December 2005

COMPARISON OF GLOMALIN AND HUMIC ACID IN EIGHT NATIVE U.S. SOILS

K. A. Nichols
USDA-ARS

S. F. Wright
USDA-ARS

0038-075X/05/17012-985-997
Soil Science

December 2005
Vol. 170, No. 12
Printed in U.S.A.

COMPARISON OF GLOMALIN AND HUMIC ACID IN EIGHT NATIVE U.S. SOILS

K. A. Nichols[1] and S. F. Wright[2]

Two important extractable fractions of soil organic matter (SOM) are humic acid (HA) and glomalin-related soil protein (glomalin). Optimizing the purity of each fraction is necessary to correlate fraction quantity and molecular characteristics with soil quality. Manipulation of extraction sequence and controlled precipitation of HA were used to evaluate co-extraction of HA and glomalin. Eight bulk soil samples (0 to 10 cm depth) were collected from four U.S. states (Colorado, Nebraska, Maryland, and Georgia). In Experiment 1, glomalin extraction (50 mM citrate, pH 8.0, at 121 °C) was followed by HA extraction (0.1 *N* NaOH at room temperature), and Experiment 2 used the reciprocal sequence. Experiment 2 HA was precipitated stepwise at pH levels 2.5, 2.0, and 1.0. Gravimetric weight, Bradford-reactive soil protein (BRSP), and immunoreactive soil protein (IRSP), along with percentages of C, N, H, and Fe, were used to compare glomalin and HA. The HA fraction from Experiment 2 contained 2-fold greater amounts of BRSP than HA from Experiment 1 and showed that pre-extraction of glomalin improved the purity of HA. The glomalin fraction from Experiment 1 contained 1.5 times the BRSP of glomalin from Experiment 2 and was twice the gravimetric weight. BRSP and gravimetric weight were concentrated in HA that precipitated at pH 2.5 or 2.0 and percentage IRSP was significantly higher in the pH 2.5 precipitate. The results indicate that glomalin should be extracted first and examined as a biomolecule separate from the humic acid mixture. Percentages C, H, N, and Fe in glomalin varied across soils and experiments. In seven soils, the changes in Fe percentage in glomalin from Experiment 1 to 2 were significantly correlated with the changes in glomalin weight and %C. Iron in glomalin from Experiment 1 was related to soil pH and clay content, whereas soil organic C was positively and significantly correlated with Experiment 1 glomalin BRSP and IRSP. In Experiment 1, a recalcitrant pool of glomalin was released by treating soil with NaOH, suggesting that a fraction of glomalin is difficult to remove from soil and glomalin extraction efficiency could be improved. Refinements to extraction and purification protocols such as pretreatment of soils with HCl and sequential extraction can facilitate studies on organic matter structure and function. (Soil Science 2005;170:985–997)

Key words: Organic matter, humic substances, soil carbon, glomalin.

ELUCIDATING the quantities and compositions of extractable soil organic fractions will help clarify processes involved in soil stabilization and C storage. Humic substances (HS), which include humic acid (HA), fulvic acid (FA), and humin, are formed by the decomposition of plant and animal debris, microfauna, biowastes, and other organic materials in the soil (Burdon, 2001; Hayes and Clapp, 2001). Soil quality factors attributed to HS include improved buffering capacity, increased moisture retention, and enhanced spring warming. HS also reportedly

[1]ARS-USDA Northern Great Plains Research Laboratory, 1701 10th Ave. SW, P.O. Box 459, Mandan, ND 58554. Dr. Nichols is corresponding author. E-mail: nicholsk@mandan.ars.usda.gov

[2]ARS-USDA Sustainable Agricultural Systems Laboratory, Bldg. 001, Room 140, BARC-West Beltsville, MD 20705.

Received Oct. 28, 2004; accepted July 18, 2005.

DOI: 10.1097/01.ss.0000198618.06975.3c

function as reservoirs of plant-available micronutrients, bind metals to alleviate both heavy metal toxicity and deficiency, bind clays and other small organic molecules to form aggregates, and act as electron shunts in microbial and abiotic redox reactions (Burdon, 2001; Fan et al., 2000; Hayes and Clapp, 2001; MacCarthy, 2001).

Analytical techniques such as nuclear magnetic resonance (NMR), gas chromatography–mass spectroscopy, and thermochemolysis have been applied to HS to identify functional groups and match function with chemistry (Hatcher et al., 2001). These techniques confirm that other molecules, such as amino acids, carbohydrates, and lipids frequently are co-extracted with HS (Hatcher et al., 2001, Kingery et al., 2000; Simpson, 2001). These associated organic molecules—proteins, carbohydrates, and lipids—have chemical compositions that may provide buffering capacity, assist in redox reactions, and bind organic matter, metals, and clays—functions that have been attributed to HS (Burdon, 2001).

Humic acid is the abundant, alkaline-soluble, and acid-insoluble dark brown to black extractable component of HS. Whether HA is a super-mixture of low molecular weight molecules (Hayes and Clapp, 2001, MacCarthy, 2001) or a high molecular weight complex (Schulten and Schnitzer, 1997) has not been resolved. Studies of HA quantity, structure, and function begin with material extracted nonspecifically from soil using NaOH or sodium pyrophosphate at room temperature (RT) (Swift, 1996).

Glomalin-related soil protein, referred to in this manuscript as glomalin, is a nonspecifically extracted, alkaline-soluble soil C pool that is linked to arbuscular mycorrhizal (AM) fungi (Rillig, 2004, Wright et al., 1996). This red-brown glycoprotein does not have co-extracted or attached tannins (Rillig et al., 2001). According to ^{1}H NMR experiments, glomalin has a consistent organic structure across soils and in sterile glomalin-free sand pot cultures that is different from HA (Nichols, 2003). The glomalin extraction protocol consists of incubating soil in 20 to 50 mM sodium citrate at an alkaline pH and 121 °C for 1-hour intervals (Wright and Upadhyaya, 1996). This is in contrast with RT extraction of HA (Swift, 1996). Glomalin precipitates from solution at pH 2.5 to 2.0 (Nichols, 2003), a pH level where a high protein fraction of HA has been isolated (Hayes and Clapp, 2001; Hayes and Graham, 2000).

Glomalin appears to have properties and functions similar to fungal hydrophobins (Nichols, 2003; Nichols and Wright, 2004; Wright and Upadhyaya, 1996), which are small, self-aggregating, hydrophobic proteins found on hyphae of many types of fungi, including ectomycorrhizal fungi (Wessels, 1997). Hydrophobins protect hyphae from solute loss, allow hyphae to grow through soil and maintain turgor pressure while crossing air- or water-filled pores (Wessels, 1997).

Glomalin sloughs from hyphae (Wright, 2000) and accumulates in soils (Rillig et al., 2001; Wright and Upadhyaya, 1996). Accumulation is thought to result from the insolubility, hydrophobicity and high Fe content of the molecule. Iron concentrations of 0.8 to 8.8% (Wright and Upadhyaya, 1998), may protect glomalin from degradation by as proposed for the role of Fe in organic matter (Hassink and Whitmore, 1997) and may increase the thermal stability and antimicrobial properties of glomalin (Paulsson et al., 1993).

The objectives of the current work were to (1) determine if extraction-protocol–defined fractions of both glomalin and HA can be isolated from the same soil sample, and if so, can they be isolated separately or are they co-extracted; (2) test sequential extraction protocols and stepwise precipitation to isolate glomalin and HA if co-extraction occurs; (3) compare C, N, H, Fe, and protein concentrations of glomalin and HA; and (4) compare amounts of glomalin and HA to soil properties, such organic C, clay, P, and Fe concentrations that may affect glomalin and HA accumulation and function.

MATERIALS AND METHODS

Soils

Bulk soil samples (0 to 10 cm depth) were collected with a shovel at two sites in each of four states: Maryland (MD), Nebraska (NE), Colorado (CO), and Georgia (GA) (Table 1). Soil was freshly collected at each site except for the NE soils that had been stored at RT for 3 years. Each site had native vegetation.

Soils were air-dried and sieved to collect material < 2 mm. Selected soil characteristics are shown in Table 1. Cation exchange capacity, pH, P, sand, and clay concentrations were measured by the Soil Testing Laboratory at the University of Maryland. Phosphorus concentration was measured with a colorimetric assay on solution extracted from soil using the Mehlich I method (Mehlich, 1953). Soil pH was measured

TABLE 1
Characteristics of eight soils extracted for glomalin and humic acid

Soil series	Baltimore (site *a*)	Baltimore (site *b*)	Sampson	Haxtun	Pacolet	Cecil	Wymore	Pawnee
Location	Maryland	Maryland	Colorado	Colorado	Georgia	Georgia	Nebraska	Nebraska
Soil type	Mollic Hapludalf	Mollic Hapludalf	Pachic Argiustolls	Pachic Argiustolls	Typic Kanhapludult	Typic Kanhapludult	Aquertic Argiudolls	Oxyaquic Vertic Argiudolls
Textural class	loam	loam	loam	sandy loam	sandy clay loam	sandy loam	silty clay loam	silt loam
Vegetation	Hardwood forest	Hardwood forest	Tall- and mid-grass prairie	Tall- and mid-grass prairie	Long-term tall fescue pasture	Hardwood forest	Tall- and mid grass prairie	Tall- and mid-grass prairie
Sand (g kg^{-1})	260	270	480	780	610[a]	750	150	210
Clay (g kg^{-1})	250	260	160	100	220[a]	110	280	200
pH	5.1	5.0	6.7	6.6	5.4	4.4	5.5	5.6
CEC[b] (cmol kg^{-1})	4.6	4.0	17.3	8.2	NA	4.2	NA	15.3
Organic C (g kg^{-1})	30.3	26.6	27.9	7.0	38.0	29.4	20.9	24.2
Fe (g kg^{-1})	15.4	16.9	4.5	2.9	17.8	4.8	6.0	5.2
P (mg kg^{-1})	23.4	29.4	232.8	123.6	30.4	21.4	42.4	20.4

[a]Values from Franzluebbers et al., 2000.
[b]NA, Insufficient amount of sample for this analysis.

in 1:1 (wt/vol) 0.01 *M* $CaCl_2$ solution. Soil texture was measured using the pipette method (Day, 1965). Total organic C, N, and H was measured by combustion with a Perkin-Elmer Series II C, H, N, S/O 2400 Analyzer (Shelton, CT) on soil treated with 0.1 *N* HCl.

Iron was extracted from soil by a modified Aqua Regia (McGrath and Cunliffe, 1985) procedure and quantified by AA (atomic absorption). Briefly, concentrated HNO_3 was added to the sample and heated to 85 to 90 °C (a temperature high enough to cause evaporation but not boiling) for 2 hours. Next, concentrated HCl (1:3 HNO_3:HCl) was added followed by incubation at 60 °C for 1 hour. After hydrolysis, samples were decanted through a Whatman 1 filter into a volumetric flask and brought to volume with deionized water (dH_2O). Iron concentration was measured with a Varian atomic absorption spectrometer (AA-400, Palo Alto, CA) with deuterium background correction.

Extraction of Glomalin

Glomalin was extracted with 50 mM sodium citrate, pH 8.0, at 121 °C for 1 hour (Wright and Upadhyaya, 1999). Samples were centrifuged and the supernatant was decanted and saved. The procedure was repeated until the supernatant was straw-colored (up to 3 more times). Supernatants from each 1-hour extraction cycle were combined and centrifuged. A 1-mL subsample was removed for protein assays (see below), and the remainder was flocculated at pH 2.0 to 2.5 by slowly adding 1 *N* HCl, the solution was placed on ice for 45 minutes, and the precipitate was pelleted by centrifuging. The pellet was dissolved in a minimum amount of 0.1 *M* NaOH and immediately dialyzed against dH_2O in dialysis tubing with molecular weight cutoff of 8,000 to 12,000 Daltons (D). Proteolysis of glomalin by exposure to 0.1 *N* NaOH was not detected by ninhydrin (unpublished data). Water in the dialysis chamber was changed at least 5 times with 8- to 12-hour incubation periods each time. Dialyzed material was centrifuged and the supernatant was collected and freeze dried. All centrifuging was carried out at 6,850*g* for 10 minutes.

Extraction of Humic and Fulvic Acids

The International Humic Substances Society method described bySwift (1996) was used to extract HA and FA. Modifications to the method were in sample size (2 g instead of 50 g) and in the purification steps. Briefly, soil was

pre-incubated in 1 *N* HCl followed by a multi-step extraction procedure: (i) extraction with 0.1 *N* NaOH at RT under N_2 overnight; (ii) centrifuging to collect the supernatant; (iii) acidification of the supernatant; (iv) precipitation of HA overnight; and (v) separation of HA (precipitate) from FA (supernatant) by centrifuging. The NaOH extraction followed by acidic separation was repeated until the solution was almost clear (two more times) to assure that all humic and fulvic acids were extracted. Protein was not detected in FA by the Bradford soil protein assay (see below), so there was no further analysis of this fraction. All centrifuging was carried out at 6,850*g* for 10 minutes.

Insoluble solid particles were removed from HA by redissolution in a minimum volume of KOH under N_2, addition of KCl (until $[K^+] \geq 3$ M), and centrifuging at 10,844*g* to remove suspended solids. HA was precipitated with HCl. After settling overnight, samples were centrifuged and the supernatant was discarded. Precipitated HA was suspended in an HCl/HF solution, incubated overnight, and collected by centrifuging at 6,850*g*. The supernatant was discarded. The HCl/HF treatment was repeated twice. Residual acid was removed by repeatedly washing the precipitate with dH_2O and centrifuging at 10,844*g* for 3 minutes.

Precipitated HA was redissolved in a minimal measured volume of 0.1 *N* NaOH. A subsample (0.5 mL) was removed for protein assays (see below), and the remaining solution was acidified rapidly to precipitate HA. Acid was removed from the precipitate by centrifuging at 10,844*g* and washing with dH_2O. The precipitate was freeze-dried.

Experiment 1: Citrate Extraction Followed by Sodium Hydroxide Extraction

Five 2-g samples per soil were citrate-extracted for glomalin followed by NaOH extraction of HA. Residual soil (soil remaining after sequential extraction of glomalin followed by HA) from all soils except Pawnee was re-extracted with citrate to determine whether the NaOH treatment to extract HA facilitated the release of a recalcitrant pool of glomalin (R. glomalin).

Experiment 2: Sodium Hydroxide Extraction Followed by Citrate Extraction

Humic acid was extracted from five, 2-g samples per soil, and the remaining soil was extracted with citrate.

Stepwise Precipitation of Glomalin in HA

A subsample of the purified and freeze-dried HA from each soil from Experiment 2 was extracted with citrate to assess co-extraction of glomalin. Trace amounts of citrate-insoluble material were collected by centrifuging at 10,844*g*, and the supernatant was titrated in steps: (1) pH 2.5 (HA2.5), (2) pH 2.0 (HA2.0), and (3) pH 1.0 (HA1.0). At each step, the precipitate was collected by centrifuging at 6,850*g* after a 30-minute incubation on ice. After step 3, the supernatant was discarded. Each precipitate was redissolved in 0.1 *N* NaOH and dialyzed against water in dialysis tubing with molecular weight cutoff of 500 D. After dialysis and centrifuging at 6,850*g* for 10 minutes, the supernatant was collected and freeze-dried. A subsample was collected for the protein assays (see below) by dissolving the freeze-dried fractions in dH_2O at neutral pH.

Protein Assays

Bradford reactive soil protein (BRSP) and immunoreactive soil protein (IRSP) (Rillig, 2004) concentrations were measured on subsamples (collected as described above) of glomalin and HA from Experiments 1 and 2, R. glomalin from Experiment 1, and the three fractions of HA collected at different pH levels in Experiment 2.

A modified Bradford protein assay (Wright et al., 1996) was used to measure BRSP concentration using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), which detects proteins >3,000 to 5,000 D (Bio-Rad Protein Assay, LIT33 Rev C). IRSP was measured by enzyme-linked immunosorbent assay (ELISA), as described by Wright and Upadhyaya (1998) with modifications in the enzyme and color developer. ExtrAvidin (Sigma-Aldrich, Inc.) phosphatase was used instead of peroxidase. Wells were rinsed with Tris [Tris (hydroxymethyl) aminomethane]-buffered saline with Tween 20 (polyoxyethylenesorbitan monolaurate) before adding the color developer, *p*-nitrophenyl phosphate in diethanolamine buffer (Wright, 1994). Absorbance was read at 405 nm after 15 minutes. Test samples were compared with a standard curve produced by dilutions of highly immunoreactive glomalin extracted from a temperate soil under native grasses. Percent immunoreactivity was calculated as amount of IRSP divided by amount of BRSP multiplied by 100.

Gravimetric Weight

All purified and freeze-dried samples—glomalin, HA, R. glomalin, HA 2.5, HA 2.0 and HA 1.0—were weighed to the nearest 0.1 mg. Gravimetric weights were reported as g kg^{-1} soil. Soil before (Initial Soil) and after (Residual Soil) extraction was dried at 70 °C, weighed, ground with a mortar and pestle, and stored under vacuum in a desiccator.

Elemental Composition

Extracted, freeze-dried organic matter fractions—glomalin, HA, R. glomalin, HA2.5, HA2.0 and HA1.0—and Initial and Residual Soil were analyzed for percentages C, N, and H. Iron was measured on glomalin, HA, R. glomalin, and on soil before extraction. Carbon, N, and H contents were measured by combustion with a Perkin-Elmer Series II C, H, N, S/O 2400 Analyzer. Iron concentration was measured by AA on samples hydrolyzed using the Aqua Regia procedure (see above). When hydrolysis was incomplete, the nonhydrolyzed material was collected by centrifuging at 10,844*g* and the dry weight was subtracted from the weight of the original sample before calculating percentage Fe.

Statistical Analysis

Gravimetric weight values were corrected for subsamples removed for protein measurement and/or secondary extractions. Means and standard error of the mean were calculated for all soils combined. All mean values were compared across experiments for all five fractions (three from Experiment 1 and two from Experiment 2) or between the three HA fractions from Experiment 2 that were precipitated at three pH levels. All means comparisons were made at the $\alpha \leq 0.05$ level by analysis of variance, using restricted maximum likelihood) after the residuals met the assumptions for normality and homogeneity of variance. When needed, data were transformed to meet the assumptions.

Pearson product-moment correlation coefficients (r) were calculated for gravimetric, BRSP and IRSP weight, and percentages C and Fe in glomalin or HA from Experiment 1 and soil concentrations of organic C, clay, Fe and P and pH level, and in a comparison of changes between Experiments 1 and 2 in glomalin %Fe, %C, and gravimetric weight. Data were checked for a normal distribution and transformed when necessary before analysis. All statistical analyses were performed using SAS software, version 8 (SAS Institute, 1999) or Statistix 8 Analytical Software (Tallahassee, FL).

RESULTS AND DISCUSSION

Glomalin and HA Isolation

Extraction-defined glomalin and HA fractions were isolated from all eight soils in both extraction sequences. Extraction of glomalin in Experiment 2 and isolation of R. glomalin after extraction of HA in Experiment 1 confirmed that glomalin is a different fraction from HA and is primarily in the "insoluble" humin fraction.

The fraction defined as R. glomalin was detected after comparing Residual Soil C values for Experiment 1 (data not shown) with Residual Soil C values from Experiment 2. High C values for Experiment 1 Residual Soil suggested that a fraction of organic matter, possibly glomalin, remained in soil even after extensive extraction with citrate and NaOH. Re-extraction of Experiment 1 Residual Soils from seven sites with hot citrate yielded the BRSP and IRSP fraction classified as R. glomalin (Table 2 and Fig. 1). The R. glomalin pool may be an encapsulated fraction of glomalin that could accumulate in soil and is only released from soil after treatment with NaOH. Further work is needed to define this glomalin fraction.

Percentages of C, H, and N in the Residual Soil decreased from the Initial Soil values by an average of 50, 80, and 33%, respectively, in Experiment 1 and 69, 80, and 66%, respectively, in Experiment 2 (Table 3), indicating that at least half of the organic matter was extracted using these procedures.

Glomalin and HA Co-Extraction Related to Extraction Sequence

Protein Values

Mean BRSP values for glomalin in Experiment 1 were higher than for any of the other fractions from Experiment 1 or Experiment 2 (Table 2). Humic acid BRSP concentrations showed significantly higher mean BRSP values in Experiment 2 than Experiment 1. When the extraction sequence maximized HA, BRSP in the glomalin fraction increased in the Sampson, Wymore, and Pawnee soils by 1, 59, and 27%, respectively, but decreased by 60 to 69% in the other five soils. The largest (3- to 4-fold) differences between BRSP in HA for the two experiments were in Maryland and Georgia

TABLE 2

Bradford-reactive soil protein and immunoreactive soil protein concentrations in glomalin, humic acid (HA), and recalcitrant glomalin (R. glomalin) extracted from eight native US soils when glomalin concentrations were maximized by extracting glomalin first (experiment 1) or when HA values were maximized by extracting HA first (experiment 2)[a]

Experiment	Fraction	Soils								Mean[b]
		MDa	MDb	Sampson	Haxtun	Pacolet	Cecil	Wymore	Pawnee	
		Total protein (g kg^{-1} soil)								
1	Glomalin	2.53	1.61	1.79	1.63	4.05	3.14	1.76	1.87	2.3 ± 0.3[a]
	HA	0.41	0.37	0.78	0.23	0.90	0.36	0.75	0.66	0.6 ± 0.1[c]
	R. glomalin[c]	NA[a]	NA	0.15	NA	2.94	1.77	0.22	ND[a]	1.3 ± 0.7[bc]
	Total	2.94	1.98	2.72	1.86	7.89	5.27	2.73		
2	HA	1.74	1.51	0.87	0.59	2.70	1.39	0.84	0.92	1.3 ± 0.2[b]
	Glomalin	0.78	0.55	1.81	0.71	1.61	1.10	2.80	2.37	1.5 ± 0.3[ab]
	Total	2.52	2.06	2.68	1.30	4.31	2.49	3.64	3.29	
		Immunoreactive protein (g kg^{-1} soil)								
1	Glomalin	1.41	0.92	0.59	0.26	1.51	0.60	0.23	0.29	0.7 ± 0.2[a]
	HA	0.01	0.01	0.28	0.07	0.27	0.13	0.00	0.00	0.1 ± 0.0[b]
	R. glomalin[c]	NA	NA	0.30	NA	0.56	0.24	0.00	ND	0.2 ± 0.1[b]
2	HA	0.13	0.15	0.14	0.08	0.13	0.12	0.11	0.12	0.1 ± 0.0[b]
	Glomalin	0.39	0.22	0.61	0.49	0.57	0.33	0.77	0.66	0.5 ± 0.1[a]

[a]NA, Samples without enough material for analysis; ND, samples not collected.

[b]Mean ± SE. Different letters within a column for each assay indicate significant differences ($P > F = 0.0014$ for total protein and <0.0001 for immunoreactive protein) according to REML after a log transformation to meet the assumptions of normality and homogeneity of variance.

soils. In Experiment 1, the glomalin fraction contained significantly more BRSP than the HA fraction (Prob > $F = 0.0009$). In Experiment 2 a significant difference between glomalin and HA BRSP could not be determined (Prob > $F = 0.7016$).

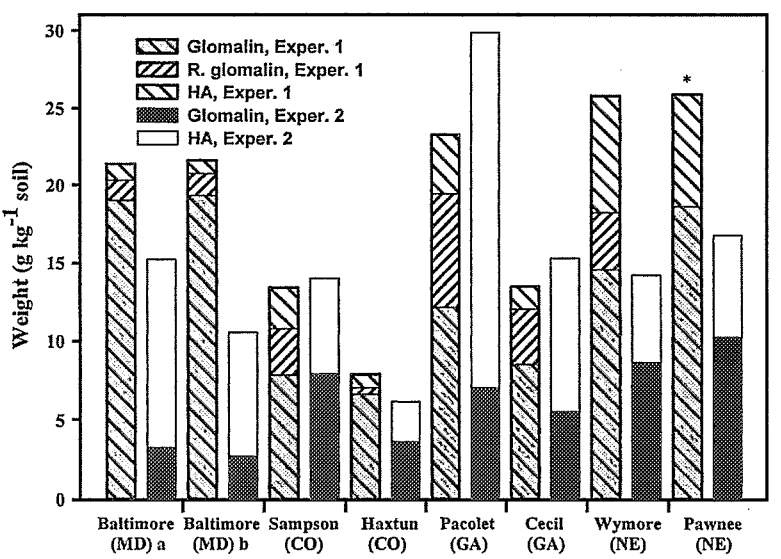

Fig. 1. Weights of glomalin, R. glomalin (extracted after NaOH treatment of soil), and humic acid (HA) extracted from eight native U.S. soils in Experiment 1: citrate extraction of glomalin before NaOH extraction of HA, or Experiment 2: citrate extraction of glomalin after NaOH extraction of HA. *R. glomalin was not extracted from the Pawnee soil.

TABLE 3

Values for elemental analysis [C, Fe, N and H] of glomalin, humic acid (HA), and recalcitrant glomalin (R. glomalin) extracted from eight native US soils in experiment 1: Citrate extraction of glomalin before NaOH extraction of HA or experiment 2: citrate extraction of glomalin after NaOH extraction of HA[a,b]

%	Fraction	MDa	MDb	Sampson	Haxtun	Pacolet	Cecil	Wymore	Pawnee	Means[c]
C	Soil - initial	3.1	2.7	2.8	0.7	3.9	3.0	2.1	2.4	2.6±0.3
C	Exp. 1 glomalin	26.5	24.6	40.7	31.0	36.9	37.3	15.1	15.4	28.4±3.5[b]
C	Exp. 1 residual glomalin	39.7	37.1	47.7	46.5	41.9	42.8	45.9	NA[c]	43.1±1.5[a]
C	Exp. 2 glomalin	40.0	39.1	48.8	44.2	39.0	40.1	47.6	46.1	43.1±1.4[a]
C	Exp. 1 HA	55.0	55.0	53.7	54.6	39.6	53.7	32.7	51.3	49.4±3.0[a]
C	Exp. 2 HA	46.0	50.9	52.1	53.6	38.7	52.8	51.6	48.9	49.3±1.4[a]
C	Exp. 1 soil – residual	1.1	1.3	0.9	0.4	1.6	1.0	1.5	1.1	1.3±0.2
C	Exp. 2 soil – residual	1.1	0.9	0.5	0.2	1.3	0.8	0.9	0.9	0.8±0.1
N	Soil – initial	0.4	0.6	0.6	0.2	0.5	0.7	0.5	0.6	0.5±0.1
N	Exp. 1 glomalin	2.3	2.1	3.9	2.7	4.1	2.9	1.5	1.4	2.6±0.4[c]
N	Exp. 1 residual glomalin	3.2	3.5	4.1	7.3	4.7	2.7	2.8	NA	4.1±0.6[a]
N	Exp. 2 glomalin	2.7	2.9	3.9	3.3	3.2	2.1	3.1	2.8	3.0±0.2[c]
N	Exp. 1 HA	3.4	3.8	4.6	4.5	3.6	3.8	2.3	3.0	3.6±0.3[ab]
N	Exp. 2 HA	3.5	4.0	4.8	4.5	3.6	3.1	4.3	3.8	4.0±0.2[ab]
N	Exp. 1 soil – residual	0.1	0.0	0.1	NA	0.3	0.2	0.2	0.2	0.1±0.0
N	Exp 2 soil – residual	0.0	0.0	0.1	0.2	0.1	0.1	0.2	0.1	0.1±0.0
H	Soil - initial	0.8	0.7	0.6	0.1	1.3	0.5	0.5	0.5	0.6±0.1
H	Exp. 1 glomalin	3.7	3.4	4.6	3.5	4.7	4.6	2.5	2.4	3.7±0.3[b]
H	Exp. 1 residual glomalin	4.7	4.6	6.0	4.6	5.4	5.3	4.4	NA	5.0±0.2[a]
H	Exp. 2 glomalin	4.5	4.5	5.5	4.6	4.4	4.5	3.9	3.5	4.4±0.2[b]
H	Exp. 1 HA	5.5	5.5	5.9	5.6	4.5	6.1	3.0	3.4	5.0±0.4[b]
H	Exp. 2 HA	4.5	4.9	5.3	5.1	4.0	5.1	4.6	3.9	4.7±0.2[b]
H	Exp. 1 soil – residual	0.3	0.3	0.2	0.1	0.9	0.4	0.4	0.4	0.4±0.1
H	Exp 2 soil – residual	0.4	0.3	0.1	NA	0.6	0.1	0.2	0.2	0.2±0.1
Fe	Exp. 1 glomalin	6.1	6.4	0.7	1.7	3.8	3.2	6.0	5.0	4.1±0.8[a]
Fe	Exp. 1 residual glomalin	2.3	NA	NA	NA	1.9	12.3	NA	NA	5.5±3.4[a]
Fe	Exp. 2 glomalin	1.9	1.8	0.2	0.3	1.5	3.1	0.3	NA	1.3±0.4[b]
Fe	Exp. 1 HA	1.0	1.2	0.1	NA	1.0	4.1	1.2	0.1	1.3±0.5[b]
Fe	Exp. 2 HA	0.1	0.1	0.4	0.1	0.2	0.2	5.3	0.1	0.8±0.7[b]

C, N, and H values for soil before (initial) and after extractions (residual) are shown.

[a]ND, not determined.

[b]NA, sufficient material to perform the analysis was not extracted.

[c]Mean ± SE in a column followed by different lowercase letters are significantly different at $\alpha = 0.05$ for separate comparisons of glomalin and HA across experiments.

Immunoreactive soil protein concentrations followed a trend similar to BRSP concentrations (Table 2). Mean values were significantly higher in the glomalin fractions than in the HA fractions from both experiments. The HA fraction had slightly higher IRSP concentrations in Experiment 2 than in Experiment 1 for all soils except the Sampson, Pacolet, and Cecil soils. In both experiments, IRSP values were significantly greater in the glomalin fractions than in the other fractions (Prob > F = 0.0030 and <0.0001, respectively).

Glomalin is a protein and therefore is excluded as a component of pure HA (Burdon,

2001; Hayes and Clapp, 2001). In these experiments, BRSP concentrations showed that both the glomalin and HA fractions contained proteinaceous material. The ELISA, which measures IRSP, uses an antiglomalin monoclonal antibody to measure glomalin concentration (Wright et al., 1996; Wright and Upadhyaya, 1996). IRSP was present in both glomalin and HA from both experiments. However, both BRSP and IRSP values indicated that when glomalin was extracted first (Experiment 1), the HA fraction contained less BRSP and IRSP than when HA was extracted first (Experiment 2).

Previous research has shown that some glomalin is soluble in an alkaline solution at RT (Wright and Upadhyaya, 1996). In addition, both HA and humin contain nonhumic substances such as lipids, carbohydrates, and proteins (Burdon, 2001; Hatcher et al., 2001; Hayes and Clapp, 2001; Kingery et al., 2000; MacCarthy, 2001; Rice, 2001; Simpson, 2001), which may resist humification by physical encapsulation in humic-mineral complexes (Hassink and Whitmore, 1997) or within soil aggregates (Bird et al., 2002; Degens, 1997).

Gravimetric Weights

Gravimetric weight of glomalin and HA varied among soils and across experiments (Fig. 1). When glomalin extraction was maximized (Experiment 1), weights were 7 to 19 g kg^{-1} for glomalin and 0.4 to 7 g kg^{-1} for R. glomalin (Fig. 1). Mean glomalin weight (13.4 g kg^{-1}) was significantly greater than R. glomalin (3.0 g kg^{-1}) or HA (3.1 g kg^{-1}) weights (Prob > F = <0.0001). Maximizing extraction of HA over glomalin (Experiment 2) resulted in HA weight increases of 62 to 92% in all soils, except Wymore (27% decrease) and Pawnee (10% decrease). Mean weight of HA (9.1 g kg^{-1}) in Experiment 2 was not significantly different from glomalin (6.2 g kg^{-1}) (Prob > F = 0.2911). Glomalin from Experiment 1 had a significantly greater weight than all other fractions from both experiments, except HA in Experiment 2 (Prob > F = <0.0001).

These results showed that when glomalin was extracted first, the combined weight of HA and glomalin was 19.5 g kg^{-1} soil (Experiment 1) compared with 15.3 g kg^{-1} soil when HA was extracted first (Experiment 2). Glomalin contributed 69% vs 41% to the total weight for Experiments 1 and 2, respectively, indicating that more glomalin appeared to be co-extracted with HA when HA is extracted first (Experiment 2).

Elemental Concentrations

Concentrations of C, H, N, and Fe in glomalin, R. glomalin, and HA extracted from soils in the same geographic region were similar, but there were wide variations across geographic regions (Table 3). HA had higher C, N, and H percentages but lower Fe percentages than the glomalin fractions across soils and across experiments indicating differences between HA and glomalin molecular structure. Elements in HA were consistent across experiments, except for

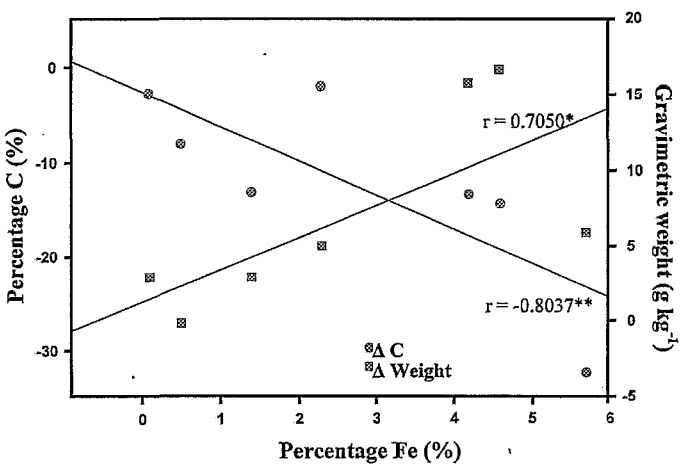

Fig. 2. Change in %Fe in glomalin from Experiment 1 to Experiment 2 compared with changes in %C (ΔC) and gravimetric weight (ΔWeight) for seven soils from four geographic regions of the United States. *,**Significance at 0.10 and 0.05, respectively.

the Wymore soils, and significant differences could not be determined. In glomalin, there were significant differences in C, H, and Fe from Experiments 1 and 2.

Carbon and H percentages in glomalin were significantly higher in Experiment 2 than Experiment 1, whereas %Fe was significantly higher in Experiment 1 than in Experiment 2 (Table 3). A significant, negative linear relationship exists between the change in Fe percentage from Experiment 1 to Experiment 2 and the change in %C, whereas a positive relationship exists between the change in %Fe and gravimetric weight (Fig. 2). A pure glycoprotein is composed primarily of C, N, H, and O, with some possibility of S. In glomalin-free sand pot cultures, glomalin collected from hyphae has C, N, and H percentages of 40.2, 5.7, and 6.5%, respectively (Nichols, 2003). Based on the chemical composition of carbohydrates and proteins, much of the remaining 47.6% of hyphal glomalin should be O. Previous research has shown that glomalin accumulates Fe and other metals, such as Al and Cu, depending on solution or soil concentration of the elements and AM fungal species (Gonzalez-Chavez et al., 2004; Nichols, 2003). Iron concentration of glomalin collected from hyphae (discussed above) is 0.2%, so the remaining 47.4% is O, possibly other metals and ash. The gravimetric weights and %C, N, and H of glomalin from Experiments 1 and 2 indicated that Experiment 1 glomalin contains a high concentration of ash material (Table 3 and Fig. 1).

Although there was a significant difference in %C between Experiments 1 and 2, the ratio of %C to the sum of C, N, and H percentages in Experiment 1 glomalin of 0.816 is similar to 0.853 for Experiment 2 (Table 3). This comparison indicated that the %C in glomalin from both experiments would be similar if ash material were removed. In addition, the ratio of glomalin Experiment 1 BRSP to Experiment 2 BRSP of 2.030 (Table 2) is similar to the ratio of 1.882 for C, N, and H weight (percentage of C, N, and H times gravimetric weight) between Experiment 1 and 2 (Table 3, Fig. 1). These comparisons indicated that the percentages of C, N, and H from both experiments reflected the amount of protein (i.e., glomalin) in the samples, and that almost all of the C, H, and N in glomalin extracts was proteinaceous material.

The higher Fe content in Experiment 1 glomalin compared with Experiment 2 glomalin may have resulted from a combination of two factors. The first factor was pretreatment of the soil in Experiment 2 with HCl before both the HA and glomalin extractions. This treatment would have removed any free or loosely bound Fe from the soil and organic matter. The second factor results from the theoretical structure and properties of glomalin. Hydrophobicity associated with native glomalin is thought to come primarily from hydrophobic amino acids (Nichols, 2003; Nichols and Wright, 2004; Wright and Upadhyaya, 1996) (also unpublished data). On hyphae and soil aggregates, these hydrophobic groups should be oriented to the surface similar to the way amino acids are oriented in fungal hydrophobins (Wessels, 1997). When exposed to high heat for prolonged periods of time, such as during the glomalin extraction procedure, proteins unfold (Creighton, 1993). When a protein cools, it refolds into a thermodynamically stable state. In an aqueous solution, such as citrate, this will be a conformation where hydrophilic groups are oriented to the surface and hydrophobic groups are folded inside (Creighton, 1993). When this happens in glomalin, Fe that is bound to native glomalin would fold into the protein and would not be removed by treatment with HCl or any other competitive chelator (Nichols, 2003; Nichols and Wright, 2004). Lower IRSP values due to the loss of the epitope for the antibody following exposure to high temperature provide further evidence for the conformational changes in glomalin during extraction (Nichols, 2003; Nichols and Wright, 2004; Wright and Upadhyaya, 1996).

Recalcitrant glomalin was higher in C, N, H, and Fe percentages than either Experiment 1 or 2 glomalin (Table 3). The high C, N, and H percentages were speculated to be because the ash content of R. glomalin would be low if previously extracted glomalin and HA co-extracted ash. The R. glomalin fraction possibly is an older fraction of glomalin and one that has accumulated higher concentrations of Fe contributing to its recalcitrance. Also, although soils were treated with HCl before extraction of R. glomalin, the treatment occurred after exposure to the high temperature citrate extraction conditions used to extract the initial Experiment 1 glomalin fraction. This may have kept the Fe in R. glomalin resulting in high Fe values. Further work is necessary to determine how R. glomalin is related to humin. There is also a need to find other extractants might be more effective in releasing all of the glomalin present in a soil.

Stepwise Precipitation of HA to Separate Glomalin and HA

Glomalin was not successfully separated from Experiment 2 HA by citrate extraction of the HA fraction. The stepwise precipitation of HA at pH levels 2.5, 2.0, and 1.0 indicated that it may be possible to separate glomalin and HA by titration between pH 2.5 and 2.0. BRSP and gravimetric weight were concentrated in HA fractions that precipitated at pH levels 2.0 and 2.5, mostly 2.0, whereas significantly higher percentage IRSP was in the HA 2.5 fraction (Table 4).

The HA fraction isolated between pH 2.5 and pH 2.0 byHayes and Clapp (2001) is distinctly different from the other HA fractions collected at other pH levels. According to ^{13}C NMR spectroscopy, this fraction consists mostly of aliphatic hydrocarbon residues and is high in both amino acids (14%) and carbohydrates (6.7%) but low in aromatic groups (Hayes and Clapp, 2001). Glomalin contains amino acid and carbohydrate residues, is low in aromatic groups, and is speculated to contain some hydrophobic, aliphatic groups (Nichols, 2003). This further suggests that the NaOH extraction method used for HA co-extracts glomalin or a glomalin-like molecule. To obtain isolated glomalin and HA fractions, glomalin should be extracted before HA.

These results concur with the conclusion by Hayes and Clapp (2001) and MacCarthy (2001) that it is currently not possible to definitively isolate pore humic acid and assure that no non-humic substances are coextracted. However, pre-treatment of the soil with HCl or a competitive chelator followed by sequential extraction of glomalin and HA and stepwise precipitation may assist isolation and separation of organic matter constituents for future analysis.

Impact of Soil Properties on Glomalin and Humic Acids

Soils examined in these experiments varied in textural and chemical characteristics (Table 1). Soil pH varied from acidic (4.4 for Cecil) to near neutral (6.7 for Sampson), and there were wide variations among soils in organic C, cation exchange capacity, Fe, and P.

Soil organic C was significantly and positively correlated with glomalin BRSP and IRSP concentrations (Table 5). Iron concentrations in glomalin were significantly correlated with soil pH and clay content, whereas Fe in HA was correlated with soil pH. Percentage C in glomalin was correlated with percentage Fe in glomalin, HA IRSP, and percentage C in HA. Humic acid HA weight was correlated with percentage Fe in the soil as well as percentage C in HA. Percentage Fe in HA was related to pH and soil P. Soil P was correlated with HA BRSP.

The results indicate that glomalin and HA concentration is affected by soil properties. Organic matter concentration is correlated with high clay content because Fe- and Al-(hydr) oxides may create bridges between organic matter and clay minerals forming organo-mineral complexes (Degens, 1997; Hassink and Whitmore, 1997). These complexes protect organic matter

TABLE 4

Weights (g kg^{-1} soil), percentage Bradford-reactive soil protein (%BRSP), percentage of BRSP that is immunoreactive (%IRSP), and percentage C for HA collected in experiment 2 and precipitated at pH levels of 2.5, 2.0, and 1.0

HA fraction	MDa	MDb	Sampson	Haxtun	Pacolet	Cecil	Wymore	Pawnee	Mean[b]
				Weight (g kg^{-1} soil)					
pH 2.5	1.15	1.11	0.53	0.23	9.69	0.04	1.59	0.40	1.8 ± 1.1[b]
pH 2.0	6.10	3.51	3.65	2.14	0.10	6.18	3.46	4.99	3.8 ± 0.7[a]
pH 1.0	0.28	0.16	0.02	0.07	0.21	0.13	0.18	0.51	0.2 ± 0.1[b]
				%BRSP					
pH 2.5	17.43	21.05	15.08	1.65	98.96	2.34	30.53	4.10	23.9 ± 11.3[a]
pH 2.0	81.58	77.78	84.64	95.74	0.45	95.34	65.52	92.83	74.2 ± 11.1[a]
pH 1.0	1.00	1.17	0.28	2.61	0.59	2.32	3.94	3.07	1.9 ± 0.5[b]
				%IRSP					
pH 2.5	11.21	11.80	18.67	6.21	9.31	3.21	11.45	16.76	11.1 ± 1.8[a]
pH 2.0	6.14	6.53	5.69	5.51	3.60	4.17	6.91	11.18	6.2 ± 0.8[b]
pH 1.0	2.74	7.10	5.77	2.78	1.51	2.08	0.43	5.14	3.4 ± 0.8[b]

[a]NA, Samples without enough material for analysis.

[b]Mean ± SE in a column and within an experiment followed by different lowercase letters are significantly different at $\alpha = 0.05$ according to REML.

TABLE 5

Correlation coefficients (*r*) comparing gravimetric (weight), total protein (BRSP), and immunoreactive protein (IRSP) weights and percentage iron (Fe) of glomalin and humic acid (HA) from experiment 1 (glomalin extracted before HA) to soil edaphic factors — pH and concentrations of organic carbon (C), iron (Fe), phosphorus (P), and clay[a]

Variables		BRSP	IRSP	C	Fe	Weight	BRSP	IRSP	C	Fe	Clay	pH	Organic	Fe	P
		Glomalin				HA					Soil				
Weight	Glomalin	−0.36	0.24	−0.17	0.15	−0.39	−0.30	−0.30	−0.07	−0.54	0.18	0.35	−0.25	−0.50	−0.20
BRSP			**0.66***	0.47	−0.02	0.06	0.30	0.52	0.00	0.45	−0.07	−0.49	**0.72†**	0.13	0.05
IRSP				0.41	0.24	−0.32	0.14	0.31	−0.21	0.16	0.33	−0.39	**0.77†**	−0.31	−0.10
C					**0.73†**	−0.46	0.01	**0.87‡**	**−0.75†**	0.20	−0.59	0.15	0.30	−0.54	0.23
Fe						0.15	−0.04	**−0.74†**	0.52	0.20	**0.82†**	**−0.65***	0.23	0.34	−0.46
Weight	HA						**0.80†**	0.02	**0.87†**	−0.42	0.36	0.08	0.18	**0.86‡**	0.53
BRSP								0.48	0.57	−0.55	0.39	0.18	0.54	0.59	**0.67***
IRSP									−0.35	−0.07	−0.42	0.29	0.42	−0.16	0.54
C										0.33	0.58	−0.09	0.09	**0.83‡**	0.17
Fe											−0.22	**−0.86†**	0.16	0.01	**−0.70***
Clay	Soil											−0.30	0.37	0.43	−0.15
pH													−0.52	−0.12	0.39
Organic C														0.06	0.34
Fe															0.07

[a]All values were tested for a normal distribution and log or sine transformed when necessary.

*, †, ‡ Denote significance at 0.10, 0.05 and 0.01, respectively.

from decomposition and are speculated to be responsible for the formation of humin (Hayes and Clapp, 2001; Rice, 2001). Multiple soil factors such as soil water, soil texture, P, Fe, and pH may influence the production of glomalin by influencing AM fungal growth. Further work will be required to show the relationship between Fe and clay content in soils, clay mineralogy, and glomalin and HA accumulation.

CONCLUSIONS

Both glomalin and HA fractions may be isolated from the same soil sample. The impact of extraction sequence on glomalin and HA BRSP, IRSP, gravimetric weight, and elemental composition followed similar trends across soils and geographic regions despite wide variations in actual values. Extraction of HA before extraction of glomalin is likely to co-extract glomalin, but protein-free HA was not achieved even by first extracting glomalin with citrate. Extractants other than the nonspecific citrate and NaOH solutions and modifications to the extraction protocol (such as pretreatment of the soil with HCl and removal of ash material from extracts) should be investigated for more thorough extraction, purification, and separation of glomalin and HA. Glomalin content of soils is related to amounts of soil organic C and clay content.

ACKNOWLEDGMENTS

This study is part of a dissertation submitted by K. Nichols to satisfy requirements for a Doctorate of Philosophy at the University of Maryland. This study was partially funded by a grant from the U.S. Department of Energy, Office of Science (DE-FG03–99ER20353), to S.F.W. We thank the following for soils used in this study: Dennis McCallister, Cynthia Johnson, and Allan Franzluebbers. We appreciate the technical assistance of Laurie McKenna and Linda Jawson.

REFERENCES

Bird, S. B., J. E. Herrick, M. M. Wander, and S. F. Wright. 2002. Spatial heterogeneity of aggregate stability and soil carbon in semi-arid rangeland. Environ. Pollut. 116:445–455.

Burdon, J. 2001. Are the traditional concepts of the structures of humic substances realistic? Soil Sci. 166:752–767.

Creighton, T. E. 1993. Proteins: Structures and Molecular Properties. W. H. Freeman and Company. New York, NY.

Day, P. R. 1965. Particle fractionation and particle-size analysis, *in* Black, C. A., ed., Methods of soil analysis, Part 1: American Society of Agronomy, Inc., Madison, WI, p. 545–567.

Degens, B. P. 1997. Macro-aggregation of soils by biological bonding and binding mechanisms and the factors affecting these: a review. Aust. J. Soil Res. 35:431–459.

Fan, T. W.-M., R. M. Higashi, and A. N. Lane. 2000. Chemical characterization of a chelator-treated soil humate by solution-state multinuclear two-dimensional NMR with FTIR and pyrolysis-GCMS. Envrion. Sci. Technol. 34:1636–1646.

Franzluebbers, A. J., S. F. Wright, and J. A. Stuedemann. 2000. Soil aggregates and glomalin under pastures in Southern Piedmont USA. Soil Sci. Soc. Am. J. 64:1018–1026.

Gonzalez-Chavez, M. C., R. Carillo-Gonzalez, S. F. Wright, and K. A. Nichols. 2004. Glomalin: A mechanism for heavy-metal sequestration by arbuscular mycorrhizal fungi. Environ. Pollut. 130: 317–323.

Hassink, J., and A. P. Whitmore. 1997. A model of the physical protection of organic matter in soils. Soil Sci. Soc. Am. J. 61:131–139.

Hatcher, P. G., K. J. Dria, S. Kim, and S. W. Frazier. 2001. Modern analytical studies of humic substances. Soil Sci. 166:770–794.

Hayes, M. H. B., and C. E. Clapp. 2001. Humic substances: considerations of compositions, aspects of structure, and environmental influences. Soil Sci. 166:723–737.

Hayes, M. H. B., and C. L. Graham. 2000. Procedures for the isolation and fractionation of humic substances. *In*: Humic Substances Seminar IV, G. Davies and E. A. Ghabbour (eds.). The Royal Society of Chemistry, Cambridge, pp. 91–109.

Kingery, W. L., A. J. Simpson, M. H. B. Hayes, M. A. Locke, and R. P. Hicks. 2000. The application of multidimensional NMR to the study of soil humic substances. Soil Sci. 165:483–494.

MacCarthy, P. 2001. The principles of humic substances. Soil Sci. 166:738–751.

McGrath, S. P., and C. H. Cunliffe. 1985. A simplified method for the extraction of the metals Fe, Zn, Cu, Ni, Cd, Pb, Cr, Co and Mn from soils and sewage sludges. J. Sci. Food Agric. 36:794–798.

Mehlich, A. 1953. Determination of P, Ca, Mg, K, Na, NH_4. North Carolina Dept. of Agriculture Soil Testing Publication No. 1–53.

Nichols, K. A. 2003. Characterization of glomalin: A glycoprotein produced by arbuscular mycorrhizal fungi. Ph.D. Dissertation. University of Maryland, College Park, MD.

Nichols, K. A., and S. F. Wright. 2004. Contributions of soil fungi to organic matter in agricultural soils. *In*: Functions and Management of Soil

Organic Matter in Agroecosystems. F. Magdoff and R. Weil (eds.). CRC Press, Boca Raton, FL, pp. 179–198.

Paulsson, M. A., U. Svensson, A. R. Kishore, and A. S. Naidu. 1993. Thermal behavior of bovine lactoferrin in water and its relation to bacterial interaction and antibacterial activity. J. Dairy Sci. 76:3711–3720.

Rice, J. A. Humin. 2001. Soil Sci. 166:848–857.

Rillig, M. C. 2004. Arbuscular mycorrhizae, glomalin, and soil aggregation. Can. J. Soil Sci. 84:355–363.

Rillig, M. C., S. F. Wright, K. A. Nichols, W. F. Schmidt, and M. S. Torn. 2001. Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forest soils. Plant Soil. 233:67–177.

SAS Institute. 1999. SAS User's Guide: Statistics, Release 8.0, SAS Inst., Cary, NC.

Schulten, H.-R., and M. Schnitzer. 1997. Chemical model structures for soil organic matter and soils. Soil Sci. 162:115–130.

Simpson, A. 2001. Multidimensional solution state NMR of humic substances: A practical guide and review. Soil Sci. 166:795–809.

Swift, R. S. 1996. Organic matter characterization *In* Methods of Soil Analysis, Part 3 Chemical methods. D.L. Sparks et al. (eds.). SSSA Book Series, No. 5, Madison, WI, pp. 1018–1020.

Wessels, J. G. H. 1997. Hydrophobins: Proteins that change the nature of the fungal surface. Adv. Microbial Physiol. 38:1–45.

Wright, S. F. 1994. Serology and conjugation of antibodies. *In*: Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties R.W Weaver et al. (eds.). SSSA Book Series, no. 5. Madison, WI, pp. 593–618.

Wright, S. F. 2000. A fluorescent antibody assay for hyphae and glomalin from arbuscular mycorrhizal fungi. Plant Soil. 226:171–177.

Wright, S. F., M. Franke-Snyder, J. B. Morton, and A. Upadhyaya. 1996. Time-course study and partial characterization of a protein on hyphae of arbuscular mycorrhizal fungi during active colonization of roots. Plant Soil. 181:193–203.

Wright, S. F., and A. Upadhyaya. 1996. Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi. Soil Sci. 161:575–585.

Wright, S. F., A. Upadhyaya, and J. S. Buyer. 1998. Comparison of N-linked oligosaccharides of glomalin from arbuscular mycorrhizal fungi and soils by capillary electrophoresis. Soil Biol. Biochem. 30:1853–1857.

Wright, S. F., and A. Upadhyaya. 1999. Quantification of arbuscular mycorrhizal fungi activity by the glomalin concentration on hyphal traps Mycorrhiza. 8:283–285.