by Elsevier B.V.

density is most closely correlated to NLFA 16:1ω5cis and (2) root colonization is most closely correlated to PLFA 16:1ω5cis.

2. Material and methods

2.1. Experimental design

Soils were collected in October 2013 from three different sites at the USDA-ARS Beltsville Agricultural Research Center, Beltsville, MD, USA. Two soils were from cultivated fields, Farming Systems Project (FSP) and South Farm (SF) following maize rotation, while the third (Vancy) was from an uncultivated site under grass. The FSP soil is classified (Soil Survey Staff, 2010) as Mattapex silt loam (fine silty, mixed, active, mesic Aquic Hapludults; Order Ultisol), while the South Farm and Vancy soils are classified as Codorus-Hatboro loam and Codorus-Hatboro sandy loam, respectively (fine-loamy, mixed, active, mesic Fluvaquentic Dystrudepts; Order Inceptisol). Physical and chemical characteristics are reported in Table 1. All soils were sieved (5 mm) and root matter was chopped into small pieces and combined with the sieved soil. The soils were stored at 4 °C until use.

The experimental design consisted of a complete randomized greenhouse trial with 3 soils and 6 replicates. The entire experiment was run twice in succession under identical growing conditions. Five maize (roundup ready DKC 61–72) seeds were sown in black plastic pots (21 cm × 17 cm diameter) filled with the previously described soils. After germination each pot was thinned to 3 seeds. The soils were fertilized with nitrogen (50 mg/kg) using ammonium nitrate in 2 split doses 2 weeks after germination. Supplemental artificial lighting was used on a 14 h light/10 h dark cycle and plants were grown at a temperature of 25 °C ± 2 °C. Pots were watered to field capacity of the soils.

Plants were harvested 48 days after germination. Rhizosphere soil was shaken off the roots which were then washed. Rhizosphere soil and roots from each replicate pot were split into 2 samples. One set of roots and soil was stored at 4 °C for root staining and spore extraction while the other set was stored at −20 °C for lipid analysis.

2.2. Microscopic determination of AMF root colonization and spore density

About 1.5–2.0 g (fresh weight) fine roots were used for staining and assessment of AMF colonization. Roots were stained with trypan blue (0.5 g trypan blue in a mixture of 876 ml lactic acid solution (Sigma–Aldrich, Milwaukee, WI, USA), 64 ml glycerol, and 60 ml H_2O) after clearing the roots in 10% KOH (Phillips and Hayman, 1970). Stained roots were observed for vesicles, arbuscules, and hyphae under a compound microscope (Nikon Eclipse E 600, Nikon, Japan) at 20× magnification and assessed for total AMF root colonization using the grid line-intersect method (Giovannetti and Mosse, 1980).

AMF spores were extracted from 20 g (fresh weight) soil by the sieving and decanting method (Gerdemann and Nicholson, 1963), with subsequent sucrose gradient centrifugation (Ianson and Allen, 1986). Healthy spores were counted in gridded petri dishes from an aliquot of spore suspension under a stereo zoom microscope (Olympus SZX12, Center Valley, PA, USA) and were expressed as number/g dry soil.

2.3. Fatty acid analysis

Solvents were HPLC or GC grade. Water was deionized to 16–18 MΩ. All other reagents were reagent grade. PLFA were analyzed as previously described (Buyer and Sasser, 2012) with a few modifications for roots and NLFA. Root samples were ground with liquid nitrogen before lyophilization and 25–30 mg dry weight was used for analysis. Lipids were extracted and the lipids fractionated by solid-phase extraction. For NLFA, an additional internal standard, 19:0 trinonadecanoin glyceride (catalog # T-165, Nu-Chek Prep, Elysian, MN, USA), was added to the extractant. The chloroform fraction from the solid-phase extraction was used for NLFA analysis while the 5:5:1 chloroform:methanol:water fraction was used for PLFA analysis. NLFA and PLFA were converted to fatty acid methyl esters by transesterification and analyzed by gas chromatography.

ELFA were analyzed by direct transesterification of soil in dilute alkaline methanol as previously described (Buyer et al., 2002) with the following modifications. 19:0 phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) was added as an internal standard with the KOH–methanol reagent, and the fatty acid methyl esters were dissolved in 0.5 ml of hexane for analysis by GC.

Gas chromatography was performed as previously described (Buyer and Sasser, 2012) on an Agilent 6890 GC (Agilent Technologies, Wilmington, DE, USA) and FAME profiles were identified using the MIDI PLFAD1 calibration mix and peak naming table (MIDI, Inc., DE, USA). Random samples were also analyzed by GC–MS (Clarus 500, Perkin-Elmer, Waltham, MA, USA) in order to confirm the fatty acid identifications.

2.4. Statistical analysis

Statistical analysis was carried out in SAS version 9.2 (Cary, NC, USA). Residuals of biomarker concentrations and spore densities were normally distributed, indicating that parametric tests were appropriate. ANOVA of biomarker concentrations and spore densities were carried out using a general linear model, while percent colonization was analyzed using a generalized linear model employing a link-logit function (Stroup, 2014). Pearson correlation coefficients were calculated in SAS.

3. Results

Microscopic observation of spores (Schenck and Perez, 1990) indicated that the predominant species in Vancy soil was *Rhizophagus irregularis* (formerly *Glomus intraradices*), while FSP and South Farm soils were dominated by *Gigaspora margarita*, *Gigaspora gigantea*, and *Glomus etunicatum* (data not shown).

While results were generally very similar for the two experiments, in a few cases there were statistically significant differences, so all results are presented separately for each experiment. In the rhizosphere (Table 2), spore counts were highest in South Farm and FSP soils and lowest in Vancy soil. Similar results were obtained for the NLFA and ELFA biomarkers, while the PLFA biomarker was

Table 1
Soil physical, chemical and nutrient characteristics of soils used in the study.

Site	Sand (%)	Silt (%)	Clay (%)	pH	C (%)	N (%)	CEC (cmol+/kg)	P[a] (μg/g)	K[a] (μg/g)	Mg[a] (μg/g)	Ca[a] (μg/g)
FSP	20.8	59.2	20.0	5.8	1.5	0.14	9.4	199	250	139	1170
South Farm	42.8	41.2	16.0	6.0	1.1	0.097	7.1	38	110	139	919
Vancy	66.8	25.2	8.0	4.9	2.6	0.18	8.5	165	109	109	720

[a]Mehlich 3 Extractant.

Table 2
Analysis of rhizosphere soils. Values are mean ± standard deviation. Different letters within a row indicate statistically significant differences ($P < 0.05$).

Method	Experiment	Soil		
		FSP	South Farm	Vancy
Spore density (spores/g)	1	31.7 ± 11.5 A	39.9 ± 13.7 A	13.7 ± 7.1 B
	2	20.2 ± 14.1 AB	34.3 ± 14.9 A	11.5 ± 8.2 B
PLFA 16:1ω5cis (nmol/g)	1	4.29 ± 0.40 A	3.65 ± 0.49 B	4.32 ± 0.39 A
	2	4.04 ± 0.27 A	3.51 ± 0.10 B	4.43 ± 0.56 A
NLFA 16:1ω5cis (nmol/g)	1	17.03 ± 5.45 A	22.42 ± 6.52 A	8.55 ± 1.17 B
	2	16.02 ± 4.41 A	18.31 ± 5.31 A	9.17 ± 1.60 B
ELFA 16:1ω5cis (nmol/g)	1	44.64 ± 11.54 A	44.00 ± 8.73 A	25.38 ± 6.22 B
	2	41.40 ± 8.12 A	44.51 ± 9.64 A	27.01 ± 5.96 B

Table 3
Analysis of roots. Values are mean ± standard deviation. Different letters within a row indicate statistically significant differences ($P < 0.05$).

Method	Experiment	Soil		
		FSP	South Farm	Vancy
Colonization (%)	1	30 ± 7 A	36 ± 7 A	41 ± 8 A
	2	34 ± 8 B	46 ± 8 A	50 ± 8 A
PLFA 16:1ω5cis (nmol/g)	1	83.44 ± 47.45 A	138.59 ± 15.20 A	132.91 ± 43.82 A
	2	76.68 ± 19.32 B	135.27 ± 26.13 A	147.70 ± 13.34 A
NLFA 16:1ω5cis (nmol/g)	1	1493.93 ± 477.90 B	2092.82 ± 680.29 B	3007.68 ± 840.43 A
	2	1716.98 ± 440.55 B	3562.34 ± 958.64 A	3534.60 ± 1088.78 A
ELFA 16:1ω5cis (nmol/g)	1	1686.58 ± 690.30 B	2481.56 ± 1364.11 AB	3598.60 ± 1160.59 A
	2	1921.72 ± 677.00 B	5236.16 ± 1512.06 A	4748.75 ± 1836.67 A

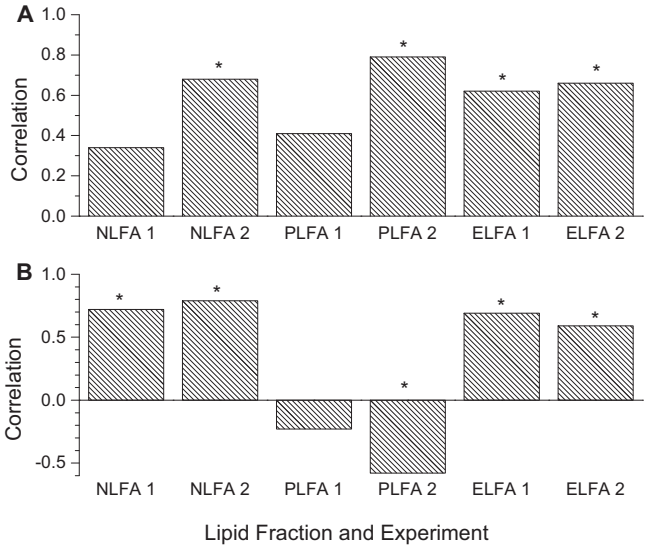

Fig. 1. Correlations between lipid biomarker concentrations and microscopic methods. A: root lipid concentrations and AMF colonization. B: rhizosphere soil lipid concentrations and spore density. Correlations are presented separately for each lipid fraction and experiment. Statistically significant correlations ($P = 0.05$) are marked with asterisks.

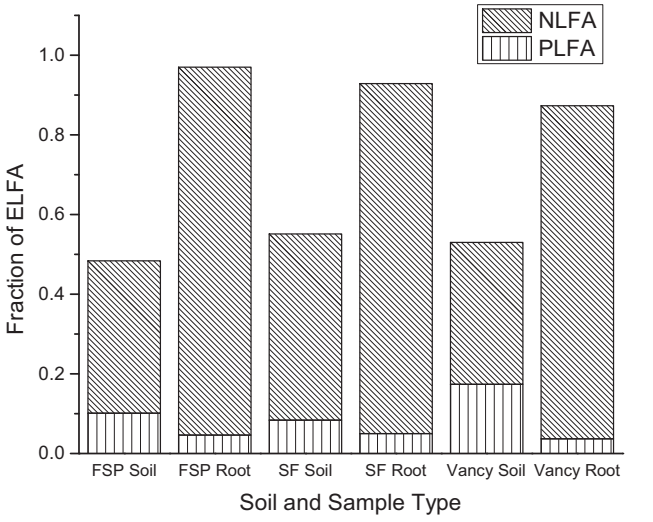

Fig. 2. Distribution of lipids among different soils. NLFA and PLFA are calculated as proportions of ELFA by soil and sample type.

lowest in the South Farm soil. In the roots (Table 3), colonization was highest in Vancy and South Farm soils and lowest in FSP soil, although the difference was statistically significant in only the second experiment. Similar results were obtained for PLFA, NLFA, and ELFA biomarkers, although the statistical significance varied somewhat.

Correlations between microscopic and lipid methods are presented in Fig. 1. In the root samples (Fig. 1A), only ELFA was significantly correlated to colonization in both experiments. PLFA and NLFA biomarkers were positively correlated to root colonization, but the correlations were statistically significant only in the second experiment. In the rhizosphere (Fig. 1B), NLFA and ELFA biomarkers were positively and significantly correlated to spore density, but the PLFA biomarker was negatively correlated. NLFA and ELFA were positively and significantly correlated to each other in both rhizosphere soil and roots in both experiments, while PLFA was positively and significantly correlated to NLFA and ELFA in roots but not in rhizosphere soil (data not shown).

Large differences were observed in the distribution of the biomarker fatty acid 16:1ω5cis between the various lipid fractions in soil and roots (Fig. 2). In soil, PLFA plus NLFA were approximately one-half of the total ester-linked fatty acids ELFA, while in roots NLFA plus PLFA were approximately 90% of ELFA. While NLFA was always much greater than PLFA, the ratio of NLFA to PLFA was higher in roots than in soil.

4. Discussion

In rhizosphere soil the biomarker NLFA 16:1ω5cis gave the highest correlation to spore density. ELFA 16:1ω5cis gave lower correlations, but they were still significant in both experiments.

PLFA 16:1ω5cis did not correlate with spore density, which is consistent with earlier results suggesting that the PLFA biomarker indicates fungal hyphal density and is confounded by its occurrence in Gram-negative bacteria. We did not measure AM fungal hyphae so we cannot evaluate the utility of the PLFA biomarker for that purpose. In roots ELFA 16:1ω5cis was the only biomarker to provide statistically significant correlations to colonization in both experiments. The remaining biomarkers all worked in one experiment but not in the other.

In theory ELFA should be equivalent to the sum of NLFA, glycolipid FA, and PLFA, and this is consistent with our finding that ELFA was always greater than the sum of NLFA and PLFA. The superiority of NLFA over ELFA for measuring spores can be explained by the inclusion of PLFA 16:1ω5cis from Gram-negative bacteria and fungal hyphae in ELFA. However, the fact that ELFA is superior to both NLFA and PLFA in correlations to root colonization is not so easily explained. Since ELFA includes NLFA, glycolipids, and PLFA, it is logical to conclude that ELFA 16:1ω5cis is summing up the contributions of spores, vesicles, and hyphae, whereas NLFA 16:1ω5cis does not include the hyphae while PLFA 16:1ω5cis does not include spores or vesicles and in addition is confounded by Gram-negative bacteria (Olsson et al., 1995; Olsson, 1999).

The PLFA biomarker was a higher proportion of ELFA in soil than in roots, while NLFA was a higher proportion of ELFA in roots than soil, and the NLFA to PLFA biomarker ratio was higher in roots than soil. These results indicate that relatively more lipid was used for energy storage in roots than in soil, which we attribute to vesicle formation in roots (Olsson et al., 1995).

Differences in spore counts and root colonization were observed between the three soils. These differences may be due to the different AMF species composition among the soils and to differences in edaphic factors between the soils, particularly pH (Coughlan et al., 2000; Rousk et al., 2009). These patterns were very similar to those observed for NLFA and ELFA but not PLFA. Thus, across three soils varying in AMF community composition, similar but not identical conclusions regarding relative spore density and root colonization can be achieved by lipid analysis and traditional microscopic methods. However, our experiment was limited to one plant species, maize, and it is possible that results would be different in other plant species.

If the only purpose of a lipid analysis is to estimate AMF spore density and plant root colonization, our results indicate that ELFA is the best choice, and can also be used for microbial community analysis as an alternative to PLFA (Drijber et al., 2000). However, if PLFA-based microbial community analysis is needed, the addition of NLFA to measure AMF spores requires little additional labor over that of doing PLFA alone.

Acknowledgements

We thank Stanley Tesch for technical assistance. Financial support from Department of Biotechnology, Govt. of India to MPS for carrying out this work during his DBT-CREST fellowship is gratefully acknowledged.

References

Allison, V.J., Yermakov, Z., Miller, R.M., Jastrow, J.D., Matamala, R., 2007. Using landscape and depth gradients to decouple the impact of correlated environmental variables on soil microbial community composition. Soil Biol. Biochem. 39, 505–516.

Bååth, E., Díaz-Raviña, M., Frostegård, A., Campbell, C.D., 1998. Effect of metal-rich sludge amendments on the soil microbial community. Appl. Environ. Microbiol. 64, 238–245.

Buyer, J.S., Roberts, D.P., Russek-Cohen, E., 2002. Soil and plant effects on microbial community structure. Can. J. Microbiol. 48, 955–964.

Buyer, J.S., Sasser, M., 2012. High throughput phospholipid fatty acid analysis of soils. Appl. Soil Ecol. 61, 127–130.

Buyer, J.S., Teasdale, J.R., Roberts, D.P., Zasada, I.A., Maul, J.E., 2010. Factors affecting soil microbial community structure in tomato cropping systems. Soil Biol. Biochem. 42, 831–841.

Clark, R.B., Zeto, S.K., 2000. Mineral acquisition by arbuscular mycorrhizal plants. J. Plant Nutr. 23, 867–902.

Coughlan, A.P., Dalpé, Y., Lapointe, L., Piché, Y., 2000. Soil pH-induced changes in root colonization, diversity, and reproduction of symbiotic arbuscular mycorrhizal fungi from healthy and declining maple forests. Can. J. For. Res. 30, 1543–1554.

Drijber, R.A., Doran, J.W., Parkhurst, A.M., Lyon, D.J., 2000. Changes in soil microbial community structure with tillage under long-term wheat-fallow management. Soil Biol. Biochem. 32, 1419–1430.

Frostegård, A., Bååth, E., Tunlid, A., 1993. Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biol. Biochem. 25, 723–730.

Gange, A.C., Bower, E., Stagg, P.G., Aplin, D.M., Gillam, A.E., Bracken, M., 1999. A comparison of visualisation techniques for recording arbuscular mycorrhizal colonisation. New Phytol. 142, 123–132.

Gerdemann, J.W., Nicholson, T.H., 1963. Spores of mycorrhizal endogone species extracted from soil by wet sieving and decanting. Trans. Br. Mycol. Soc. 46, 235–244.

Giovannetti, M., Mosse, B., 1980. An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol. 84, 489–500.

Grigera, M.S., Drijber, R.A., Wienhold, B.J., 2007. Increased abundance of arbuscular mycorrhizal fungi in soil coincides with the reproductive stages of maize. Soil Biol. Biochem. 39, 1401–1409.

Ianson, D.C., Allen, M.F., 1986. The effect of soil texture on extraction of vesicular–arbuscular mycorrhizal spores from arid soils. Mycologia 78, 164–168.

Larsen, J., Bødker, L., 2001. Interactions between pea root inhabiting fungi examined using signature fatty acids. New Phytol. 149, 487–493.

Lehman, R.M., Taheri, W.I., Osborne, S.L., Buyer, J.S., Douds Jr., D.D., 2012. Fall cover cropping can increase arbuscular mycorrhizae in soils supporting intensive agricultural production. Appl. Soil Ecol. 61, 300–304.

Li, X.L., George, E., Marschner, H., 1991. Extension of the phosphorus depletion zone in VA mycorrhizal white clover in a calcareous soil. Plant Soil 136, 41–48.

Moore-Kucera, J., Dick, R.P., 2008. PLFA profiling of microbial community structure and seasonal shifts in soils of a Douglas-fir chronosequence. Microb. Ecol. 55, 500–511.

Olsson, P.A., 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiol. Ecol. 29, 303–310.

Olsson, P.A., Bååth, E., Jakobsen, I., Söderström, B., 1995. The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. Mycol. Res. 99, 623–629.

Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing roots and staining parasitic and vesicular–arbuscular fungi for rapid assessment of infection. Trans. Br. Mycol. Soc. 55, 158–161.

Rasmann, C., Graham, J.H., Chellemi, D.O., Datnoff, L.E., Larsen, J., 2009. Resilient populations of root fungi occur within five tomato production systems in southeast Florida. Appl. Soil Ecol. 43, 22–31.

Rousk, J., Brookes, P.C., Bååth, E., 2009. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. Appl. Environ. Microbiol. 75, 1589–1596.

Schenck, N.C., Perez, Y., 1990. Manual for the Identification of VA Mycorrhizal Fungi, 3rd ed. Synergistic, Gainesville, FL.

Schutter, M.E., Dick, R.P., 2000. Comparison of fatty acid methyl ester (FAME) methods for characterizing microbial communities. Soil Sci. Soc. Am. J. 64, 1659–1668.

Smith, S., Read, D., 2008. Mycorrhizal Symbiosis. Academic Press, New York.

Soil Survey Staff, 2010. Keys to Soil Taxonomy, 11th ed. USDA-Natural Resources Conservation Service, Washington, DC.

Stroup, W.W., 2014. Rethinking the analysis of non-normal data in plant and soil science. Agron. J. 106, 1–17.

Van Aarle, I.M., Olsson, P.A., 2003. Fungal lipid accumulation and development of mycelial structures by two arbuscular mycorrhizal fungi. Appl. Environ. Microbiol. 69, 6762–6767.

Vestberg, M., Polojärvi, A., Pitkänen, T., Kaipainen, S., Puolakka, E., Keskitalo, M., 2012. Neutral lipid fatty acid analysis is a sensitive marker for quantitative estimation of arbuscular mycorrhizal fungi in agricultural soil with crops of different mycotrophy. Agric. Food Sci. 21, 12–27.

Zelles, L., 1997. Phospholipid fatty acid profiles in selected members of soil microbial communities. Chemosphere 35, 275–299.

Zelles, L., Bai, Q.Y., Ma, R.X., Rackwitz, R., Winter, K., Beese, F., 1994. Microbial biomass, metabolic activity and nutritional status determined from fatty acid patterns and poly-hydroxybutyrate in agriculturally managed soils. Soil Biol. Biochem. 26, 439–446.