



Short communication

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

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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.

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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 16:1 ω 5cis 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 16:1 ω 5cis, 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 16:1 ω 5cis and 18:1 ω 5cis 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

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density is most closely correlated to NLFA 16:1 ω 5cis and (2) root colonization is most closely correlated to PLFA 16:1 ω 5cis.

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 \times 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 \pm 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 H₂O) 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 \times 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 M Ω . 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 trionadecanoin 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 (μ g/g)	K ^a (μ g/g)	Mg ^a (μ g/g)	Ca ^a (μ g/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

^aMehlich 3 Extractant.

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