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SHORT NOTE

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Quantification of arbuscular mycorrhizal fungi activity by the glomalin concentration on hyphal traps

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Abstract Strips of horticultural film (16–32 cm^2) were used to trap extraradical hyphae emanating from roots of sudangrass [*Sorghum sudanense* (Piper) Staph] enclosed in 40-μm mesh bags and colonized by *Gigaspora rosea* FL 224-1, *Glomus intraradices* EY 113/114, or *Glomus caledonium* UK 301-1. Strips of film were placed at opposite sides of 17–21 replicate sand culture pots for each isolate and were removed after 12–14 weeks of plant growth. To extract glomalin, a strip was cut into small pieces and submerged in 2 ml of 20 mM citrate, pH 7.0 and then autoclaved for 60 min. A quantitative enzyme-linked immunosorbent assay (ELISA) detected 0.005–0.04 μg glomalin in the volume of extract tested. The Bradford protein assay detected 1.25–5 μg of protein in the volume of extract tested. Both assays gave results ranging from 5–40 μg glomalin/cm^2 of film. Protein assay values were correlated with ELISA values ($r=0.6091$, $P\leq 0.001$, $n=118$). Analysis of variance indicated that isolates differed in Bradford protein values ($P=0.001$), but not ELISA values ($P=0.154$). Spatial variability of glomalin deposition ca. 7 cm from roots on opposite sides of pots was indicated by significant paired T tests ($P<0.05$) for protein values for each of the three isolates and ELISA for two isolates. These results indicate that hyphal traps, Bradford protein assay and ELISA are useful to assess hyphal activity over a growing season.

Key words Hyphae · Protein · *Gigaspora gigantea* · *Glomus intraradices* · *Glomus caledonium*

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Introduction

Recent discoveries suggest that glomalin, a glycoprotein (Wright et al. 1998) produced in copious amounts by arbuscular mycorrhizal (AM) fungal hyphae plays a major role in aggregate stabilization (Wright and Upadhyaya 1998). Glomalin on hyphae and in soil was not discovered until a chelating agent at neutral to alkaline pH and heat (121 °C) were used to solubilize the naturally insoluble compound (Wright et al. 1996; Wright and Upadhyaya 1996). There is now a need for a rapid assay to measure differences in temporal and spatial activity of AM glomalin production in soil to relate to cropping practices that will lead to greater aggregate stability. Current methods to assess hyphal concentration in soil require either tedious microscopic examination for hyphal length based upon hyphal diameter as the taxonomic criterion for AM fungi (Miller et al. 1995) or chemical analysis for chitin or ergosterol (Frey et al. 1994). Ergosterol and chitin are not practical parameters for field-collected samples due to their non-specificity for AM fungi (Frey et al. 1994). Also, we have noted (unpublished data) that hyphae of some *Glomus* spp. lyse as plants senesce, and, thus, a measure of hyphal length in soil may not reflect the presence of these species. Here we report the use of plastic horticultural film to trap hyphae, the extraction of glomalin from traps, and the quantification of glomalin by two assays.

Materials and methods

Pot cultures of sudangrass [*Sorghum sudanense* (Piper) Staph] inoculated with *Gigaspora rosea* FL 224-1, *Glomus intraradices* EY 113/114, and *Glomus caledonium* UK301-1 were tested. The isolates were from the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi, J. B. Morton (curator), West Virginia University, Morgantown, WVa, USA. A 1:1 mixture of coarse sand (Q4) and crushed coal of the same particle size was used as the growth medium. The sand was pre-extracted to remove glomalin. Approximately 8 kg of sand was

placed in a large autoclavable container, covered with 50 mM citrate, pH 8.0 and heated at 121 °C for 90 min. The citrate solution was poured off and the sand was thoroughly washed with tap water followed by deionized water, dried then sterilized by autoclaving for 1 h on 2 consecutive days. The coal, tested and found to be free of glomalin, was sterilized as described above.

Twelve seeds and 100 spores were placed in approximately 300 ml of sterile potting mix enclosed in a 40 μm nylon mesh in the center of a 15-cm-diameter pot. The remaining pot volume was filled with the sterile sand-coal mix. A low phosphorus nutrient solution (Millner and Kitt 1990) was supplied at timed intervals during the 16-h daily light cycle (200 μmol/s/m^2) through drip irrigation.

A 20-mil-thick polyethylene horticultural film with micro-funnel holes (16 holes cm^2) (WeedBlock, Easy Gardner, P.O. Box 21025, Waco, Tex., USA), was cut into strips ca. 8.5 × 3 cm. Strips were inserted vertically at opposite outside edges of a pot so that the 3-cm edge of the film was 1 cm below the potting mix surface.

At 12–14 weeks, a sterile spatula was used to move the sand away from the strip before it was removed. Strips were cut into small pieces, placed into glass vials (18-cm diameter, flat-bottom), and 2 ml of 20 mM citrate, pH 7.0 was added. Extraction of glomalin was carried out at 121 °C for 60 min (Wright and Upadhyaya 1996). The citrate extractant containing solubilized glomalin was poured off and analyzed. Three 6 × 3 cm strips that had not been in pots were used as controls.

Glomalin concentration on the plastic film was determined by the Bradford protein assay using 96-well plates as previously described for hyphae and soil extracts (Wright et al. 1996; Wright and Upadhyaya 1996). Extracts were centrifuged at 10 000 *g* for 5 min to remove insoluble material. Bovine serum albumin standards were used in a range of 1.25–5.0 μg/well. The volume of extract tested was 50 or100 μl, and blanks containing 50 or 100 μl of 20 mM citrate were used to correct for citrate in test samples.

The enzyme-linked immunosorbent assay (ELISA) is a modification of the previously reported protocol (Wright and Upadhyaya 1998). Briefly, 1 μl of sample was added to 49 μl phosphate-buffered saline (PBS) in a well of a 96-well polyvinyl chloride plate. Samples were dried at 37 °C. Wells were blocked with 2% (w/v) non-fat milk in PBS for 15 min. The following were incubated for 1 h each at room temperature with extensive washing with PBS + Tween 20 between reagents: monoclonal antibody (MAb) 32B11, biotinylated antimouse IgM antibody with a long spacer arm (Jackson ImmunoResearch, Inc., West Grove, Pa., USA), and streptavidin peroxidase. Color development was with ABTS [2,2' azino-bis(3 ethylbenzthiazoline-6-sulfonic acid)]. A standard curve for 0.005–0.04 μg of glomalin on each 96-well plate was processed along with the samples. Glomalin used to prepare the standard curve had been extracted from a soil and consistently gave the same ELISA value as glomalin extracted from fresh hyphae of various AM fungi. Replicate assays for protein and ELISA were run on the extract of each strip. Concentrations are reported for 1 cm^2 of strip to account for slight differences in sizes of strips.

Presence of hyphae on the horticultural film was assessed by immunofluorescence, using MAb 32Bll. The method was used for assessment of glomalin on hyphae attached to root pieces (Wright et al. 1996).

Results and discussion

Horticultural plastic strips were effective traps for hyphae and glomalin as revealed by immunofluorescence (Fig. 1). Hyphae from all isolates were attached to both sides of the plastic film and showed typical bright green fluorescence. Glomalin that had presumably sloughed from hyphae was seen as fluorescent material attached to the plastic. Spores showed only yellow autofluorescence. Some sand particles had a smooth green autofluorescence that was easily distinguished from the rough immunofluorescent material typical of glomalin on hyphae or attached to the plastic.

The quantitative ELISA was sensitive and results for standards were reproducible. The ELISA test was more sensitive than the protein assay for individual strips, i.e. reproducible standard curves for ELISA were 0.005–0.04 μg/well of glomalin and 1.25–5.0 μg/well of BSA for the Bradford assay.

The relationship between ELISA and Bradford protein for all strips tested showed a significant linear correlation ($r = 0.6091$, $P \leq 0.001$, $n = 118$). Values for both assays ranged from less than 5–40 μg glomalin/cm^2. Control strips had protein and ELISA values of 0.006 ± 0.001 and 0.004 ± 0.002 μg/cm^2, respectively. Mean values for the results of assays on isolates are shown in Table 1. The general trend was for higher

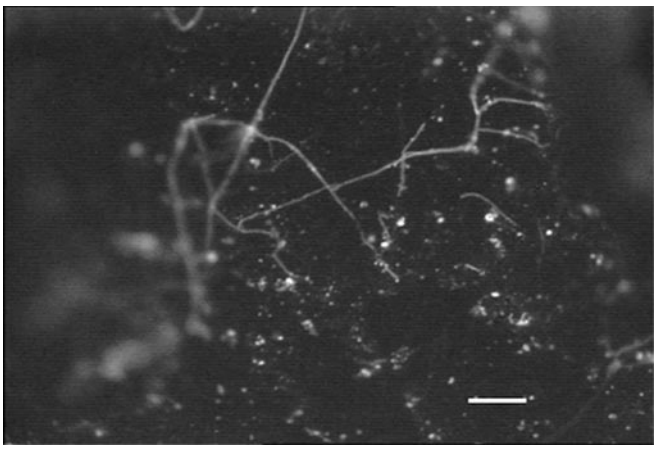

Fig. 1 Typical immunofluorescence of arbuscular mycorrhizal hyphae and glomalin attached to plastic horticultural mesh; *bar* 60 μm

Table 1 Mean values of Bradford protein and enzyme-linked immunosorbent (ELISA) assays for glomalin in extracts of hyphal traps removed from pot cultures 12–14 weeks after inoculation. Means whithin a column followed by the same letter are not significantly different ($P = 0.05$)

Isolate	Trap area (cm^2)	Trap number	Protein (μg/cm^2)	ELISA
Glomus caledonium UK 301-1	20–32	42	22.8 a	16.8 a
Glomus intraradices EY113/114	19–27	34	15.9 b	14.8 a
Gigaspora rosea FL224-1	16–27	42	20.7 a	14.0 a

Bradford protein values than ELISA values. Because pot cultures varied in age by only 2 weeks, an analysis of variance of the means for individual strips was run to compare isolates. There was a significant difference in the protein mean values among isolates ($P=0.001$) but not for ELISA ($P=0.154$). The immunoreactive portion of total protein varied from 68 to 93%. Other isolates that we tested had ca. 100% immunoreactive protein (Wright et al. 1996 and unpublished data). Differences in the ratio of Bradford protein to ELISA need to be studied on individual isolates over time to determine whether culture conditions or isolates vary in the production of the immunoreactive fraction.

Variation in deposition of glomalin on strips at opposite sides of pots was tested by a paired *T*-test on all replicates of each isolate. There were significant *T* test values ($P<0.05$) for protein deposition by all isolates. ELISA *T* test values were significant ($P<0.03$) except for *Glomus caledonium* where $P=0.13$. These results indicate spatial variability in deposition of glomalin ca. 7 cm from roots.

The Bradford protein assay is easy and rapid. Concentration of glomalin in extracts tested for the current work were within a range detectable using either 50 or 100 μl of extract. However, lower concentrations of glomalin on strips may be encountered and would require concentration of the extract. The ELISA requires precision pipetting equipment for 1–2 μl of extract, the monoclonal antibody against glomalin, a variety of reagents, and more time than the protein assay.

We propose the use of both ELISA and Bradford protein assay for field experiments to determine the activity of glomalin-producing hyphae. Evidence to date indicates that glomalin is a very stable molecule in soil under no-tillage management of corn (manuscript submitted), but degradation of the molecule undoubtedly also is a factor under field conditions. Glomalin assays from a variety of soils show that the immunoreactive fraction is more closely related to aggregate stability than total glomalin (Wright and Upadhyaya 1998). By measuring both total and immunoreactive glomalin from traps, we may be able to get more information about both deposition of these fractions over short periods of time and degradation of the molecule after deposition. A measure of glomalin produced over a growing season in a defined area by the AM fungi community could be used to indicate agricultural management practices contributing to glomalin accumulation. Plastic strips enclosed in soil-filled bags of nylon mesh that exclude roots could be used to indicate the concentration of glomalin from extraradical hyphae in field soil. The total area of traps and size of traps required for comparisons over time in field experiments needs further study because spatial variability is inherent in hyphal distribution. Other types of horticultural film may work as well as the polyethylene film used in this study, but preliminary testing of a product is recommended.

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