

ABSTRACT

Title of dissertation: CHARACTERIZATION OF GLOMALIN, A
 GLYCOPROTEIN PRODUCED BY ARBUSCULAR
 MYCORRHIZAL FUNGI

Kristine Ann Nichols, Doctor of Philosophy, 2003

Dissertation directed by: Professor Jay Scott Angle
 Department of Natural Resource Sciences and Landscape
 Architecture

Glomalin is an insoluble glycoprotein produced by hyphae of arbuscular mycorrhizal (AM) fungi. It is resistant to degradation and is found in large amounts in soil. Classical operationally defined extracts of soil organic matter include a large proteinaceous fraction. Therefore, clarification of glomalin as a separate fraction of extractable soil organic matter (SOM) is needed. Proof that glomalin accumulates over long periods of time has not been attempted. The overall hypothesis tested for this dissertation is that

AM fungi are the source of an abundant, unique and important SOM component. The quantity of glomalin in soils was compared with particulate organic matter (POM), glomalin, humic acid (HA), and fulvic acid (FA) which were sequentially extracted from 5 – 8 undisturbed U.S. soils, aggregates and agriculturally managed soils that differed in tillage, crop rotation, and/or fertilizer amendment. Each fraction was extracted with the appropriate procedure: glomalin in pH 8.0 citrate at 121° C, POM by floatation in NaCl solution, and HA and FA in NaOH with acidic separation. Organic matter fractions were evaluated for total and immunoreactive protein and/or gravimetric and C weights. Percentages of C, N and H were used to characterize each fraction. Glomalin structure was examined by proton nuclear magnetic resonance (¹H NMR), removal of iron and separation of amino acid and carbohydrate groups. Glomalin accumulation in pot cultures was assessed at 14-week intervals in a 294-day experiment. Glomalin was unique in protein, C, H, and N contents compared with HA, FA and POM. Glomalin contributed ca. 20% of soil organic carbon. A recalcitrant glomalin pool was discovered that might have a functional role in water-stability of aggregates. ¹H NMR spectra of glomalin were unique compared with HA spectra. Extracted glomalin had tightly bound iron, organic matter, amino acids and carbohydrates. Sustainable agricultural management practices — reduced tillage, increased crop diversity, and reduced synthetic amendments — increased aggregate stability of bulk soil and glomalin and POM concentrations. Glomalin production under controlled conditions was affected by irradiance. These results provide evidence that glomalin is a separate and unique fraction of SOM and is important in terrestrial carbon sequestration and sustainable agricultural practices.

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ARBUSCULAR MYCORRHIZAL FUNGI

by

Kristine Ann Nichols

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Advisory Committee:

Professor Jay S. Angle, Chair
Professor Allen P. Davis
Professor Emmanuel K. Dzantor
Dr. Walter F. Schmidt
Professor Raymond R. Weil
Dr. Sara E. Wright

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LIST OF ABBREVIATIONS

Al	aluminum
AM	arbuscular mycorrhizal
°C	degrees Celsius
C	carbon
Ca	calcium
Cd	cadmium
Cu	copper
cm	centimeter(s)
FA	fulvic acid
Fe	iron
g	gram(s)
h	hour(s)
H	hydrogen
HA	humic acid
HCl	hydrochloric acid
HF	hydrofluoric acid
L	liter(s)
<i>M</i>	molar
min	minute(s)
mL	milliliter(s)
mm	millimeter(s)
<i>mM</i>	millimolar
μl	microliter(s)
μg	microgram(s)
μ <i>M</i>	micromolar
<i>N</i>	normal
N	nitrogen
NaCl	sodium chloride
NaOH	sodium hydroxide
OM	organic matter
P	phosphorus
Pb	lead
POM	particulate organic matter
s	second(s)
SOM	soil organic matter
Zn	zinc

CHAPTER 1

GLOMALIN – A GLYCOPROTEIN PRODUCED BY ARBUSCULAR MYCORRHIZAL FUNGI

Background

Plant and soil health are dependent upon the interactions of biological, physical, and chemical components. The rhizosphere, or root zone, is the location of the greatest flow of energy and minerals among these components (Wright and Millner, 1994). In this highly productive region, a vital symbiosis exists between roots of 80% of all vascular plant species and soil-borne arbuscular mycorrhizal (AM) fungi (Smith and Read, 1997). This mutualistic association has existed for more than 400 million years or since plants first moved from an aquatic to terrestrial environment (Morton, 1990; Simon et al., 1993; Taylor et al., 1995). In this symbiosis, plants benefit by increased uptake of immobile nutrients in soil and improved soil structure (Wright and Upadhyaya, 1998), while the fungus receives photosynthetic carbon and other essential nutrients from the host (Smith and Read, 1997).

Arbuscular mycorrhizal hyphae may colonize up to 80% of plant host root length (Millner and Wright, 2002). At arbuscules within root cells, hexose sugars from the plant are exchanged for nutrients acquired and transported by the fungus. Typically between 12 to 27% of photo-assimilated carbon is given to AM fungi (Tinker et al., 1994). Carbon cost to the plant is balanced by access to a greater volume of soil through fungal hyphae (thread-like projections). Hyphae have a much larger surface area to volume ratio than root hairs and fan out up to 8 cm beyond nutrient depletion zones around roots (Douds and Millner, 1999; Millner and Wright, 2002). This allows AM fungi to scavenge even highly immobile nutrients, such as phosphate. In addition, the fungal cell membrane is capable of concentrating solutes against a gradient (Bolan, 1991; George et al., 1992). The high carbon cost of P uptake is compensated for by an

increase in photosynthetic capability of the host through increased leaf surface area and photosynthetic efficiency (Bolan, 1991; George et al., 1992). Mycorrhiza is the most efficient mechanism for P acquisition, especially under stress conditions. The mycorrhizal symbiosis operates on a continuum between a mutually beneficial relationship and an almost parasitic relationship (where plant host growth declines with AM colonization). Although these fungi are not plant host specific, host and fungal genotypes and soil abiotic and biotic variables have been shown to influence the nature of the symbiosis (Bethlenfalvay et al., 1982; Brundrett, 1991; Gianinazzi et al., 1995; Varma, 1995).

The mycorrhizal symbiosis provides a number of benefits to the plant host. Rapid growth of fine, ephemeral hyphae in microsites containing high concentrations of nutrients such as P, N, Fe, Cu and Zn (Clark and Zeto, 1996; Douds and Millner, 1999; Pawlowska et al., 2000) provides an efficient mechanism for nutrient uptake. The mycorrhizal relationship reduces the growth of plant pathogens, especially fungal pathogens, by increasing host resistance (i.e. triggering a defense response), altering root exudations to stimulate the growth of microbes antagonistic to pathogens, competing for photosynthetic carbon, and reducing the number of infection sites (Borowicz, 2001; Hooker and Black, 1995). The type of pathogen (nematode or fungal), pathogen species, mode of action (necrotrophic or wilt for fungal pathogens and migratory or sedentary for nematodes), and pathogen density help to determine the severity of disease (Borowicz, 2001). As with other benefits in the mycorrhizal relationship, the magnitude and direction of effects on disease resistance are dependent upon host genotype, AM species and isolate, timing of AM colonization, other soil

organisms and abiotic factors. Mycorrhizal fungi may hyper-accumulate toxic heavy metals, such as Cd and Pb, and keep them from the plant host (Gonzalez-Chavez et al., 2002; Diaz et al., 1996). Metal uptake depends upon soil fertility, metal concentration, soil pH, host plant, and AM species and may interfere with P nutrition in the host plant (Gonzalez-Chavez et al., 2002; Diaz et al., 1996).

In addition to improving plant health, mycorrhizal fungi also contribute to soil health. Fungal hyphae improve soil structure by helping to form water-stable soil aggregates (Miller and Jastrow, 1990; Rillig and Steinberg, 2002; Tisdall et al., 1997). Mycorrhizal fungi also improve rhizosphere health by stimulating root exudation which promotes the growth of other soil microbes (Borowicz, 2001; Paul and Clark, 1996). Arbuscular mycorrhizal fungi contribute directly to SOM by accounting for 5 to 50% of the total microbial biomass in soil (Olsson et al., 1999).

Glomalin, a glycoprotein produced by AM fungi

The identification of glomalin, a glycoprotein produced by arbuscular mycorrhizal fungi, has led to a reevaluation of fungal contributions to SOM and aggregate stability. Glomalin was identified at the USDA in the early 1990's during work to produce monoclonal antibodies reactive with AM fungi. One of these antibodies reacted with a substance on hyphae of a number of AM species (Wright et al., 1996). This substance was named glomalin after Glomales, the order to which AM fungi belong. Several other typical soil fungi, such as *Rhizoctonia*, *Gaeumannomyces*, *Endogone*, *Mucor*, and *Phytophthora*, were tested for cross-reactivity with the antibody against glomalin but were not immunoreactive (Wright et al., 1996). The glomalin fraction is operationally defined by its extraction procedure but is further characterized

by total and immunoreactive protein assays (Wright et al., 1996). Glomalin has been found in abundance (typically, 2 to 15 mg g⁻¹ and up to >60 mg g⁻¹) in a wide range of soil environments (acidic, calcareous, grassland and cropland) (Wright and Upadhyaya, 1998; Wright et al, 1999) and appears to be as ubiquitous as AM fungi themselves (Carlile and Watkinson, 1996; Olsson et al., 1999; Wright and Upadhyaya, 1998).

Glomalin was revealed on AM fungal hyphae using an indirect immunofluorescence procedure that employs the antibody against glomalin and a second antibody tagged with fluorescein isothiocyanate (FITC) molecule (Wright, 2000). Evidence that glomalin is produced by AM fungi, not plant roots, was obtained early in the investigation of the reaction of the monoclonal antibody against glomalin. In a blind experiment, immunofluorescence correctly identified glomalin only on roots that were later described as having AM colonization by J.B. Morton (West Virginia University). In more recent work with an axenic culture of transformed carrot roots, glomalin was extracted from hyphae in a root free zone (Rillig and Steinberg, 2002). Glomalin also is routinely extracted from hyphae up to 7 cm away from roots in pot cultures where hyphae are separated from roots by a 38- μ m nylon mesh bag (Wright and Upadhyaya, 1999). Immunofluorescence assays show that glomalin coats AM fungal hyphae, sloughs from hyphae onto colonized roots, organic matter, soil particles, horticultural or nylon mesh, and glass beads, and is found on arbuscules within root cells (Wright et al., 1996; Wright and Upadhyaya, 1999; Wright, 2000).

Several 'pools' of glomalin have been identified based on solubility characteristics: (i) easily extractable glomalin (EEG), (ii) total glomalin (TG) and (iii) a 'scum' at the air-water interface that occurs during harvesting of hyphae from pot-

cultured AM fungi. The EEG 'pool' is extracted using 20 mM citrate, pH 7.0, for 0.5 h (Wright and Upadhyaya, 1998). Total glomalin is extracted with 50 mM citrate, pH 8.0, in 1-h intervals (Wright and Upadhyaya, 1998). When mature sand-based pot cultures are submerged in water, an unattached fraction of glomalin forms tan-colored foam on the surface of water. This 'scum' is apparently a sloughed component of glomalin and is very hydrophobic. We speculate that 'scum' floats on soil water until it attaches to soil or organic matter particles, but the chemistry of this interaction currently is not defined. Hydrophobic and/or cationic interactions may be the mechanisms by which glomalin becomes deposited on soil or organic particles and mesh or glass beads (Wright and Upadhyaya, 1996; unpublished data). Glomalin may move in and out of these operationally defined pools (i.e. EEG becomes scum and scum becomes TG). Steinberg and Rillig (2003) found that following soil incubation to measure decomposition, EEG increased while TG decreased. They speculated that partial degradation decreases sorption of glomalin to soil particles, which may increase solubility and the amount in the EEG pool.

Typically, glomalin concentration in these pools is measured by a Bradford total protein assay (i.e. TG and EEG) and immunoreactive protein (i.e. IRTG and IREEG) assays (Wright et al., 1996). The Bradford protein assay is non-specific and will detect any proteinaceous material. Bradford concentrations are based on comparison with a bovine serum albumin (BSA) standard curve. The immunoreactive protein assay, or enzyme-linked immunosorbent assay (ELISA), uses the monoclonal antibody specific for glomalin, but certain artificial conditions may reduce immunoreactivity. ELISA values are determined by comparison to 100% immunoreactive glomalin extracted from

hyphae or soil (Wright et al., 1996). The total protein assay measures concentrations ranging between 1.25 to 5.0 μg while ELISA numbers range from 0.005 to 0.04 μg (Wright and Upadhyaya, 1999). Since the range of Bradford values is 100 times greater than ELISA numbers, values of over 100% could be supported.

Comparisons of the total and immunoreactive pools of glomalin extracted from soil or pot culture show that not all of the extracted material is immunoreactive. Reduction in immunoreactivity may be due to exposure to conditions that affect the site of binding of the antibody. The reactive site for a monoclonal antibody is very specific (Goding, 1986). In this case, the monoclonal antibody was generated against glomalin on spores of AM fungi and not against extracted glomalin. Therefore, some reactivity is lost probably because of conformational changes during the high temperature (121°C) and long time period (at least 0.5 to 1.0 h) used in the extraction procedure (unpublished data). In the soil, organic matter, metals (such as iron), clay minerals, and other substances may bind to glomalin causing conformational changes or masking the reactive site. Degradation is another factor in soil extracts and may result in a decline in immunoreactivity (Wright and Upadhyaya, 1999). In addition, conformational changes may occur in the molecule when it sloughed from the hyphae and is in the scum pool due to self-aggregation via hydrophobic interactions.

Glomalin is present on extramatrical hyphae of all AMF tested to date (i.e. representatives from all known genera, except *Sclerosystis*) (Wright et al., 1996). As hyphae degrade, this hydrophobic, highly stable glycoprotein sloughs off to coat organic matter and other soil particles. Wright et al. (1996) hypothesize that glomalin forms a conglomeration with root fragments and organic matter, thus protecting it from

degradation by microorganisms. As a result, Wright and Upadhyaya (1998) found a strong correlation between glomalin concentration and soil aggregation.

Glomalin is dark red-brown color and soil after extraction loses the brown color associated with organic matter. The brown color of glomalin was hypothesized to be due to incorporation of iron as a structural component and may play a role in accumulation and/or function (Wright and Upadhyaya, 1998). Following microwave digestion, atomic absorption analysis indicated that the amount of iron in the molecule varies from 0.8-8.8% (Wright and Upadhyaya, 1998). Cultures grown in media that contains low levels of iron had little glycoprotein accumulation and one AM fungal isolate, *Gigaspora gigantea* (MA453A), failed to grow under these Fe-limited conditions. These results are corroborated by field surveys in which glomalin concentration and aggregate stability are low in high pH soils, where iron is less available (Wright and Upadhyaya, 1998). Information on the dynamics of glomalin accumulation on external hyphae, auxiliary cells, spores, or internal structures (intraradical hyphae, arbuscules, vesicles) and soil aggregation has yet to be obtained.

The correlation between glomalin concentration and soil aggregation also may be influenced by iron. Iron- and Al-(hydr)oxides are speculated to be involved in aggregate formation by bridging organic matter to clay minerals and to contribute to the persistence of aggregates (Bird et al., 2002; Wright and Upadhyaya, 1998). Kemper and Chepil (1965) found that aggregate stability is a function of organic matter, clay and free iron oxides.

Foundations for the production of a glomalin (-type) molecule

Evolution of land plants and the environment of early earth

In the early Devonian, at least 400 million years ago, plants moved from the oceans onto land (Taylor et al., 1995). The environment of this early earth was very harsh with few plant-available nutrients and little structure to the soil (Lewis, 1987). Weathering of silicates in an abiotic environment was very slow, due mostly to mechanical and chemical weathering, which in turn were hampered by rapid wind and rain erosion in the absence of crust stabilizing algae, lichens, and more advanced biota (Schwartzman and Volk, 1989). Devonian land plants were morphologically very simple with few leaves, branches, or roots. Evolutionary progress was expressed in variant genotypes with greater nutrient-extraction ability and habits promoting water retention (Pirozynski and Malloch, 1975; Schwartzman and Volk, 1989; Taylor et al., 1995). In this evolutionary process, plants did not readily evolve root hairs to improve efficiency of adsorption. Instead, they exploited their relationship with organisms, such as fungi and algae, to combat nutrient deprivation (Pirozynski and Malloch, 1975; Taylor et al., 1995).

Recent evidence of similar sequences in the SSU rRNA region between several *Glomus* species and *Geosiphon pyriforme* indicates that this may be the precursor for the AM symbiosis in early land plants (Gehrig et al., 1996). *Geosiphon pyriforme* forms a structure for nutrient exchange in its cyanobacterium host, *Nostoc punctiforme*, which is analogous to the arbuscule found in AM fungi (Gehrig et al., 1996).

Ecological, geological, and cytological evidence has shown that there were explosive periods of morphological innovation during the Devonian Period (Gensel and

Andrews, 1987; Schwartzman and Volk, 1989). During this time, photosynthetic free oxygen became more abundant creating the ozone layer to protect land plants from UV radiation (Pirozynski and Malloch, 1975). This allowed plants to flourish on land and the number of genera of plants increased from 1 to 28 with plants changing from tiny creeping structures to a diverse array of 0.5-9 m high structures with leaves, roots, reproductive systems, and secondary growth (Gensel and Andrews, 1987). Algae and fungi have been implicated in the formation of calcareous crusts and biomineralization that made nutrients plant available and increased the rate of soil development (Stubblefield and Taylor, 1988). Soil and plant development would lower P_{CO_2} and surface temperatures for the evolution of more complex life forms (Schwartzman and Volk, 1989).

In mutualistic relationships, both plant host and fungal invader obtain benefits that outweigh the inherent costs of the symbiosis. The mycorrhizal symbiosis that subsequently evolved optimizes Liebig's "Law of the Minimum" (Read, 1991). Carbon, the limiting nutrient for AM fungi, was supplied in abundance by plants whose growth was limited by access to soil minerals, which the fungal hyphae could readily supply (Read, 1991).

As protected root inhabitants, endomycorrhizal fungi have changed little from their ancient relatives, because they have not been subjected to the selective pressures that have caused morphological changes among the plants (Lewis, 1987; Morton, 1990; Stubblefield and Taylor, 1988). Indeed, the fossil record shows that the fungal structures found in the roots of Devonian plants are almost exactly the same as the structures found in the roots of modern plants (Stubblefield and Taylor, 1988; Taylor et

al., 1995). The intimate relationship between plant and fungi is best appreciated if it is realized that these organisms belong to different kingdoms that have evolved separately but in complete interdependence (Wessels, 1997).

In satisfying the need in soils for a persistent material capable of performing a number of functions necessary for plant growth, nature has adopted a simple and elegant solution whereby plants benefit vitally from the ubiquitous, immediately surrounding organic medium resulting from the decay of the plants' predecessors (MacCarthy, 2001). The general functions of humic substances could, in principle, be satisfied by a suite of biomolecules that possess the requisite combination of hydrophilic, acidic, complexing, and sorptive properties, but many of these molecules do not survive for long periods in the environment (Burdon, 2001; MacCarthy, 2001). Therefore, the formation of humic substances or, more likely, glomalin would provide the organic environment that plants need for productive growth. Glomalin would have evolved this function, because supposedly it was first formed when there was no other organic matter in the soil.

Hydrophobins – Proteins that function at an interface

Fungi grow by tunneling their way through moist living and dead substrata. To tunnel effectively, fungi need to build turgor pressure via wall synthesis and protein secretion. To escape a liquid environment (i.e. produce aerial structures such as fruiting bodies or cross pores in soil), fungi need to lower surface tension. Hydrophobins are proteins produced by fungal saprophytes, pathogens, and mutualists (Wessels, 1997; Whiteford and Spanu, 2002). They are produced on the surface of fungal hyphae and help fungi tunnel and escape their typical environment. The level of surface activity of

hydrophobins is in the range of commercial synthetic surfactants, making hydrophobins some of the most surface-active molecules known.

Hydrophobins were first identified by the discovery of mRNAs abundantly transcribed during development processes. Biochemical methods used to detect and measure extractable proteins were not effective in the discovery of hydrophobins because these molecules are highly insoluble. Class II hydrophobins are not even soluble in hot SDS solutions and do not show up in conventional SDS-PAGE analysis.

Hydrophobins are a group of proteins that have a signal sequence for secretion and are easily translocated through the hyphal wall to the surface at the growing hyphal tip by the flow of semi-fluid wall polymers (Wessels, 1997). These proteins self-assemble into amphipathic films at air-water interfaces. The formation of an amphipathic membrane at an air-water interface decreases the surface tension of water (72 to 24 mJ m^{-2}) more than any other known protein (Wessels, 1999). The amphipathic membrane also allows them to convert hydrophobic surfaces to hydrophilic surfaces and visa versa.

A protein is classified as a hydrophobin based on containing eight cysteine residues and a two hydrophobic amino acid sequences, but there is very little amino acid sequence homology even among hydrophobins produced by the same organisms or with the same function in different organisms. Disulfide bridges formed between the cysteine residues prevent premature assembly by keeping the hydrophobin molecule properly folded until it reaches the hyphal surface. Typically four “loops” are found in hydrophobins: two that have mostly hydrophobic residues and two that are hydrophilic (Kershaw and Talbot, 1998; Wessels, 1997). Some hydrophobins are glycosylated,

which may enhance interaction with the hydrophobic surface by inducing the formation of hydrophobic α -helices and/or act as a hydrophilic domain for anchoring (Whiteford and Spanu, 2002). This glycosylation is typically high in mannose.

In the monomer state hydrophobins are not surface active and are harmless to the cellular membranes of the producing fungus. When the monomers self-assemble, they produce a rodlet layer. This rodlet layer is produced at any interface – air-water, water-hydrophobic liquid or solid. Recent evidence, using hydrophobin mutants, shows that rodlet layers are instrumental in generating surface hydrophobicity, but the individual rodlets are too small to contribute to hydrophobicity alone (Kershaw and Talbot, 1998). Lipid interactions in rodlet layer production and in linking rodlets to the underlying cell wall has been proposed as the mechanism in the formation of hydrophobic layers (Kershaw and Talbot, 1998).

Hydrophobins are among the most abundant proteins (> 10% of protein synthetic activity) produced by fungi with many of them being secreted (i.e. fungi sometimes export into the medium more than half of the protein that they make) (Wessels, 1997; Wessels, 1999). Hydrophobins are typically produced at a level of 0.06 to 0.17 g L⁻¹ of media (Askolin et al., 2001). With the introduction of two additional copies of the gene into *Trichoderma reesi*, the hydrophobin was produced at a rate of 0.6 g L⁻¹ of media or 4.3 mg g⁻¹ hyphae without interference in fungal growth or severe foaming (Askolin et al., 2001).

Hydrophobins play a number of key roles in fungal development and the interactions of fungi with the environment and other organisms, particularly with plants. Many roles have been found for hydrophobins in addition to helping in the emergence

of aerial fungal structures. Hydrophobins may: (1) coat spores to prevent clumping and ensure easy dissemination, (2) facilitate the attachment of hyphae to hydrophobic surfaces such as plant or insect cuticles as a precursor to infection, (3) coat hyphae to protect against microbial attack, (4) allow proper gas exchange in fungal air channels, (5) assist in hyphal wall construction, (6) assist in transport between fungus and host in mutualistic relationships, (7) prevent desiccation or water-logging of aerial structures, and (8) assist in communication between a fungus and a plant to elicit various responses.

In ectomycorrhizal fungi, hydrophobins accumulate during active root colonization (i.e. levels increased several fold) where it may play a role in the attachment of hyphae to root surfaces or in hyphal aggregation to form the hyphal mantle (Martin et al., 1999; Tagu et al., 1996; Whiteford and Spanu, 2002). Cell wall proteins, such as hydrophobins, may play additional roles in cytoskeletal changes that result in specific modification of the host tissues to create a favorable niche for the mycobiont (Martin et al., 1999).

Molecules, called repellents, are similar to hydrophobins in that they contain a number of hydrophobic amino acids alternating with hydrophilic, but they do not contain the characteristic eight cysteine residues (Kershaw and Talbot, 1998; Whiteford and Spanu, 2002). Repellents are components of walls of aerial hyphae in *Ustilago maydis*. These proteins do not self-assemble into rodlets like hydrophobins, but they aggregate into a layer with a hydrophobic side and a hydrophilic side. A similar type of oligopeptide is found in the filamentous bacterium *Streptomyces coelicolor* (Kershaw and Tablot, 1998), which highlights the importance of aerial development to the fungal

lifecycle and the need to coat these aerial structures with an amphipathic membrane that can assist in binding hyphae to surfaces.

Transferrins – A class of iron-binding glycoproteins

Lactoferrin, or lactotransferrin, is a member of a group of iron-binding glycoproteins, called transferrins. Transferrins are extracellular proteins that have an affinity for binding heavy metals, especially iron. Lactoferrin is a ~80 kD protein or a dimer with 650 to 700 amino acid residues that is found in all external secretions (including tears and sweat) of most animals but is highest in milk (1-7 mg/ml) (Iyer and Lonnerdal, 1993). It is composed of a single polypeptide chain with 17 disulfide bridges due to a high concentration of cysteine (Paulsson et al., 1993). As a glycoprotein, lactoferrin is ~5% carbohydrate with poly-N-acetyllactosamine glycans that contribute to its stability and help it to survive intact through the digestive system (Iyer and Lonnerdal, 1993). This molecule functions in iron binding and transport, as a growth factor for lymphocytes, as a bacteriostatic molecule, and as a regulator in immune response. Its chelating capability is critical for its biological functions and the overall stability of the molecule (Iyer and Lonnerdal, 1993; Paulsson et al., 1993). This protein is resistant to proteolysis by trypsin or trypsin-like enzymes, high heat (up to ~70°C), and/or low pH with iron-saturated lactoferrin being the more resistant form (Iyer and Lonnerdal, 1993; Paulsson et al., 1993). If the glycosyl groups are removed through deglycosylation, the overall function and binding capabilities of this molecule are not disrupted (Iyer and Lonnerdal, 1993).

Heterogeneity exists in the carbohydrate attached to lactoferrin from different tissues in the same species and from different species (Iyer and Lonnerdal, 1993;

Nagasako et al., 1993; Paulsson et al., 1993). Despite structural homology, protein from different species has different binding characteristics. Iron saturation gives the molecule an overall positive charge and may produce several bands in a SDS-PAGE gel depending on iron concentration and/or degree of degradation or deglycosylation (Nagasako et al., 1993)

Crystallographic imaging shows that this molecule folds into two globular lobes, each containing an iron-binding site consisting of four conserved residues: aspartic acid, two tyrosines, and a histidine. When iron binding occurs, this dimer changes conformation and the N-terminal domains fold around these iron atoms in a hinge-like motion, keeping iron irreversibly bound over a pH range from 4-7. One dimer can also bind up to 1400 more iron atoms with additional iron-binding at surface electron donating groups, such as imidazole, thiol, and indole found in histidine, cystine, and tryptophan residues (Nagasako et al., 1993). These atoms are less tightly bound and may be released into solution. This protein typically has a creamy color but will be red to salmon-pink when saturated with iron. Lactoferrin is able to stabilize the bioavailable ferrous iron and keep it from being changed into the insoluble ferric iron, thereby keeping iron soluble at low pH and high iron concentration.

Despite homologous structure and function among lactoferrin proteins produced by different organisms, the iron binding and antibody binding properties are highly specific (Iyer and Lonnerdal, 1993). This specificity also extends to iron-binding, where lactoferrin from one organism cannot bind iron in another organism. Even transferrin produced by the same organism, which is 60% homologous in structure and sequence to lactoferrin, does not have the same binding ability and cannot replace

lactoferrin on receptors found within the gastrointestinal tract (Iyer and Lonnerdal, 1993). This protein is resistant to proteolysis, high heat, and low pH, which made it difficult to hydrolyze for amino acid sequencing, but these characteristics stabilize the protein in the biological system.

The bacteriostatic activity of the molecule also is not completely understood. Initially, it was thought that iron-sequestration by lactoferrin keeps biologically needed iron away from bacteria. However, there are some types of bacteria, such as *Staphylococcus aureus*, *Aeromonas hydrophila*, and *Shigella flexneri*, which have specific receptors that bind with lactoferrin and extract the iron. Veken et al. (1996) found that the bacterium, *Pasteurella multocida*, uses these receptors to bind to transferrin and hemoglobin, but cannot bind to the structurally similar lactoferrin to utilize iron. Apolactoferrin (lactoferrin without iron) is often more bacteriostatic than lactoferrin with iron. Rather than competing for iron, some transferrins bind to bacterial cells and cause the lipopolysaccharide (LPS) layer from the cell wall of enteric gram-negative bacteria to be released from the rest of the layers in the cell wall. Releasing the LPS layer increases susceptibility of bacterial cells to antibacterial agents such as lysozyme and rifampicin (Iyer and Lonnerdal, 1993).

A protein assay dye reagent was used to spectrophotometrically determine protein concentration by comparison with a standard curve created either from commercial lactoferrin or from bovine serum albumin (BSA). If immunoassays are to be used, monoclonal antibodies are often created for the type of lactoferrin that is being examined since the antibody-antigen reaction is so specific for this protein.

Iron-binding occurs concomitantly with binding of two bicarbonate ions that play an important structural role. Gerstein et al. (1993) analyzed the mechanisms involved in the motion of the protein upon iron-binding that keeps the iron tightly bound. Crystallographic structures of the open and closed protein and comparison with other instances of hinged domain closure and with sliding motion closure have been used to determine how this mechanism works. Lactoferrin is a two lobed protein and upon iron-binding, the protein closes with the two N-terminal domains moving together as if on 'hinges'. These 'hinges' involve three large angular changes that cause most of the motion. The remaining motion is due to smaller changes in the neighboring residues. The motion from these changes passes through the two β -strands and links the domains together. A crucial feature of this mechanism is the few packing constraints that exist in this molecule. The domains make different packing contacts in the open and closed forms, resulting in a see-saw motion. When the protein closes, the residues with interface on one side are close-packed and buried, while the other side is now exposed. When the protein opens, the residues are in the opposite configuration. This hinge mechanism closes the protein and keeps the iron tightly bound. Iron concentration is about 700 times higher than the amount due to binding alone (Nagasako et al., 1993). This suggests that lactoferrin may help to regulate iron in biological systems by binding to it non-specifically and stabilizing ferrous iron (which is normally insoluble, since iron is only soluble in the ferric state).

Lactoferrin is an iron-binding glycoprotein, and the structure has been fairly well analyzed through the use of amino acid sequencing and x-ray crystallography, but the function continues to be debated. It appears to play a regulatory role in iron

metabolism that varies with developmental age and physiological state. It also appears to have growth stimulatory, immunological, and bacteriostatic effects, but these mechanisms are not very well understood, although they are not directly related to the iron binding capacity. The iron-binding capacity and the glycosyl groups associated with this protein add stability to the protein which allow it to be biologically active within organisms and to pass intact through the digestive system.

Characterization of glomalin

Glomalin extracted from soil is very similar to glomalin extracted from single-species pot cultures. Samples have been examined using SDS-PAGE (Rillig et al., 2001b; Wright et al., 1996; Wright and Upadhyaya, 1996), NMR (Rillig et al., 2001b), capillary electrophoresis (CE) (Wright et al., 1998), and C, H, N analysis by combustion (Rillig et al., 2001b). There are minor variations in elemental constituents of glomalin among samples, but CE and SDS-PAGE demonstrated that glomalin extracted from soil is similar to glomalin from hyphae. Rillig et al. (2003) and Steinberg and Rillig (2003) examined decomposition of glomalin following moist soil incubation in the dark at 18°C. One of the incubation studies (Steinberg and Rillig, 2003) showed that hyphal length declined by 60% after 150 days of incubation while TG declined by 25%, IRTG disappeared almost completely, EEG did not change, but IREEG increased five-fold. In the other study (Rillig et al., 2003), the TG declined by 48 to 81% and the EEG declined by 51 to 88% after 413 days of incubation. Using ¹⁴C data, Rillig et al. (1999) calculated a turnover time for glomalin of 7 to 42 y. However, the incubation studies suggest that a long-lived, recalcitrant glomalin fraction exists with a much longer turnover time.

Soil organic matter

In soil, plant- and microbially-produced organic carbon is found in two pools: (1) the labile, “light” or particulate organic matter (POM) fraction and (2) the recalcitrant, “heavy” or humic fraction. The POM fraction represents fresh or partially decomposed plant material, while the humic fraction is more completely decomposed material. The POM fraction is similar in chemical composition to plant material (Cambardella and Elliott, 1992) and may be separated by floatation in high-density liquids, such as NaCl or sodium polytungstate (Wolf et al., 1994; Gale and Cambardella, 2000). Changes in POM concentration are correlated with changes in soil fertility due to tillage practices or environmental factors (Cambardella and Elliott, 1992). As POM degrades further, it is transformed into humic substances.

The "heavy" fraction contains three types of humic substances: (1) humic acid (HA), (2) fulvic acid (FA), and (3) humin. Historically, solubility characteristics and color have separated humic substances into the acid- and alkaline-soluble, yellow to light brown FA fraction; the alkaline-soluble, dark brown to black HA fraction; and the insoluble, black humin. The low turnover rate, especially in the HA and humin fractions, makes these components important in sustainable agriculture and C sequestration. Humic substances are considered important in sustainable agriculture because they enhance water-holding capacity, permeability, soil aggregation, buffering capacity, and cation exchange capacity.

Typically, humic substances account for the majority of SOM [about 70 to 80 % of the soil organic carbon (SOC)] (Hayes and Clapp, 2001; Hayes and Graham, 2000). These substances are derived from biological and chemical transformations of organic

debris (i.e. POM) and are higher in C but lower in O and H than POM (Hayes and Graham, 2000; Swift, 1996). The composition of humic substances varies slightly from sample to sample according to types of plant material and microbial populations. Frequently, because biochemical compounds such as amino acids, carbohydrates, and lipids, often are complexed to and extracted with humic substances, the extract is operationally but not structurally defined (Clapp and Hayes, 1999; Hayes and Graham, 2000). Low molecular weight humic substances can be bridged together via the introduction of iron salts to form high-molecular weight substances (Hatcher et al., 2001). Despite being chemically extracted in alkaline solution and separated by acidification, neither HAs nor FAs are pure compounds. Some residues or impurities in HAs may be removed by increasing the ionic strength of the extraction solution or by using XAD resins (Hayes and Clapp, 2001). These impurities contribute to the high concentrations for HAs reported for most soils.

The recent discovery of glomalin has led to a reexamination of SOM components (Wright et al., 1996; Wright and Upadhyaya, 1996). Glomalin is a ubiquitous and abundant glycoproteinaceous molecule (Rillig et al., 2001b; Wright and Upadhyaya, 1999). However, unlike POM or humic substances, glomalin is not derived from the decomposition of plant- or microbially-produced material. Glomalin forms a hydrophobic sheath on hyphae that may keep material from being lost from across the hyphal membrane and/or may protect the hyphae from microbial attack (Wright and Upadhyaya, 1998; S. F. Wright, personal communication). Its presence in soil helps to stabilize aggregates, which in turn help to protect the molecule from degradation (Wright and Upadhyaya, 1998). Glomalin appears to be highly correlated with

aggregate stability (Wright and Upadhyaya, 1998) and with carbon sequestration in the soil by helping to physically protect organic matter within aggregates (Rillig et al., 1999; Rillig et al., 2001a).

Soil aggregation

Loss of topsoil due to erosion is a serious problem in agroecosystems. Pimentel et al. (1995) estimated that during the last 40 years nearly one-third of the world's arable land was lost to erosion with a current rate of 10 million hectares per year. Soil aggregates are important for: (1) maintaining soil porosity, which provides aeration and water infiltration rates favorable for plant and microbial growth, (2) increasing stability against wind and water erosion, and (3) storing carbon by protecting organic matter from microbial decomposition (Bird et al., 2002; Hassink and Whitmore, 1997; Rillig et al., 1999). Since both aggregate stability and SOM decline upon cultivation, it may be that SOM (i.e. POM, humic substances, microbially-produced molecules, and fungal hyphae) plays a role in aggregate formation, but the exact mechanism is not understood. Aggregate formation is a complex process of physical and chemical interactions (Kemper and Chepil, 1965; Miller and Jastrow, 1990; Tisdall and Oades, 1982).

Electron microscopy shows that aggregates are a conglomeration of soil minerals (clay particles, fine sand and silt), small plant or microbial debris, bacteria, free amorphous organic matter and organic matter strongly associated with clay coatings (Chenu et al, 2000; Six et al., 2001). Fungal hyphae may initiate aggregate formation by providing the framework upon which organic matter collects (Miller and Jastrow, 1990; Tisdall et al., 1997). Chemical processes then contribute to aggregate formation and stability by coating with hydrophobic polymers, binding mineral particles

with organic polymers, bridging organic matter to clay particles by polyvalent cations, and gluing with polysaccharides (Degens, 1997; Piccolo and Mbagwu, 1999; Chenu et al., 2000). Drying and wetting actions, shrinking and swelling of clays, freeze-thaw cycles, compaction, and enmeshing by fungal hyphae and/or fine roots physically stabilize aggregates (Chaney and Swift, 1986; Degens, 1997).

In reduced or no-till systems, Chaney and Swift (1986) found that the stubble and mulch litter promote aggregate formation because fungal decomposition of organic matter produces gluing agents, such as polysaccharides and mucigels. Caesar-TonThat and Cochran (2002) found that ligninolytic basidiomycetes produce large quantities of hydrophobic polysaccharides, glycolipids or glycoproteins that bind to and stabilize soil particles in water-stable aggregates. However, many of the polysaccharides produced by microbial degradation will glue aggregates together quickly but are water-soluble and ephemeral and do not contribute to the long-term stability of aggregates (Chaney and Swift, 1986; Six et al., 2001).

Without the protection of hydrophobic coatings, soil aggregates may be disrupted by rainfall because of slaking, the differential swelling of clays, mechanical dispersion by the kinetic energy of raindrops, and physiochemical dispersion. The aliphatic molecules involved in aggregate formation increase water stability and long-term survival of aggregates, because attractive forces between these molecules are much stronger internally than externally (Degens, 1997; Piccolo and Mbagwu, 1999; Chenu et al., 2000). Soil organic matter containing high concentrations of aliphatic groups, such as HA, may increase aggregate stability and the long-term stabilization of organic materials (Piccolo and Mbagwu, 1999). These aliphatic, hydrophobic groups and

polymers are the major contributors to the water-stability of aggregates. They increase the contact angle for water penetration, which restricts infiltration and slaking, lowers wettability and increases the internal cohesion of aggregates (Chenu et al., 2000).

Glomalin contributes to the stabilization of aggregates by sloughing off hyphae onto the surrounding organic matter, binding to clays (probably via cation bridging by iron), and providing a hydrophobic coating (Wright and Upadhyaya, 1999). This is demonstrated in a number of experiments, where total and, especially, immunoreactive concentration of glomalin are positively correlated with percent water-stable soil aggregates in both agricultural and native soils (Bird et al., 2002; Rillig et al., 2003; Wright and Anderson, 2000; Wright and Upadhyaya, 1998; Wright et al., 1999).

Glomalin under elevated CO₂

Several studies were conducted to compare glomalin concentrations to aggregate stability under elevated CO₂ conditions. In a native grassland ecosystem in northern California, TG and IRTG concentrations increased with higher CO₂ concentrations, along with hyphal length at one site, and aggregate stability in 1-2 and 0.25-1 mm aggregate size fractions (Rillig et al., 1999). Long-term exposure to elevated atmospheric CO₂ conditions from a natural CO₂ spring in New Zealand formed by a thermal vent resulted in a linear increase in percent root colonization by AM fungi, soil hyphal length, TG and EEG along a CO₂ gradient (Rillig et al., 2000). In a *Sorghum* field, aggregate stability, hyphal length and EEG increased with elevated CO₂ (Rillig et al., 2001a). In both the grasslands (Rillig et al., 1999) and *Sorghum* field (Rillig et al., 2001a), aggregate stability was correlated with glomalin concentrations. These studies

show that under elevated CO₂ conditions, photosynthetic carbon is allocated belowground and glomalin may provide a significant sink to trap carbon in the soil.

Objectives and experimental design

Glomalin is a ubiquitous, abundant glycoprotein produced by hyphae of arbuscular mycorrhizal (AM) fungi. This research had three major objectives in the characterization of glomalin: (1) to validate that glomalin is a unique and major pool of soil organic matter (SOM), (2) to measure glomalin accumulation in a pot culture system, and (3) to examine the glomalin molecule.

Because of its abundance, reddish-brown color and solubility characteristics, glomalin may be co-extracted with humic substances which are widely studied for their role in sustainable agriculture and as a carbon sink. These similarities resulted in two central questions for the proposed research: (1) is glomalin uniquely different from humic substances, and (2) is glomalin present in amounts high enough to play a role in SOM processes, such as aggregate stability? Experiments where glomalin, humic substances (HA and FA) and particulate organic matter fractions are extracted from both native and managed agricultural soils will be conducted to answer these questions. Comparisons will be made among fractions using amounts measured by total and immunoreactive protein assays and gravimetric and C weight. Typically, glomalin is quantified by protein concentration. However, this measurement does not provide a method for comparison to HA, FA and POM. The gravimetric and C weights are used to make that comparison. In addition, glomalin is comprised of more than just proteinaceous material. Other groups, such as carbohydrates and iron, are a part of the glomalin molecule. Therefore, protein weight is an underestimation of glomalin

concentration while gravimetric weight is a direct measurement of the mass of purified glomalin.

Glomalin is extremely stable and continues to accumulate in the soil over time. To directly measure this accumulation, a pot culture experiment utilizing repeated culturing the same pot over a 294-day period with three 14-week intervals. These pot culture experiments are a direct measure of glomalin accumulation to see how much glomalin may be added at one time and how that amount continues to increase with additional culturing.

Examining the composition of the glomalin molecule will help in the understanding of its functional role, reactions with other soil particles, and ability to accumulate and act as a carbon sink. Proteins are the most complex macromolecules known, and glycoproteins are especially complex in structure, composition, and functional roles (Varki, 1993). In addition to containing a peptide and carbohydrate residues, glomalin also binds metals, such as iron, and may be associated with organic matter and clay minerals.

The ecological (organic matter comparison), developmental (pot culture production), and structural (molecular composition) components will be combined to provide an understanding of the role(s) of glomalin in the mycorrhizal symbiosis. This work will also provide a foundation for future studies relating glomalin to soil structure and global climate change issues.

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CHAPTER 2

ISOLATION AND COMPARISON OF EXTRACTABLE SOIL ORGANIC MATTER FRACTIONS

Abstract

The majority of soil organic matter (SOM) is comprised of humic substances [humic acid (HA), fulvic acid (FA) and humin], particulate organic matter (POM) and glomalin. The hypothesis that methods to extract POM, humic substances and glomalin are specific for these SOM fractions was tested by sequential extractions from the same soil sample. Bulk soil samples (0 to 10 cm depth) from six native soils – two each from Colorado (CO), Maryland (MD) and Georgia (GA) – were analyzed. Particulate organic matter was extracted first with high density NaCl solution, followed by extraction of glomalin with a citrate solution at 121°C, and then by extraction of HA and FA in a NaOH solution. Glomalin, HA and FA were similarly extracted from the POM fraction. Total protein and carbon, nitrogen and hydrogen concentrations showed that glomalin and HA were, for the most part, separate fractions. However, because of a lack of a definitive assay for HA and the identification of immunoreactive protein (using an antibody against glomalin) in the HA fraction, the question of co-extraction of glomalin with HA remains unresolved. The POM fraction also contained glomalin.

Introduction

Soil organic matter (SOM) is important for soil stabilization, nutrient cycling and carbon (C) storage. Humic substances (HS) – humic acid (HA), fulvic acid (FA) and humin – contribute 70 to 80% of the soil organic carbon (SOC) (Hayes and Graham, 2000). Generally, HS are defined as highly condensed plant material and microbial byproducts produced during the decomposition of plant residues (Burdon, 2001; Hayes et al., 2001; MacCarthy, 2001). Operationally, HS are divided into fractions based on solubility characteristics: alkaline-soluble HA, acid- and alkaline-

soluble FA and mostly insoluble humin. Fractions of HS are structurally heterogeneous (Burdon, 2001; MacCarthy, 2001).

Humin is a highly condensed fraction of organic matter that is bound tightly to clays or in organometallic complexes and is mostly insoluble (Hayes and Graham, 2000; Rice, 2001). Humic and fulvic acids are extracted in NaOH solution and separated by acidification. Amino acids, carbohydrates, lipids and metals that often are complexed to and extracted with HS are treated as contaminating material (Clapp and Hayes, 1999; Hayes and Graham, 2000; Schulten and Schnitzer, 1997). Some residues or impurities in HAs are removed by increasing the ionic strength of the extract solution or by using resins (Swift, 1996; Hayes and Graham, 2000). These impurities contribute to the high weights reported for HA in most soils. Using ^{13}C NMR, Schnitzer and Schuppli (1989) found that of the 92 mg g^{-1} of material from a Melfort [Black Chernozem (similar to a Mollisol)] soil extracted with NaOH and operationally defined as humic acid about 59% was ash material.

Other major fractions of SOM are particulate organic matter (POM) (Gale and Cambardella, 2000; Stevenson, 1994) and glomalin (Wright and Upadhyaya, 1998). Particulate organic matter consists of insoluble plant debris (mostly roots) that floats in high-density inorganic liquids such as sodium chloride (NaCl) or sodium polytungstate (Gale and Cambardella, 2000; Wolf et al., 1994). Glomalin, a glycoproteinaceous substance produced by arbuscular mycorrhizal (AM) fungi, is found in abundance (2 to $14 \text{ g protein kg}^{-1}$ soil) in a variety of soils (Wright et al., 1996; Wright and Upadhyaya, 1996; Rillig et al., 2001).

Arbuscular mycorrhizal fungi colonize 80% of vascular plant species and are found worldwide in almost every soil (Trappe, 1987). Glomalin is produced on AM hyphae and accumulates in soils (Wright and Upadhyaya, 1999; Rillig et al., 2001). In its native state, glomalin exists as an insoluble compound on hyphae and in soil. As hyphae degrade, glomalin sloughs off onto soil particles providing a hydrophobic coating that contributes to soil stabilization (Wright and Upadhyaya, 1996; 1998). A citrate buffer (pH 7.0 to 8.0) at high heat (121°C) is used to extract glomalin (Wright et al., 1996; Wright and Upadhyaya, 1996). This extraction procedure operationally defines glomalin which is quantified by protein assays on the extract solution (Wright et al., 1996; Wright and Upadhyaya, 1998).

Glomalin is classified as a glycoprotein because it binds to lectins (Wright et al., 1996) and by identification of N-linked oligosaccharides on the molecule with capillary electrophoresis (Wright et al., 1998). Extracted glomalin is flocculated by acidification or with saturated ammonium sulfate and is soluble in an alkaline solution (Wright et al., 1996). Glomalin extracted from sand-based pot cultures of various AM fungi is equivalent to glomalin extracted from soil according to protein banding on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, ELISA, glycoprotein assays, C, N and H concentrations and NMR spectra (Wright and Upadhyaya, 1996; Wright et al., 1998; Rillig et al, 2001; unpublished data).

The objectives of this study were to: (1) determine if the four major SOM fractions (POM, glomalin, HA and FA) can be isolated separately from a soil sample as defined by extraction technique and concentrations of C, N and H, and (2) quantify and compare SOM fractions using common measures for HA, FA and POM (gravimetric

and C weights) and by the standard measures for glomalin (total and immunoreactive protein assay values).

Materials and methods

Soils

Bulk soil samples (0-10 cm depth) were collected with a shovel at two locations in three U.S. states [Maryland (MD), Colorado (CO) and Georgia (GA)]. The following series were sampled: Baltimore (MD) (at two sites designated as 'a' and 'b'), Sampson and Haxtun (CO), and Pacolet and Cecil (GA). The soils were all acidic loams – Mollic Hapludalfs (Baltimore series), Pachic Argiustolls (Sampson and Haxtun series) and Typic Kanhapludults (Pacolet and Cecil series). All soils were collected fresh from sites that had native vegetation. Soils were air-dried and sieved to collect particles < 2 mm. Five replicate 2-g samples were extracted from each soil sample.

Soil organic matter extractions

Density separation of particulate organic matter

Particulate organic matter was removed by floatation in a high-density solution using a method modified from Wolf et al. (1994). Briefly, soil samples were covered with a NaCl solution (12%, w/v), vortexed, and allowed to settle for 30 min. (See Appendix A1 for detailed extraction procedures.) After the mineral fraction had settled, the solution was carefully decanted. Floating organic matter (POM fraction) was collected on a 0.053 mm screen. This procedure was repeated a total of five times. The POM fraction was washed with distilled water to remove salt, rinsed from the screen into pre-weighed weigh boats and dried at 70°C. Soil minus POM was washed with distilled water, pelleted by centrifugation, and dried at 70°C.

Citrate extraction of glomalin

Glomalin was extracted from the POM fraction and soil minus POM using 50 mM sodium citrate, pH 8.0, at 121°C for 1 h (Wright and Upadhyaya, 1999). (See Appendix A2 for detailed extraction procedures.) Samples were centrifuged and the supernatant was decanted and saved. The procedure was repeated until the supernatant was straw-colored (up to three more times). Supernatants from each 1 h extraction cycle were combined and centrifuged. Glomalin was flocculated at pH 2.0 to 2.5 by adding 1 N HCl, the solution was placed on ice for 45 min, and the precipitate was pelleted by centrifugation. The pellet was dissolved in a minimum amount of 0.1 N NaOH and dialyzed extensively against deionized (dH₂O) in tubing with molecular weight cut-off (MWCO) of 8,000 to 12,000 Daltons (D). Dialyzed material was centrifuged and the supernatant was collected and freeze dried. All centrifugations were at 6850 × g for 10 min.

Sodium hydroxide extraction of humic and fulvic acids

The International Humic Substances Society method described by Swift (1996) was used to extract HA and FA. (See Appendix A3 for detailed extraction procedures.) Modifications to the method were in sample size (2 g instead of 50 g) and in the purification steps. Briefly, soil samples were pre-incubated in HCl followed by a multi-step NaOH extraction procedure: (i) extraction under N₂ overnight; (ii) centrifugation to collect the supernatant; (iii) acidification of the supernatant; (iv) precipitation of HA overnight; and (v) separation of HA (precipitate) from FA (supernatant) by centrifugation. The NaOH extraction followed by acidic separation was repeated until

the solution was almost clear to assure that all humic and fulvic acids were extracted. All centrifugations were at $6850 \times g$ for 10 min.

Insoluble solid particles were removed from HA by: (i) re-dissolution in a minimum volume of KOH under N_2 ; (ii) addition of KCl (until $[K^+] \geq 3 \text{ M}$); (iii) centrifugation at $10844 \times g$ to remove suspended solids; and (iv) precipitation of HA from the supernatant with HCl. After settling overnight, samples were centrifuged and the supernatant was discarded. The HA precipitate was: (i) suspended in an HCl-HF solution, (ii) incubated overnight, and (iii) collected by centrifugation at $6850 \times g$. 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 $10844 \times g$ for 3 min.

The HA precipitate was re-dissolved 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 $10844 \times g$ and washing with DH_2O . The precipitate was freeze-dried.

The FA fraction (i.e. acid soluble material) was dialyzed against water until the pH was neutral using tubing with a MWCO of 8,000 to 12,000 D. Insoluble material was pelleted by centrifugation at $6850 \times g$ for 10 min and the supernatant was freeze-dried.

Sequential extraction

Extraction procedures were conducted sequentially on the same soil sample. (See Appendix B for a diagram of the extraction sequence.) First, the POM fraction was

separated from the mineral fraction. For each soil, POM from the five 2-g samples was combined to facilitate more accurate quantification of glomalin, HA and FA. Both the POM and soils minus POM were extracted for glomalin followed by co-extraction of HA and FA. The POM fraction material that remained after extraction of glomalin, HA and FA was classified as Residual POM.

Quantitative measurements

Protein assays

Total and immunoreactive protein concentrations were measured on subsamples of HA, FA and glomalin. (See Appendix C for detailed methods.) For glomalin and FA, the subsample was taken prior to purification by dialysis, and the HA subsample was collected from re-dissolved HA precipitate prior to freeze-drying as discussed above.

A modified Bradford protein assay (Wright et al., 1996) was used to measure total protein (TP) concentration. Samples were diluted with phosphate buffered saline (PBS) and reacted with Bio-Rad® (Hercules, CA) Bradford protein dye reagent. Absorbance was read at A_{595} after 5 min. Protein concentration was determined by comparison with a bovine serum albumin (BSA) standard curve and reported as g protein kg^{-1} soil.

Immunoreactive protein (IRP) concentration was measured by 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 the color developer, *p*-nitrophenyl phosphate in diethanolamine buffer (Wright, 1994), was

added. Absorbance was read at A_{405} after 15 min. Test samples were compared to a standard curve produced by dilutions of highly immunoreactive glomalin extracted from a temperate soil under native grasses. Immunoreactive protein concentrations were reported as g kg^{-1} soil. Percent immunoreactivity was calculated as amount of IRP divided by amount of TP times 100 and reported as the range and the mean for all soils.

Gravimetric weight

The POM fraction and Residual POM were dried at 70°C and weighed to the nearest 0.1 mg. All freeze-dried samples of HA, FA and glomalin were weighed to the nearest 0.1 mg. Gravimetric and protein weights were compared by dividing the calculated protein weight by the measured gravimetric weight times 100. Comparisons of TP and IRP to gravimetric weight for glomalin and HA from soils and glomalin from POM (P. glomalin) were reported as the mean and SE for all soils.

Carbon, nitrogen and hydrogen concentrations

Carbon, nitrogen, and hydrogen concentrations were measured by combustion with a Perkin-Elmer Series II C, H, N, S/O 2400 Analyzer. Freeze-dried glomalin, HA and FA fractions from replicate 2-g soil samples were combined to give a bulk sample of each fraction. All fractions plus POM prior to extraction, Residual POM and soil before (Initial) and after (Residual) all extractions were stored in a desiccator under vacuum until analyzed. Gravimetric weights were multiplied by percentage C to give a C weight (g kg^{-1}) in each fraction.

Statistical analysis

Means and standard errors (SE) were calculated for protein weights from each soil. Gravimetric weight values were corrected for subsamples removed for protein

assays. Carbon, nitrogen and hydrogen concentrations for each fraction (Residual POM, glomalin, HA and FA) were means and SEs for all soils combined. Mean comparisons were made at $\alpha \leq 0.05$ by ANOVA (Analysis of Variance) using REML (Restricted Maximum Likelihood) after meeting the assumptions for normality and homogeneity of variance for the residuals. When needed, the log transformation was made to meet the assumptions. All statistical analyses were performed using SAS software, ver. 8 (SAS Institute, 1999).

Results

Protein concentrations

Total protein concentration in glomalin from soil was significantly higher than protein in all other fractions for all soils except Sampson (Table 2A). Protein was detected in P. glomalin in measurable amounts ($>0.1 \text{ g kg}^{-1}$), but not in HA or FA extracted from POM. Fulvic acid fractions had no measurable protein.

Immunoreactivity of proteinaceous material in glomalin extracted from soil ranged from 10 to 45% (mean = 25%). Percentages of IRP in HA ranged from 1 to 65% (mean = 18%). Glomalin in the POM fraction had 5 to 19% IRP (mean = 14%). (See table in Appendix D for IRP values in the three major soluble fractions.)

Comparison of protein to gravimetric weight

Glomalin and HA averaged $25 \pm 4\%$ and $55 \pm 15\%$ proteinaceous material on a weight basis, respectively, for all soils. (See table in Appendix E for gravimetric weight values for the fractions extracted from all six soils.) In the POM fraction, glomalin was $100 \pm 54\%$ proteinaceous. Immunoreactive protein was $6 \pm 2\%$, $10 \pm 3\%$ and $8 \pm 5\%$ of the gravimetric weight for glomalin and HA from soil and P. glomalin, respectively.

Carbon, nitrogen and hydrogen concentrations

Humic acid contained significantly more C, N and H than any other fraction, including glomalin (Table 2B). Humic acid from soil had a C concentration similar to HA from POM but was higher in N. Percentages C, N and H in P. glomalin were similar to percentages in glomalin from soil. Fulvic acid in POM had higher C contents and C to N ratio than FA in soil. Statistically, FA and Residual POM had similar C contents but Residual POM was lower in N with a higher C to N ratio.

Carbon weights of SOM components

Glomalin in soil and Residual POM were the dominant C-containing fractions extracted (up to 3 and 2 g C kg⁻¹ soil, respectively). (See table in Appendix F for carbon weight values in each fraction from all six soils.) In the Sampson soil, the Residual POM fraction contained the most C while in the Baltimore soils, Residual POM and P. glomalin accounted for smaller amounts of C (Fig. 2A). The majority (43%) of the SOC initially present in these soils was non-extractable (humin). Of the total extracted C, glomalin from soil and P. glomalin combined accounted for 43% followed by Residual POM, HA and FA with 21, 7 and 3%, respectively.

Discussion

Total protein values for glomalin these six soils averaged 2.53 g kg⁻¹ (Table 2A) and were consistent with previously reported values for glomalin from a variety of temperate soils (2 to 14 g kg⁻¹ soil) (Wright and Upadhyaya, 1998). Glomalin was expected to be a major fraction of SOM because: (1) glomalin coats the surface of AM fungal hyphae (Wright, 2000; Wright et al., 1996; Wright and Upadhyaya, 1999) and hyphal lengths can be >100 m g⁻¹ in an undisturbed prairie soil (Miller et al., 1995), (2)

glomalin resists degradation by proteases, high heat, and low pH (Wright and Upadhyaya, 1998) and (3) glomalin has a turnover time of at least 7 to 42 y (Rillig et al., 2001) and maybe up to 200 y (R.M. Miller, personal communication).

High glomalin and low HA contributions to total extractable C (Fig. 2A) were inconsistent with reports of humic substances as the major C-containing fractions of OM in most soils (Hayes and Graham, 2000; Stevenson, 1994). Contaminants (i.e. amino acids, carbohydrates, lipids and metals) that are typically co-extracted with HA (Clapp and Hayes, 1999; Hayes and Graham, 2000; Schulten and Schnitzer, 1997) and are found in humin (Hayes and Graham, 2000; Rice, 2001) suggest that a compound such as glomalin could be present in these fractions. Protein assay values, especially the IRP values, indicated that glomalin was in the HA fraction.

Glomalin contained high concentrations of protein (Table 2A). However, only about one-fourth of the gravimetric weight of glomalin from soil was proteinaceous whereas almost all of the glomalin in POM from the Baltimore site b, Sampson and Haxtun soils was proteinaceous. This indicated that the extraction of glomalin from soil either co-extracted some other substance with similar solubility characteristics as glomalin (soluble at alkaline pH, survived high temperature, and was acid-insoluble after extraction), such as HA, or the extracted glomalin contained a tightly-bound, non-proteinaceous substance. Lower percentage C values for glomalin from soil compared to glomalin from the POM fraction (Table 2B) suggested that it is unlikely that a large amount of co-extracted organic material was included in the gravimetric weight of glomalin from soil. In addition, NMR spectroscopy shows that glomalin does not have co-extracted or attached tannins (Rillig et al., 2001).

Iron possibly contributes to high gravimetric weight and correspondingly low C values for glomalin from soil. Previous analysis of glomalin indicates a wide range of iron in glomalin from soils (0.8 to 8.8%) (Wright and Upadhyaya, 1998). This is in contrast to lower values for iron in glomalin from pot cultures (0.2%) (Chapter 7). Glomalin may accumulate iron over time in soil, and this characteristic could account for the resistance to decomposition and formation of stable complexes within soil aggregates. Iron-saturated transferrins, such as lactoferrin, are glycoproteins that are thermal stable and have antimicrobial properties (Paulsson et al., 1993). Organometallic complexes formed by Fe- and Al-hydroxides protect organic matter from decomposition in stable soil aggregates (Tisdall and Oades, 1982; Rice, 2001). Ongoing work is examining the composition of glomalin more fully with iron targeted as important for glomalin stability and function.

Plant-derived particulate matter was easily recognized as unique because it was insoluble particulate matter that was separated from the extractable components in POM (glomalin, HA and FA). Likewise, FA was readily distinguished from other fractions because it is both acid- and alkaline-soluble, a lighter color than glomalin and HA, and had essentially no proteinaceous residues. The purification procedure used for FA was not optimized to retain all of the low-molecular weight components in this fraction. Solubility of FA in acid and the lack of proteinaceous material in FA extract prior to purification eliminated this fraction as a source of glomalin.

Distinguishing between glomalin and HA was more difficult because both were dark-colored, alkaline-soluble and acid-insoluble. Operationally, glomalin and HA can be separated by origin and extraction method. Glomalin originates from hyphae of AM

fungi and is extracted in a citrate solution at 121°C (Wright et al., 1996; Wright and Upadhyaya, 1996) while HA is derived from chemically and biologically degraded organic matter and is solubilized in NaOH solution (Burdon, 2001; Hayes et al, 2001). However, both glomalin and HA contained proteinaceous and immunoreactive material. It is possible that even after extensive extraction with citrate, some glomalin remained in the soil. Subsequent treatment of soil with NaOH to extract HA may have resulted in co-extraction of a 'recalcitrant' glomalin fraction as indicated by the presence of IRP in HA. Across all soils 18% of the protein in HA was IRP. Co-extraction of non-glomalin proteins with HA also is a possibility.

Immunoreactivity is a useful indicator of the presence of glomalin in the protein fraction extracted from soils using alkaline citrate at high heat, but previous work shows that values for Bradford protein and ELISA may not be equivalent. Values for percentage of IRP in glomalin and HA from soil and *P. glomalin* were within the lower range of values obtained from other studies where multiple 1-h extractions were required to remove glomalin from soils. Samples from Georgia pasture soils (n = 192) (similar to the Cecil and Pacolet series included in this study) ranged from 19 to 76% immunoreactive glomalin (Franzluebbers et al., 2000). Glomalin extracted from a Weld silt loam soil (Akron, Colorado) under perennial grass (n = 9) was 27 to 87% immunoreactive (Wright and Anderson, 2000). Extraction conditions such as the concentration of citrate and length of exposure to heat during extraction at least partially influences immunoreactivity (Wright and Upadhyaya, 1996). In this study, immunoreactivity probably was compromised in order to optimize the amount of glomalin extracted from a sample.

Co-extraction of HA and glomalin in hot, alkaline citrate solution is a possibility. However, percentage C and H in glomalin indicate that a large amount of HA was not co-extracted (Table 2B). Humic acid from soil averaged 53% C and 5.6% H. These values are much less than 35% C and 4.1% H for glomalin from soil and 40% C and 4.5% H for P. glomalin. Results indicated that POM contained essentially no HA. Therefore, percentage C in P. glomalin probably reflects a value for glomalin not contaminated with HA. Also, the value for percentage C in P. glomalin is similar to that of glomalin extracted from AM fungal hyphae produced in single-species pot cultures. Isolates of AM fungi [*Gigaspora rosea* Nicolson and Schenck FL224 (INVAM) and *Glomus etunicatum* Becker and Gerdemann BR220 (INVAM)] had 44% and 38% C, respectively (Chapter 6). Based on C concentrations in P. glomalin and HA, if glomalin from soil were contaminated with HA, percentage C should have been higher, not lower than P. glomalin.

In these six soils, the amount of C represented by adding up C weights for all seven of the extracted fractions plus the non-extractable C that remained in the residual soil, accounted for an average of 67% of the total organic C across all soils (Fig. 2A). Unaccounted-for-C possibly was lost in the many extraction and purification procedures used in this study. Also, minor C fractions such microbial biomass C or hot-water extractable carbohydrates were not measured.

Glomalin accounted for a higher percentage of the SOC than HA even though the HA molecule has a higher concentration of C. Using protein assays, and concentrations of C, H, and N, glomalin and HA were, for the most part, shown to be separate OM fractions. However, because of a lack of a definitive assay for HA and the

identification of immunoreactive material in the HA fraction by ELISA, the question of co-extraction of glomalin with HA remains. MacCarthy (2001) stated that it may be difficult to definitively isolate humic and non-humic substances from each other because of shared functional groups and solubility characteristics. In this study, similarities in extraction methodology (i.e. alkaline solution extraction and acidic separation), physical properties such as dark brown color and functional properties such as soil aggregate stabilization and metal accumulation and the low amounts of HA measured compared to glomalin makes co-extraction a possibility. Ongoing research is investigating co-extraction of glomalin and HA and better methods to separate these constituents of SOM.

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Table 2A. Total protein (g protein kg⁻¹ soil) in glomalin extracted from the particulate organic matter (POM) fraction (P. glomalin) and glomalin and humic acid (HA) from soil minus POM. †

Fraction	Baltimore a	Baltimore b	Sampson	Haxtun	Pacolet	Cecil	Mean §
P. Glomalin	0.13c	0.19c	1.69a	0.59b	0.23b	0.65b	0.58±0.24b
Glomalin	2.25±0.04a	2.19±0.06a	1.70±0.02a	1.14±0.03a	2.87±0.03a	1.55±0.08a	1.95±0.25a
HA	0.98±0.03b	0.95±0.08b	0.28±0.00b	0.06±0.01c	0.32±NDb	0.20±NDc	0.46±0.16b
Prob>F ‡	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0012	<0.0001

† Mean ± SE were determined for fractions extracted from soil but not for fractions extracted from POM that was combined before extraction.

‡ Significant differences were determined according to REML. ND = not determined.

§ Mean ± SE for all six soils.

Table 2B. Percentages C, N and H and ratio of C to N for glomalin, humic acid (HA) and fulvic acid (FA) extracted from both the particulate organic matter (POM) fraction and soil minus POM and for the initial soil sample prior to extractions and residual soil material remaining after all extractions averaged across six soils in the U.S. †

	Fraction	% C	% N	% H	C to N	N‡
	Initial soil	2.85±0.48	0.22±0.08	0.64±0.15	19.12±8.09	6
	POM	23.12±1.54	1.18±0.09	3.24±0.10	20.43±2.86	6
POM fraction	Glomalin	39.57±1.50	2.75±0.28	4.45±0.13	15.07±1.42	6
	HA	49.62±2.34	2.80±0.26	5.33±0.26	17.96±1.39	3
	FA	40.33±0.57	1.97±0.34	5.64±0.04	21.19±3.90	2
	Residual POM§	28.13±3.46c	1.29±0.17c	3.50±0.44b	22.30±1.92a	6
Soil minus POM	Glomalin	34.55±2.14b	3.00±0.29b	4.07±0.18b	11.75±0.63b	6
	HA	53.19±0.40a	3.89±0.39a	5.64±0.17a	14.38±1.79b	5
	FA	26.11±2.86c	2.31±0.33b	4.27±0.41b	11.80±0.83b	6
	Residual soil	1.23±0.23	0.07±0.02	0.34±0.08	15.72±3.81	6
Prob>F		<0.0001	<0.0001	0.0007	<0.0001	

† Means and standard errors in a column followed by different lowercase letters represent significant differences in fractions. Significant differences (P=0.05) were determined according to REML on the four major organic matter fractions – Residual POM and glomalin, HA and FA from soil.

‡ Number of soils used to calculate mean and standard error.

§ Residual POM is the residual POM after extraction of glomalin, HA and FA.

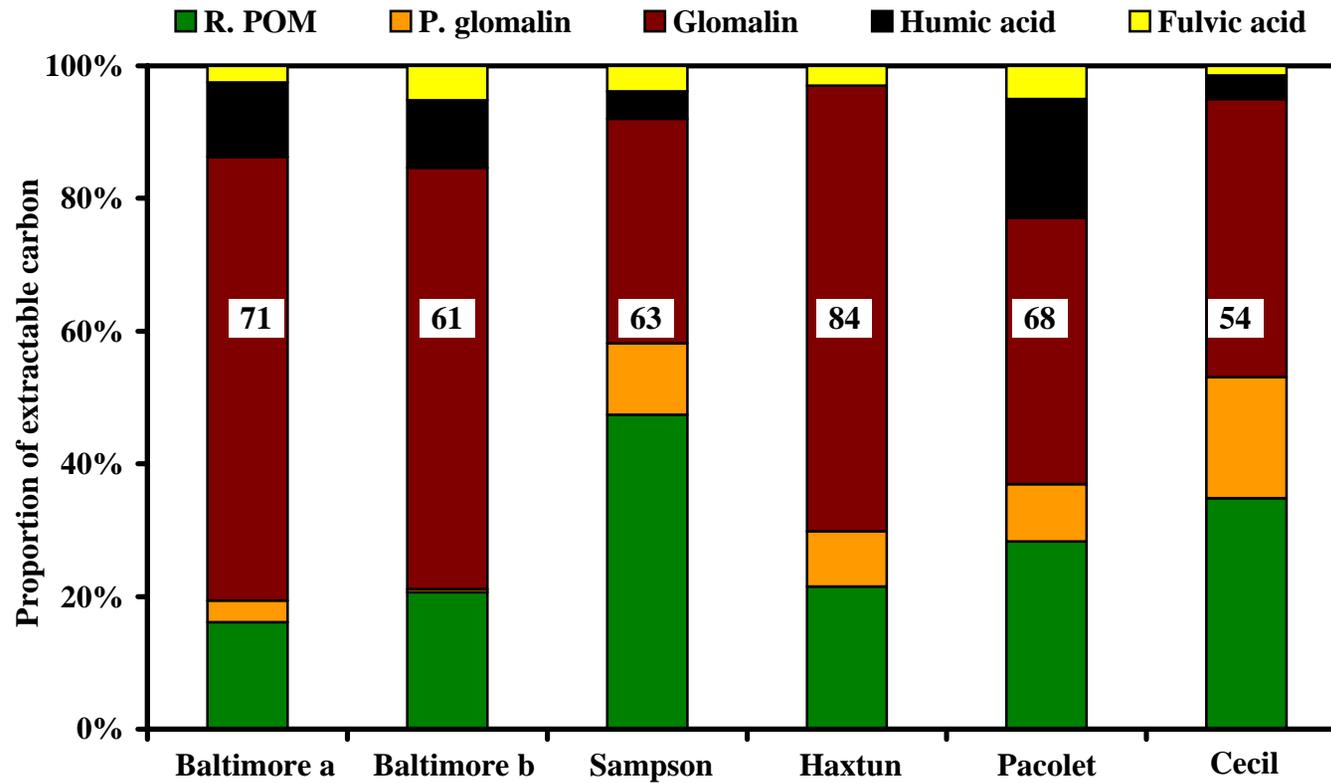


Figure 2A. Proportion of extractable carbon in each organic matter fraction – Residual particulate organic matter (R. POM), glomalin in POM (P. glomalin), glomalin, humic acid and fulvic acid – extracted from six native soils in the U.S. The proportion of total extractable carbon to total organic carbon in each soil is the number in the center of each bar.

CHAPTER 3

COMPARISON OF EXTRACTION SEQUENCES TO OPTIMIZE THE PURITY OF HUMIC ACID AND GLOMALIN

Abstract

Glomalin and humic acid (HA) are alkaline-soluble and acid-insoluble fractions of soil organic matter that differ in optimal temperature for extraction – glomalin at 121 °C and HA at RT. The hypothesis of this study was that HA and glomalin could be isolated from soil samples as essentially pure fractions by manipulating the extraction sequences to favor one or the other of these fractions. Bulk samples (0 to 10 cm depth) from eight soils – two soils from each of four U.S. states (Colorado, Nebraska, Maryland and Georgia) – were tested. Extraction Sequence (ES) 1 was citrate-extraction of glomalin followed by NaOH-extraction of HA to maximize glomalin amounts and ES 2 was NaOH-extraction followed by citrate-extraction to maximize HA amount. Comparisons between glomalin and HA fractions from the two extraction sequences were by Bradford total protein, immunoreactive protein and gravimetric weights and concentrations of carbon, hydrogen, nitrogen and iron. HA protein values from ES 2 were two times greater than from ES1. Low-protein HA (0.23 to 0.90 g protein kg⁻¹soil) was obtained by a two-step procedure: extraction of glomalin followed by extraction of HA (ES 1). Glomalin concentrations measured by the total protein assay were higher in ES 1 compared to ES 2 for all except the Nebraska soils. A recalcitrant pool of glomalin was released from soil after NaOH-extraction of HA in ES 1. Significant correlations were found between iron, organic C and clay concentrations in soil and protein concentrations, percentage iron and gravimetric weight of glomalin from ES 1.

Introduction

Soil organic matter (SOM) influences soil structure, water holding capacity, water and oxygen infiltration rates, soil erosion, pesticide efficacy and soil fertility. A

previous study (Chapter 2) showed that the majority of SOM is comprised of glomalin, Residual POM and humic substances (HS), such as humin, humic acid (HA) and fulvic acid (FA). Of these, glomalin and POM were the largest fractions isolated and HA was present in concentrations lower than expected. Glomalin and HA are distinctly different from POM (insoluble plant debris) and FA (acid- and alkaline-soluble). Therefore, POM and FA were neither collected nor examined in this study. Both HA and glomalin were extracted in alkaline solutions (glomalin in citrate, pH 8.0, at 121 °C and HA in 0.1 N NaOH at RT) and separated from the extraction solution and other contaminants by acidification. Since glomalin was extracted prior to HA in Chapter 2, it was possible that HA was co-extracted with glomalin. This would account for the high glomalin amounts and corresponding low amounts of HA.

Glomalin is a red-brown, glycoproteinaceous substance produced by arbuscular mycorrhizal (AM) fungi (Wright et al., 1996; 1998; Wright and Upadhyaya, 1996). Native glomalin is hydrophobic and insoluble unless exposed to an alkaline buffer [citrate, borate, or pyrophosphate at pH 7.0 to 9.0 (see Appendix G)] at high temperature (121°C) (Wright et al., 1996; Wright and Upadhyaya, 1996). Hydrophobic interactions appear to bind glomalin into a complex structure that is consistent across soils (Rillig et al., 2001; Wright et al., 1996; Wright and Upadhyaya, 1998; 1999; Chapter 7). Glomalin apparently provides a hydrophobic coating on AM hyphae to reduce solute loss (Wright and Upadhyaya, 1996). Other fungi, including some ectomycorrhizal fungi, produce self-aggregating small proteins called hydrophobins that protect aerial hyphae at air-water interfaces (Wessels, 1997). Glomalin is different in at least one way from hydrophobins because it contains iron (0.8 to 8.8%) (Wright and

Upadhyaya, 1998). Iron may protect glomalin and other organic matter from degradation by forming bridges to clay minerals and other types of organic matter.

Humic substances (HS) are formed by the decomposition of plant and animal debris, microfauna, biowastes, and other organic materials in the soil and are non-proteinaceous (Burdon, 2001; Hayes and Clapp, 2001). In soil, HS are thought to provide acidic and basic buffering capacity; retain soil moisture; contribute to soil warming; act as a reservoir of plant-available micronutrients; bind to metals, clays and other small organic molecules to form aggregates; and act as an electron shunt in microbial and abiotic redox reactions (Burdon, 2001; Fan et al., 2000; Hayes and Clapp, 2001; MacCarthy, 2001). However, many of the above functions are not supported by the chemistry of small molecules formed from the degradation of plant and microbial debris (i.e. HS) (Burdon, 2001; Hayes and Clapp, 2001). Therefore, there is a gap between the molecular structure of classic HS – decomposition products – and organic substances that provide buffering capacity, assist in redox reactions, and bind organic matter, metals, and clays.

Modern analytical techniques such as NMR (nuclear magnetic resonance), GC-MS (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, which are not considered part of HS, are frequently extracted with HS and provide many of the functional properties of HS. Techniques, such as 2D NMR, show that most of the N in HA is in the form of amino acids (Kingery et al.,

2000; Simpson, 2001) and that aliphatics are underrepresented and aromatics are overrepresented in older models of HA structure (Burdon, 2001; Hatcher et al., 2001).

Humic acid (HA) is the alkaline-soluble and acid-insoluble dark brown to black component of HS. Whether HA is a supermixture of low molecular weight molecules (Hayes and Clapp, 2001, MacCarthy, 2001) or a high molecular weight complex (Schulten and Schnitzer, 1997), this fraction is defined operationally as an alkaline soluble material. Elucidating the quantities and composition of HA and glomalin will help clarify the processes involved in soil stabilization and carbon storage. To do this, procedures must be found to determine how much glomalin or HA is co-extracted with each extraction procedure.

In this study, eight soils – two from each of four U.S. states – were extracted for glomalin and HA using Extraction Sequence (ES) 1 – glomalin followed by HA and ES and ES 2 – HA before glomalin. (See Appendix H for diagrams of the sequences.) A highly proteinaceous fraction has been found in HA collected at pH 2.0-2.5 and not in HA collected at pH 1.0 (Clapp and Hayes, 2001; Hayes and Graham, 2000; M.H.B. Hayes, personal communication). This indicates the presence of glomalin in this fraction. Therefore, a subsample of HA extracted during ES 2 was redissolved and precipitated at pH 2.5, 2.0 and 1.0. These HA precipitates were analyzed the same as glomalin and HA collected in Sequences 1 and 2. Because glomalin and HA are operationally defined by extraction procedures (Swift, 1996; Wright et al., 1996), both fractions from the two experiments were carefully examined for co-extraction using total and immunoreactive protein assays (which are specific for glomalin) and C

concentrations (which are higher in HA than in glomalin). Nitrogen, iron and hydrogen concentrations were used to further define glomalin and HA.

The objectives of this study were to: (1) Determine the extent of co-extraction of glomalin and HA by measuring protein concentrations in glomalin and HA isolated by extraction sequences that maximize one fraction or the other, (2) Compare selected chemical characteristics (such as C, N, Fe, H and protein concentrations) of glomalin and HA from both experiments, and (3) Compare amounts of glomalin and HA to soil characteristics [such as clay, P and Fe concentrations] that may affect glomalin and HA accumulation and function.

Materials and Methods

Soils

Bulk soil samples (0-10 cm depth) were collected with a shovel at two locations in four states – Maryland (MD), Nebraska (NE), Colorado (CO) and Georgia (GA). The following series were sampled: Baltimore (MD) (at two sites – MD A and MD B), Sampson and Haxtun (CO), Pacolet and Cecil (GA), and Wymore and Pawnee (NE). Soil was freshly collected at each site except for the NE soils which had been stored at RT for 3 y. Each site had native vegetation. Soils were air-dried and sieved to remove fragments >2-mm. Glomalin and HA were extracted from 10 g (five 2-g duplicate subsamples) of each soil.

Selected variables were measured on soils prior to extraction (Table 3A). Cation exchange capacity (CEC), pH level, and P, sand and clay concentrations were measured by the Soil Testing Laboratory at the University of Maryland (Table 3A). (See Appendix I for values from other elements measured in these soils.) Phosphorus

concentration was measured by colorimetric assay on solution extracted from soil using the Mehlich I procedure. Soil pH was measured in 1:1 (w/v) 0.01 M CaCl₂ solution. Total C, N, and H and organic C were measured by combustion with a Perkin-Elmer Series II C, H, N, S/O 2400 Analyzer (Shelton, CT) at USDA-ARS. Organic C was measured on soil treated with 0.1 N HCl to remove carbonate carbon, rinsed with dH₂O (de-ionized water), pelleted by centrifugation at 6850 × g, and dried at 70°C.

Iron was extracted from soil by a modified Aqua Regia (McGrath and Cunliffe, 1985) procedure and quantified by Atomic Absorption (AA) at USDA-ARS. Briefly, concentrated HNO₃ was added to the sample and heated to 85-90°C (a temperature high enough to cause evaporation but not boiling) for 2 hrs. Next, concentrated HCl (equaling 1 part HNO₃:3 part HCl) was added followed by incubation at 60°C for 1 hr. After hydrolysis, samples were decanted through a Whatman 1 filter into a volumetric flask and brought to volume with dH₂O. Iron concentration was measured with a Varian Atomic Absorption Spectrometer (AA-400, Palo Alto, CA) with deuterium background correction.

Soil organic matter extractions

Citrate extraction of glomalin

Samples were extracted with 50 mM sodium citrate, pH 8.0, at 121°C for 1 h (Wright and Upadhyaya, 1999). (See Appendix A2 for a detailed description of extraction and purification.) Extraction was repeated until the supernatant was straw-colored (up to three more times). All supernatants from each 1 h extraction were combined. Extracts were purified by precipitation, solubilized in NaOH and dialyzed

against dH₂O. Dialyzed material was centrifuged and the supernatant was collected and freeze dried.

Sodium hydroxide extraction of humic acid

HA was extracted according to a method recommended by the International Humic Substances Society (Swift, 1996) with slight modifications. Incubation conditions and solution concentrations used were as described by Swift (1996). (See Appendix A3 for a detailed description of HA extraction.) Modifications of the method were primarily in sample size (2 g instead of 50 g) and the purification procedures. FA was not collected.

Following a pre-incubation with HCl, soil extracted using a multi-step NaOH extraction procedure: (i) neutralization with NaOH under N₂, (ii) extraction overnight with NaOH and (iii) acidification to precipitate HA. The NaOH extraction followed by acidic separation was repeated two more times (until the solution was almost clear) to assure that all HA was extracted. All centrifugations were at 6850 × g for 10 min. Insoluble solid particles were removed from HA by: (i) re-dissolving in a minimum volume of KOH under N₂; (ii) adding KCl (until [K⁺] ≥ 3 M); (iii) centrifugation at 10844 x g to remove suspended solids; and (iv) precipitation with HCl. After settling overnight, samples were centrifuged again and the supernatant was discarded. The precipitated HA fraction then was: (i) suspended in 0.1 N HCl and 0.3 N HF, (ii) incubated overnight, and (iii) centrifuged at 6850 x g with the supernatant discarded. The HCl/HF treatment was repeated twice. Residual acid was removed by repeatedly washing the precipitated HA with dH₂O and centrifuging at 10844 × g for 3 min.

After removal of insoluble particles and ash material, the HA precipitate was re-dissolved in a known minimum volume of 0.1 N NaOH. A subsample (0.5 mL) was removed for protein assays (see below). The remaining solution was acidified immediately to precipitate HA. Acid was removed by centrifuging at 10844 x g and rinsing with water. The precipitate then was freeze-dried.

Extraction sequences

Extraction Sequence 1: Glomalin followed by humic acid

Soils were citrate-extracted for glomalin followed by NaOH extraction of HA (see Appendix H1). Residual soil (soil remaining after sequential extraction of glomalin followed by HA) was re-extracted with citrate to determine whether the NaOH treatment to extract HA facilitated the release of a recalcitrant pool of glomalin. This recalcitrant glomalin (R. glomalin) was collected from all soils except the Pawnee soil (which was not citrate extracted for R. glomalin).

Extraction Sequence 2: Humic acid followed by glomalin

Humic acid was extracted from soils with NaOH, and the remaining soil was extracted with citrate to obtain glomalin (see Appendix H2). Following purification of HA (described above), a subsample of the freeze-dried HA fraction from each soil was extracted with citrate to assess co-extraction of glomalin. The supernatant from citrate extraction of the original HA potentially contained glomalin and HA that was solubilized during the citrate-extraction procedure. The material in this citrate solution was precipitated in steps to determine differences in protein concentration by pH level: (1) pH 2.5 (HA 2.5), (2) pH 2.0 (HA 2.0), and (3) pH 1.0 (HA 1.0). At each step, precipitate was collected by centrifugation at $6850 \times g$ after 30-min incubation on ice.

Following step 3, the supernatant was discarded. Each precipitate was re-dissolved in 0.1 N NaOH and dialyzed against water in dialysis tubing with MWCO of 500 D. After dialysis, the supernatant collected by centrifugation at $6850 \times g$ for 10 min was freeze dried.

Quantitative measurements

Protein assays

Total and immunoreactive protein concentrations were measured on subsamples of glomalin and HA from ES 1 and ES 2, R. glomalin from ES 1 and the three fractions of HA collected at different pH levels in ES 2. (See Appendix C for detailed descriptions of the protein assays). For glomalin and R. glomalin, the subsamples were taken prior to dialysis. For HA, the subsample was collected from re-dissolved HA precipitate as discussed above. HA fractions from ES 2 (HA 2.5, HA 2.0 and HA1.0) were solubilized in water at neutral pH. A subsample was removed from each, and the remaining solution was freeze-dried.

A modified Bradford protein assay (Wright et al., 1996) was used to measure total protein (TP) concentration. Samples were diluted in PBS (phosphate buffered saline) and reacted with Bio-Rad® (Hercules, CA) Bradford protein dye reagent. Absorbance was read at A_{595} after 5 min. Protein concentration was determined by comparison with a bovine serum albumin (BSA) standard curve and reported as g protein kg^{-1} soil.

Immunoreactive protein (IRP) concentration was measured by 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 A_{405} after 15 min. Test samples were compared to a standard curve produced by dilutions of highly immunoreactive glomalin extracted from a temperate soil under native grasses. Immunoreactive protein concentrations were reported as g kg^{-1} soil. Percent immunoreactivity was calculated as amount of IRP divided by amount of TP times 100.

Gravimetric weight

All purified and freeze-dried samples – glomalin, HA, R. glomalin, pH fractionated HA – were weighed to the nearest 0.1 mg. Gravimetric weights were reported as g kg^{-1} soil. Soil after extraction (Residual) was dried at 70°C , weighed and ground with a mortar and pestle for elemental analysis.

Elemental composition

Extracted, freeze-dried organic matter fractions – glomalin, HA, R. glomalin, pH fractionated HA – and Residual soil (stored in a desiccator under vacuum) were analyzed for C, N, H and/or Fe contents. 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 glomalin and HA samples hydrolyzed with Aqua Regia (see above). Hydrolysis was incomplete for a limited number of samples, so the weight of the original sample was corrected for non-hydrolyzed material collected by centrifugation at $10844 \times \text{g}$.

Statistical analysis

Gravimetric weight values were corrected for subsamples removed for protein measurement and for secondary extractions. Means and SEs were calculated for all soils combined. For all mean weight values (i.e. TP, IRP and gravimetric weight) and elemental concentrations (C, N, H and Fe) in glomalin, HA and R. glomalin, two statistical comparisons were made: (1) across sequence for all five fractions (three from ES 1 and two from ES 2) and (2) within Sequences 1 and 2, individually. The first analysis compared glomalin to HA, and the second analysis tested whether extraction sequence affected glomalin or HA individually. All means comparisons for weights and elemental concentrations were made at the $\alpha \leq 0.05$ level by ANOVA (Analysis of Variance) using REML (Restricted Maximum Likelihood) after the residuals met the assumptions for normality and homogeneity of variance. When needed, the log transformation was made to meet the assumptions.

Pearson product-moment correlation coefficients (r) were calculated for gravimetric, TP and IRP weight and percentage Fe in glomalin or HA from ES 1 and soil concentrations of organic C, clay, Fe and P and pH level. Significance was measured at the $\alpha = 0.05$ level. All statistical analyses were performed using SAS software, ver. 8 (SAS Institute, 1999).

Results

Quantitative values

Protein concentrations

Glomalin from ES 1 and ES 2 contained the highest TP concentrations (Table 3B). Protein concentrations in glomalin from ES 2 were 45 to 61% lower than in glomalin from ES 1 for five soils. In the remaining three soils, protein concentration in glomalin from ES 2 was 23 to 62% higher from ES 1.

Humic acid protein concentrations increased in all soils from ES 1 to ES 2. The largest (3 to 4 fold) increases were in the MD and GA soils. Proteinaceous material in HA was concentrated in the fractions that precipitated at pH 2.0 and 2.5. When examined within an extraction sequence, the glomalin fraction contained significantly more protein than HA ($P = 0.0009$) in ES 1. In ES 2, a significant difference between glomalin and HA TP could not be determined ($P = 0.2317$).

Immunoreactive protein concentrations followed a trend similar to TP concentrations. Mean values were significantly higher in the glomalin fractions from both experiments (Table 3C). In the HA fraction, IRP concentrations increased from ES 1 to ES 2 for all soils, except for the Sampson, Pacolet and Cecil soils. Immunoreactive protein concentration was significantly greater in the glomalin fractions than that in HA and R. glomalin fractions from ES 1 and HA from ES 2 ($P = 0.0030$ and <0.0001 , respectively).

Gravimetric weights

When glomalin extraction was maximized (ES 1), weight of glomalin was 7 to 19 g kg⁻¹ soil and weight of R. glomalin was 0.4 to 7 g kg⁻¹ soil (Fig. 3A). Maximizing

HA over glomalin (ES 2) resulted in HA weight increases of 62 to 92% in all soils except Wymore and Pawnee where HA decreased by 27 and 10%, respectively. The Pacolet soil had the largest amounts of R. glomalin in ES 1 and HA in ES 2.

Mean weight of glomalin in ES 1 (13.4 g kg⁻¹ soil) was significantly greater than for R. glomalin and HA (3.0 and 3.1 g kg⁻¹ soil), respectively (P <0.0001). Mean weight of HA (9.1 g kg⁻¹ soil) in ES 2 was not significantly different from glomalin (6.2 g kg⁻¹ soil) (P = 0.2911). Glomalin from ES 1 weighed significantly more than glomalin from ES 2 (P <0.0001).

The HA precipitate collected at pH 2.0 in ES 2 had the highest weight of all three sequentially precipitated fractions in seven soils. In the Pacolet soil, the highest weight of precipitate was obtained at pH 2.5 (Fig. 3B).

Comparison of gravimetric to total protein weight

The total gravimetric weight of glomalin was an average of 20 and 31% proteinaceous for ES 1 and ES 2, respectively. For HA, the total gravimetric weights were 27 and 16% protein for ES 1 and ES 2, respectively. Proteinaceous material was in the HA fractions that precipitated at pH levels 2.0 and 2.5, and averaged 41 and 55% of the gravimetric weight, respectively, for these precipitates.

Elemental composition

In all eight soils, especially the Wymore and Pawnee soils, the percentage C in glomalin increased in ES 2 compared to ES 1 (Table 3D). The R. glomalin fractions had C values comparable to glomalin in ES 2. Percentage C in the HA fraction declined from ES 1 to 2 in all soils, except the Cecil and Wymore soils. The HA fraction that

precipitated at pH levels 2.5 and 2.0 had the highest C values, and, in some cases, these values were even greater than in the original HA sample.

Mean values for elemental concentrations of initial soils, glomalin, HA, R. glomalin and soil after extractions (Residual) are presented in Table 3E. Within ES 1, HA had a significantly higher C than glomalin, but not R. glomalin. Both glomalin and R. glomalin contained more than 4% Fe while HA had 1.2% Fe. In ES 2, HA contained significantly more C and N than glomalin, but there was no significant difference in Fe and H concentrations. The HA fractions collected at pH levels 2.0 and 2.5 in ES 2 were significantly higher in C and H than the fraction collected at pH 1.0 ($P \leq 0.004$).

Glomalin from ES 1 had significantly lower concentrations of C and H than R. glomalin and glomalin from ES 2. Also, glomalin from ES 2 was lower in N than glomalin in ES 1 or R. glomalin. Glomalin and R. glomalin in ES 1 contained more Fe than glomalin in ES 2. Humic acid did not differ significantly in percentage C, Fe, N or H across Sequences 1 and 2.

Comparison of glomalin and soil characteristics

Iron concentration in the soil was significantly and positively correlated with gravimetric weight, IRP and percentage Fe in glomalin from ES 1 and soil organic C and clay content (Table 3F). Glomalin weight also was correlated with Fe in glomalin and clay content. Additionally, organic C was related significantly to glomalin TP and IRP. Humic acid TP concentration was positively correlated with HA weight and soil P concentration.

Discussion

Procedures operationally defined to extract HA or glomalin successfully separated these SOM fractions from the eight soils tested. However, it is currently not possible to isolate humic and nonhumic substances definitively from each other and assure that no co-extraction has occurred (MacCarthy, 2001). Since co-extraction was possible, the differences in weight, protein, and elemental values for glomalin and HA in Experiments 1 and 2 showed that the typical NaOH extraction of HA was more likely to co-extract glomalin than the citrate, high temperature glomalin extraction to co-extract HA.

Glomalin was found in lower amounts and HA in greater amounts in ES 2 than in ES 1 (Fig. 3A). It was speculated that glomalin was co-extracted with HA in ES 2. Higher TP values in HA from ES 2 supported this hypothesis (Table 3B and 3C). The amount of glomalin in HA was estimated to be at least 2 to 14% (mean = 8%) of weight of HA. This estimate was calculated as:

$$\frac{\% \text{ TP in HA from ES 2} - \% \text{ TP in HA from ES 1}}{\text{gravimetric weight of HA in ES 2}} \times 100 \quad (1)$$

Subtraction of HA TP in ES 2 from HA TP in ES 1 corrected for soil proteins or peptides other than glomalin that may have been co-extracted with HA. This calculated value underestimated the amount of glomalin in HA since TP values are only a fraction of gravimetric weight in glomalin. A value somewhere between 8% (calculated from the change in TP) and 80% [calculated from the change in total weight (for six soils, not including Wymore and Pawnee)] actually may represent the amount of glomalin that was co-extracted with HA in ES 2.

Isolation of R. glomalin in ES 1 and the extraction of glomalin in ES 2

confirmed that operationally defined glomalin was different from HA and was part of the 'insoluble' humin fraction. Recent examination of humin shows that it contains more nonhumic substances such as lipids, carbohydrates, and proteins than previously hypothesized (Hayes and Clapp, 2001; MacCarthy, 2001; Rice, 2001). These proteins resist humification by becoming physically encapsulated in humic-mineral complexes (Hatcher et al., 2001). The R. glomalin pool and its similarity to operationally-defined humin indicated that glomalin has a fraction with a slow decomposition rate and may accumulate to high amounts in soil.

Cecil (GA), Sampson and Haxtun (CO) soils contained the lowest concentrations of OM, clay and glomalin (Table 3A). High P concentrations, near neutral pH and low Fe values in the Sampson and Haxtun soils could explain the low glomalin amounts. High P can reduce the growth of AM fungi (Bolan, 1991), and glomalin accumulation is low in calcareous and low Fe soils (Wright and Upadhyaya, 1998). However, the low amount of glomalin in the acidic, low P, high Fe Cecil (GA) soil was contrary to expected levels if P and Fe are controlling factors. The only factor that these three soils have in common that could explain the low OM and glomalin concentrations was the low clay content.

Organic matter concentration is correlated with high clay content. Iron- and Al-(hydr)oxides create bridges between organic matter and clay minerals to form organo-mineral complexes (Hassink and Whitmore, 1997). These complexes protect organic matter from decomposition and are speculated to be responsible for the formation of humin (Hayes and Clapp, 2001; Rice, 2001). In soil, P, Fe and pH may influence the

production of glomalin by impacting AM fungal growth, but overall accumulation of glomalin may be tied to clay content, type of clay, and the presence of Fe- and Al-(hydr)oxides.

Elemental (C, Fe, N and H) percentages in glomalin, R. glomalin and HA showed that these fractions represented different molecules (Table 3E). Glomalin was different from HA because glomalin from ES 1 contained significantly less C and more Fe than HA. Recalcitrant glomalin had similar C values as HA, but a significantly higher Fe concentration (Table 3E).

Carbon, Fe, N and H values for glomalin, R. glomalin and HA were examined to determine if extraction sequence influenced molecular composition and if these changes support the hypothesis of co-extraction. For HA from six of the eight soils, C values declined slightly from ES 1 to ES 2. This supports the hypothesis of co-extraction of glomalin with HA (Table 3D). Using the percentage C values for glomalin from ES 1 and the estimated amount of glomalin in each fraction, it was possible that the slight reduction in HA-C in ES 2 was due to co-extraction of glomalin. Another way to look at the possible co-extraction is to examine HA-C values (mean = 49.7%) in ES 2. This value is almost equal to estimated HA-C values (mean = 49.5%) when 10% of the mass in HA was estimated to come from co-extracted glomalin as discussed above.

For glomalin, C, Fe, N and H contents were significantly affected by extraction sequence (Table 3E). Variation in C does not support the hypothesis of co-extraction of HA in the glomalin extract. If this occurred, glomalin from ES 1 would not be significantly lower in C than glomalin from ES 2 and R. glomalin. Glomalin from ES 2 had significantly less Fe than glomalin from ES 1, which probably decreased the weight

and thereby gave the higher C concentrations. In ES 1, glomalin was 28% C and 4% Fe which equals a total atomic mass of 5.6. If the Fe concentration were reduced to 1%, glomalin would require 42% C for an equivalent mass. The percentage C in glomalin in ES 2 was 43% (Table 3E). Therefore, the additional amount of iron in glomalin from ES 1 would account for the significant difference in percentage C in glomalin between Sequences 1 and 2 and co-extraction of HA would not.

In ES 2, iron in glomalin was not lost by hydrolysis because structural decomposition and concomitant reduction in C and protein values did not occur. Instead, treating the soil with a strong acid (HCl) prior to extraction of HA and glomalin probably reduced Fe concentration in glomalin just as treatment with acid or a strong chelator reduces Fe concentration in HA (Fan et al., 2000; Hatcher et al., 2001; Simpson, 2001). Iron is tightly bound to glomalin but is not a major part of the native glomalin molecule. Glomalin freshly produced on hyphae has Fe concentrations of \leq 0.3% (Chapter 7).

In the recalcitrant glomalin pool (which had high Fe values), high heat used in extraction of the initial glomalin possibly helped 'fuse' Fe on glomalin in such a way that it could not be removed by the acid treatment as it was in ES 2. Similar types of fusion have been found for aromatic compounds in char material produced by burning (Hayes and Clapp, 2001) and other research by our lab has shown that some of the extraction buffer may be incorporated as part of the glomalin molecule with the high heat used for extraction (Chapter 7).

Recalcitrant glomalin also had a high percentage C (higher than glomalin in ES 1 and similar to glomalin in ES 2). Presumably, this pool of glomalin has resided in the

soil for a long time. Although resistant, glomalin is not immune to decomposition. Hydrolysis and oxidative chemical degradation in the soil and during the extraction procedure can induce significant changes in organic matter (Hayes and Clapp, 2001; MacCarthy, 2001). This type of degradation, or humification, would leave behind more long chain hydrocarbons (with a higher C percentage) than more labile carbohydrates and amino acids (with a lower C percentage).

Contamination of HA with glomalin during the typical NaOH extraction was expected (Chapter 2). Glomalin is extracted routinely in alkaline solutions (Wright et al., 1996; Appendix G), and a small fraction of glomalin is soluble after prolonged incubation in an alkaline solution at RT (unpublished data). In addition, proteinaceous and carbohydrate residues have frequently been found in the HA fraction that are co-extracted with the HA molecule but are not part of its structure (Burdon, 2001; Hatcher et al., 2001; Hayes and Clapp, 2001; Simpson, 2001). In ES 2, stepwise titration at pH 2.5, pH 2.0 and pH 1.0 was used in an attempt to separate HA and glomalin because a highly proteinaceous fraction is isolated at pH 2.5 to 2.0 (Hayes and Graham, 2000; Hayes and Clapp, 2001) and glomalin precipitates in this pH range (Wright et al., 1996). Total protein concentrations in the pH 2.5 and pH 2.0 precipitated fractions showed that glomalin was present. The HA 1.0 fraction was very low in protein, but the C, N and H content differed greatly from the relatively low-protein HA from ES 1. Therefore, HA 1.0 was probably not representative of HA.

The fraction that is isolated between pH 2.5 and pH 2.0 by Hayes 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. Further studies of these residues are presented in Chapter 7. This study demonstrated that the NaOH extraction method used for HA will co-extract glomalin or a glomalin-like molecule. To obtain isolated glomalin and HA fractions, glomalin must be extracted prior to HA. The R. glomalin fraction also needs to be examined further along with the organic (humins) fraction that remains in the soil. Soil organic matter is an important component to soil health. A greater understanding of the molecules that comprise organic matter will increase understanding of its functional roles and how to maintain and/or accumulate more of it in soil.

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Table 3A. Characteristics of eight soils extracted for glomalin and humic acid. †

	Baltimore (site <i>a</i>)	Baltimore (site <i>b</i>)	Sampson	Haxtun	Pacolet	Cecil	Wymore	Pawnee
Soil Type	Mollic Hapludalf	Mollic Hapludalf	Pachic Argiustolls	Pachic Argiustolls	Typic Kanhapludult	Typic Kanhapludult	Aquertic Argiudolls	Oxyaquic Vertic
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‡	750	150	210
Clay (g kg ⁻¹)	250	260	160	100	220‡	110	280	200
pH	5.1	5.0	6.7	6.6	5.4	4.4	5.5	5.6
CEC (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

† NA = insufficient amount of sample for this analysis.

‡ Values from Franzluebbbers et al., 2000.

Table 3B. Total protein (g protein kg⁻¹ soil) in glomalin, humic acid (HA), recalcitrant glomalin (R. glomalin), and HA fractions precipitated at pH levels 2.5, 2.0 and 1.0 extracted from eight native U.S. soils when glomalin concentrations were maximized by extracting glomalin first (Extraction Sequence 1) or when HA values were maximized by extracting HA first (Extraction Sequence 2). †

Extraction Sequence	Fraction	MD A	MD B	Sampson	Haxtun	Pacolet	Cecil	Wymore	Pawnee	Mean‡
1	Glomalin	2.53	1.61	1.79	1.63	4.05	3.14	1.76	1.87	2.3±0.3a
	HA	0.41	0.37	0.78	0.23	0.90	0.36	0.75	0.66	0.6±0.1c
	R. glomalin¶	NA	NA	0.15	NA	2.94	1.77	0.22	ND	1.3±0.7bc
	HA	1.74	1.51	0.87	0.59	2.70	1.39	0.84	0.92	1.3±0.2b
2	pH 2.5§	0.55	0.48	0.31	0.02	5.56	0.06	0.80	0.07	1.0±0.7
	pH 2.0§	2.59	1.77	1.74	0.94	0.03	2.48	1.71	1.59	1.6±0.3
	pH 1.0§	0.03	0.03	0.01	0.03	0.03	0.06	0.10	0.05	0.0±0.0
	Glomalin	0.98	0.69	2.27	0.89	2.02	1.38	4.67	3.72	2.1±0.5ab

† NA = quantity of sample was insufficient for analysis; ND = samples not collected.

‡ Mean ± SE. Different letters within a column indicate significant differences (P = 0.0016) according to REML.

§ Fractions of HA collected by step-wise precipitation.

¶ R. glomalin = recalcitrant glomalin released from soil after NaOH extraction of HA.

Table 3C. Immunoreactive protein (g protein kg⁻¹ soil) in glomalin, humic acid (HA), recalcitrant glomalin (R. glomalin), and HA fractions precipitated at pH levels 2.5, 2.0 and 1.0 extracted from eight native U.S. soils when glomalin concentrations were maximized by extracting glomalin first (Extraction Sequence 1) or when HA values were maximized by extracting HA first (Extraction Sequence 2). †

Extraction Sequence	Fraction	MD A	MD B	Sampson	Haxtun	Pacolet	Cecil	Wymore	Pawnee	Mean‡
1	Glomalin	1.41	0.92	0.59	0.26	1.51	0.60	0.23	0.29	0.7±0.1a
	HA	0.01	0.01	0.28	0.07	0.27	0.13	0.00	0.00	0.1±0.0c
	R. glomalin¶	NA	NA	0.30	NA	0.56	0.24	0.00	ND	0.3±0.1bc
2	HA	0.13	0.15	0.14	0.08	0.13	0.12	0.11	0.12	0.1±0.0c
	pH 2.5§	0.06	0.06	0.06	0.00	0.52	0.00	0.09	0.01	0.1±0.1
	pH 2.0§	0.16	0.12	0.10	0.05	0.00	0.10	0.12	0.18	0.1±0.0
	pH 1.0§	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0±0.0
	Glomalin	0.39	0.22	0.61	0.49	0.57	0.33	0.77	0.66	0.5±0.1ab

† NA = samples without enough material for analysis; ND = samples not collected.

‡ Mean ± SE. Different letters in a column indicate significant differences (P <0.0001) according to REML.

§ Fractions of HA collected by step-wise precipitation.

¶ R. glomalin = recalcitrant glomalin released from soil after NaOH extraction of HA in ES 1.

Table 3D. Carbon percentages in glomalin, humic acid (HA), recalcitrant glomalin (R. glomalin), and HA fractions precipitated at pH levels 2.5, 2.0 and 1.0 extracted from eight native U.S. soils when glomalin concentrations were maximized by extracting glomalin first (Extraction Sequence 1) or when HA values were maximized by extracting HA first (Extraction Sequence 2). †

Extraction Sequence	Fraction	MD A	MD B	Sampson	Haxtun	Pacolet	Cecil	WymorePawnee	
1	Glomalin	26.52	24.63	40.69	31.01	36.71	37.12	15.06	15.54
	HA	54.96	55.01	53.72	54.58	39.59	50.65	29.49	51.91
	R. glomalin¶	39.29	37.11	47.67	46.51	41.91	42.76	45.86	ND
	HA	45.98	50.91	52.08	53.62	38.72	52.79	52.50	51.21
	pH 2.5§	49.86	48.40	46.55	NA	51.03	51.34	49.26	37.86
2	pH 2.0§	50.53	50.06	50.23	51.53	NA	NA	49.12	49.95
	pH 1.0§	44.56	30.84	25.58	33.76	37.51	NA	40.78	37.74
	Glomalin	39.99	39.08	48.80	44.17	38.98	40.11	47.59	47.89

† NA = samples without enough material for analysis; ND = samples not collected.

§ Fractions of HA collected by step-wise precipitation.

¶ R. glomalin = recalcitrant glomalin released from soil after citrate extraction was followed by NaOH extraction.

Table 3E. Elemental analysis [carbon (C), iron (Fe), nitrogen (N) and hydrogen (H)] of soil before (Initial) and after (Residual) extraction, glomalin, humic acid (HA), recalcitrant glomalin (R. glomalin), and HA fractions precipitated at pH levels 2.5, 2.0 and 1.0 extracted from eight native U.S. soils in Extraction Sequence 1: citrate extraction of glomalin prior to NaOH extraction of HA or Extraction Sequence 2: citrate extraction of glomalin after NaOH extraction of HA. †

	Fraction	C	Fe‡	N	H	n§
		%				
Extraction Sequence 1	Glomalin	28.41±3.44b	4.1±0.8a	2.56±0.36a	3.68±0.31b	8 (8)
	HA	48.74±3.29a	1.2±0.5b	3.54±0.30a	4.89±0.42a	8 (7)
	R. Glomalin	43.02±1.48a	5.5±3.4a	4.01±0.62a	5.01±0.22a	7 (3)
	Residual soil	1.26±0.19	ND	0.12±0.03	0.38±0.09	8
Prob>F		0.0003	0.0282	0.0763	0.0191	
Extraction Sequence 2	HA	49.73±1.78a	0.8±0.6a	3.96±0.20a	4.65±0.19a	8 (8)
	pH 2.5	47.16±1.96	ND	4.14±0.26	4.63±0.22	6
	pH 2.0	50.39±0.32	ND	4.06±0.17	4.94±0.16	7
	pH 1.0	35.82±2.40	ND	4.15±0.77	3.28±0.33	7
	Glomalin	43.33±1.51b	1.3±0.4a	2.98±0.19b	4.39±0.21a	8 (7)
Soil - Residual		0.83±0.12	ND	0.10±0.03	0.23±0.07	8
Prob>F		0.0158	0.0627	0.0028	0.3752	
Soil – Initial		2.87±0.35	0.92±0.22	0.21±0.06	0.63±0.11	8

† Means \pm SE in a column and within an experiment followed by different lowercase letters are significantly different at $\alpha = 0.05$ according to REML.

‡ ND = not determined.

§ n = number of soils used for C, N and H. Values in parentheses were for Fe.

Table 3F. Correlation coefficients (r) comparing gravimetric (weight), total protein (TP), or immunoreactive protein (IRP) weights of glomalin† and humic acid† (HA) and percentage iron (Fe) in glomalin to soil edaphic factors – pH and concentrations of organic carbon (C), iron (Fe), phosphorus (P) and clay.‡

Variables	TP	IRP	Fe	Weight	TP	Clay	pH	Organic C	Fe	P
	Glomalin			HA						
Weight – Glomalin	-0.1263	0.3022	0.8992*	0.1918	0.0366	0.8041*	-0.4568	0.4262	0.6409*	-0.1650
TP – Glomalin		0.6698*	0.0080	0.0688	0.2977	-0.0693	-0.4943	0.7510**	0.4264	0.0520
IRP – Glomalin			0.2810	-0.2729	0.1640	0.3525	-0.3693	0.8530***	0.8705*	-0.1160
Fe – Glomalin				0.1934	-0.0403	0.8219*	-0.6440	0.5014	0.6296*	-0.4590
Weight – HA					0.7850*	0.3731	0.0587	0.1942	-0.0934	0.4945
TP – HA						0.3913	0.1820	0.4575	0.1957	0.6742*
Clay							-0.2954	0.5777	0.7017*	-0.1509
pH								-0.5369	-0.5031	-0.3922
Organic C									0.8743*	-0.0713
Fe										-0.2451

† Glomalin and HA samples were those from Extraction Sequence 1 (i.e. glomalin extracted before HA).

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

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

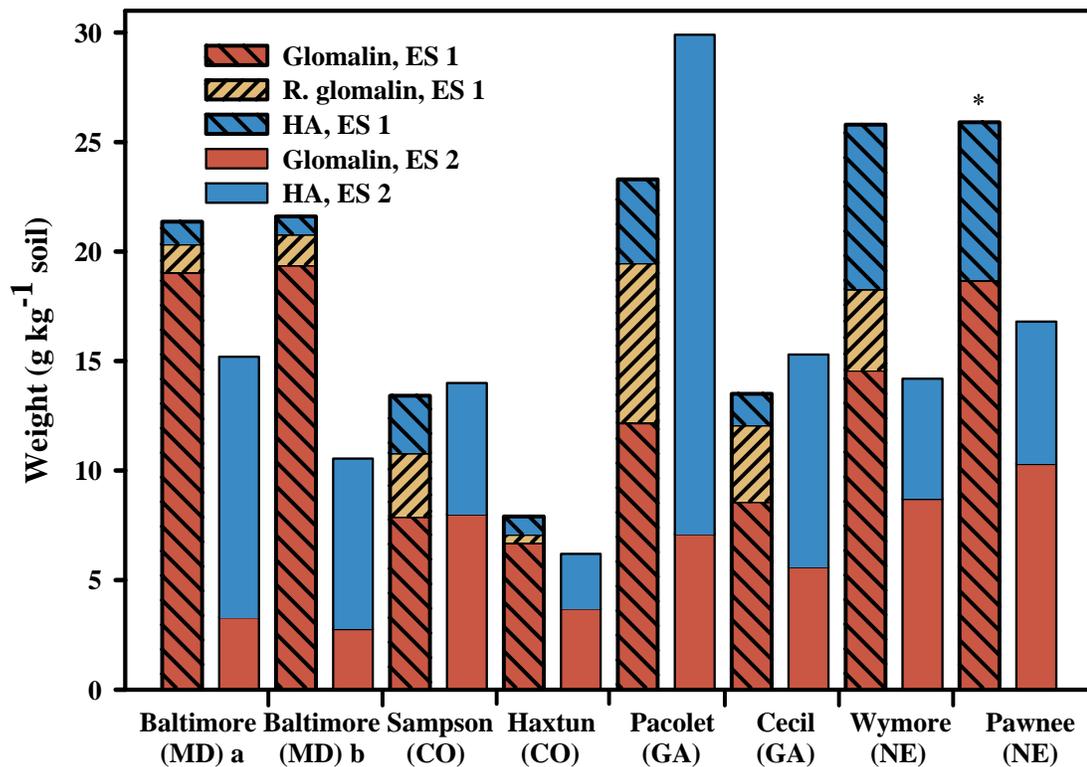


Figure 3A. Weights of glomalin (glomalin and R. glomalin) and humic acid (HA) extracted from eight native U.S. soils in Extraction Sequence (ES) 1: citrate extraction of glomalin prior to NaOH extraction of HA or ES 2: citrate extraction of glomalin after NaOH extraction of HA.

* R. glomalin was not extracted from the Pawnee soil.

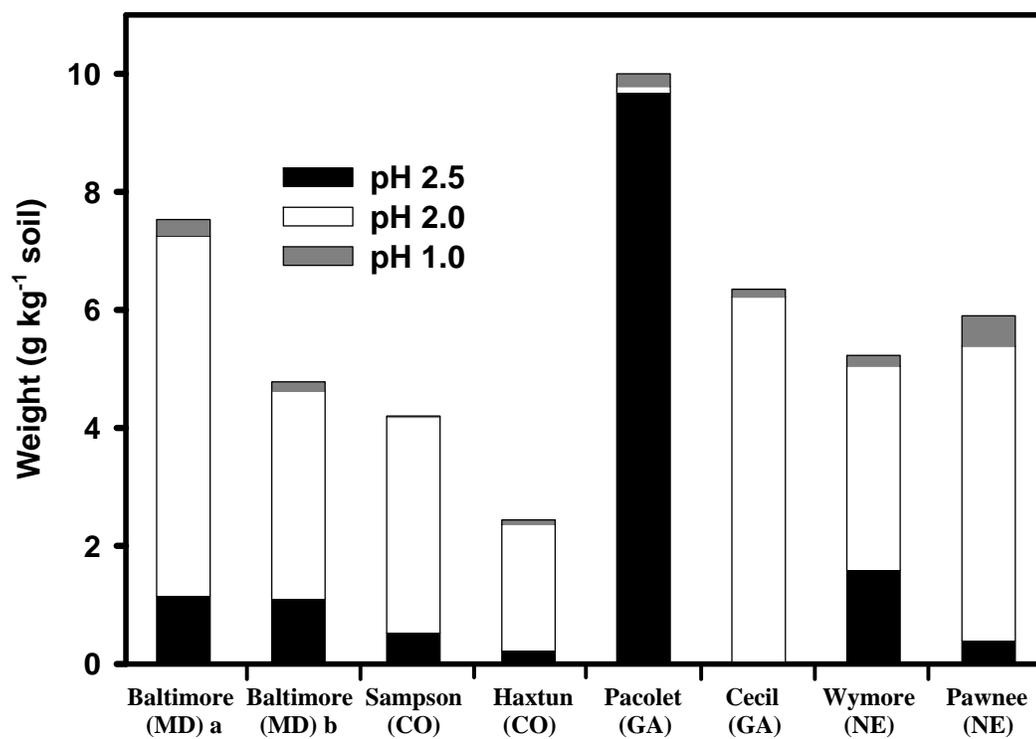


Figure 3B. Weights of humic acid (HA) fractions extracted from eight native U.S. soils and precipitated at pH levels 2.5, 2.0 and 1.0 in Extraction Sequence 2: NaOH extraction of HA before citrate extraction of glomalin.

CHAPTER 4

SOIL ORGANIC MATTER DISTRIBUTION IN SOIL AGGREGATES

Abstract

Aggregation is a soil quality factor that positively affects water infiltration rates, resistance to erosion, and nutrient cycling. The fraction or type of carbon compound influences the persistence and water-stability of aggregates. The hypothesis that extractable organic matter fractions are related to aggregate stability was tested in five native U.S. soils. Three aggregate size classes (1-2, 0.5-1, and 0.25-0.5 mm) were separated by dry sieving. The percentage of water-stable aggregates (WSA) was measured by wet-sieving. Within each aggregate size class, the major labile and recalcitrant carbon fractions — Residual POM, glomalin, humic acid (HA), and fulvic acid (FA) — were extracted. The weight of organic carbon in each fraction was measured. All three aggregate size classes from the five soils were highly stable (most >90%). The highest amounts of carbon were found in the humin (i.e. unextractable carbon remaining after all other fractions had been extracted), Residual POM and glomalin fractions. The amount of Residual POM declined with decreasing aggregate size, while the more recalcitrant fractions [i.e. humin and a recalcitrant fraction of glomalin (R. glomalin)] increased. Total soil carbon, R. glomalin carbon, and FA carbon were significantly correlated with WSA. The R. glomalin, humin, HA, FA and total carbon weights were related to iron concentration in the aggregates which indicated that these organic matter fractions are stabilized within organo-mineral complexes formed by iron bridging organic matter to clay particles. These organo-mineral complexes are important in the long-term stability of aggregates and may help native and agricultural soils sequester carbon.

Introduction

Soil aggregation is a process in which the forces binding soil particles together are stronger than the forces between adjacent clusters of particles (Degens, 1997; Tisdall and Oades, 1982). A well-aggregated soil maintains porosity and aeration that is favorable to plant and microbial growth, water infiltration, stability against water and wind erosion, and protects organic matter from microbial decomposition (Bird et al., 2002; Degens, 1997; Hassink and Whitmore, 1997). A number of biochemical (inorganic and organic) and physical processes are involved in the formation and stability of aggregates.

Biochemical processes produce binding agents and stabilizing compounds such as polysaccharide glues, aliphatic polymers, and organo-mineral complexes formed by polyvalent cations bridging organic matter to clay particles (Degens, 1997; Diné and Nolan, 2000; Piccolo and Mbagwu, 1999; Chenu et al., 2000). Drying and wetting actions, shrinking and swelling of clays, flocculation of clay minerals, freeze-thaw cycles, compaction, and enmeshing by fungal hyphae and/or fine roots physically stabilize aggregates (Chaney and Swift, 1986; Degens, 1997; Franzluebbers et al., 2000; Tisdall and Oades, 1982).

Organic constituents of aggregates are plant or microbial debris, fungal hyphae, bacteria, free amorphous organic matter (OM) and OM strongly associated with clay particles (i.e. clay coatings) (Chenu et al., 2000). This organic matter contributes directly and indirectly to aggregate stability (Chenu et al., 2000; Tisdall and Oades, 1979). Indirectly, organic matter provides energy for microorganisms, and directly, the products produced by microbial degradation act as binding agents in aggregate

formation. Three classes of organic binding agents that have been identified are: (1) transient, polysaccharides, (2) temporary roots and hyphae, and (3) persistent compounds associated with polyvalent cations (Jastrow et al., 1998; Tisdall and Oades, 1982).

Plants and soil biota in concert influence aggregation by (1) root growth, exudation and architecture, (2) production of organic acids, (3) metabolism of root exudates, (4) decomposition of organic debris, (5) physical entanglement by roots and hyphae, (6) production of soil-binding agents by roots, bacteria, filamentous fungi and actinomycetes, and (7) earthworm activity (Rillig et al., 2002; Schreiner and Bethlenfalvay, 1995). In addition to belowground contributions, plants form canopies that protect the soil surface from disturbance and increase organic matter inputs and moisture content (Bird et al., 2002). Both plant roots and fungal hyphae may initiate aggregate formation by enmeshing or cross-linking organic matter and coarse fragments, acting as nucleation sites, and/or by supplying substrates to the microbial community (Bethlenfalvay et al., 1999; Degens, 1997; Jastrow et al., 1998; Schreiner and Bethlenfalvay, 1995; Tisdall and Oades, 1982).

A rapidly developing hyphal network is related to an equally rapid increase in water-stable aggregates (WSA) (Bethlenfalvay et al., 1999). The majority of the hyphae in soil is from arbuscular mycorrhizal (AM) fungi (Miller et al., 1995; Schreiner and Bethlenfalvay, 1995). These fungi have several advantages over other soil fungi: (1) AM fungi utilize photosynthetic carbon which eliminates the carbon limitations that affect saprophytic growth, (2) grazers prefer saprobic hyphae over AM hyphae, (3) AM fungal hyphae have an affinity for binding soil organic matter, (4) hyphae have the

ability to enter micropores within the soil matrix to form aggregates and acquire nutrients, (5) active AM hyphae may enhance aggregate stability by extracting water and causing a parallel reorientation of embedded clays (similar to what has been seen on plant roots), and (6) an amorphous, polysaccharide-containing organic substance has been found coating the surface of AM fungal hyphae and attached sand grain-sized particles (Gupta and Germida, 1988; Rillig et al., 2002; Schreiner and Bethlenfalvay, 1995; Tisdall and Oades, 1979; Wright and Upadhyaya, 1996).

Analysis of the amorphous substance on hyphae of AM fungi showed that: (1) clay particles attach firmly to it, (2) it contains sugars, mostly glucose and some galactose, xylose and possibly mannose, and (3) it does not contain phenolics, but may have proteins, aromatics or nucleotides (Schreiner and Bethlenfalvay, 1995; Tisdall and Oades, 1979). The same could be said for glomalin (Wright et al., 1996). In addition, glomalin is a hydrophobic substance that protects hyphae from nutrient and water loss (Wright et al., 1996), which is similar to a class of proteins, called hydrophobins, found on hyphae of a variety of fungi, including ectomycorrhizal fungi (Wessels, 1999). Hydrophobins form an amphipathic membrane that protects aerial hyphae from moisture changes (Wessels, 1999). Glomalin is a biomolecule that resists decomposition and has been correlated with WSA in a number of soils (Bird et al., 2002; Rillig et al., 2002; Wright et al., 1999; Wright and Upadhyaya, 1998).

Aggregate stability is a function of the strength of the physiochemical attractions between the organic and mineral components and the lability, size and location of organic binding agents (Dinel and Nolin, 2000; Jastrow et al., 1996). Labile fractions (i.e. polysaccharides) may increase aggregate formation initially but a decrease in

aggregation is seen after only a few weeks (Degens, 1997; Piccolo and Mbagwu, 1999). Polysaccharides are ephemeral (i.e. water-soluble and readily degraded by microbes) and of small molecular size (Chaney and Swift, 1986; Six et al., 2001). Therefore, they are unlikely to be important binding agents in aggregates with diameters of several millimeters and are less important to aggregate stability under high organic matter contents (Jastrow et al., 1996; Kemper and Koch, 1966; Tisdall and Oades, 1982). Transient binding agents and entanglement by temporary roots and hyphae are not enough to form persistent, water-stable aggregates. Instead, cementing agents that are resistant to microbial degradation by being either biochemically-resistant or physically inaccessible are required along with hydrophobic, water-stable compounds.

The formation of organo-mineral complexes produces clay “skins” or iron oxide coatings on aggregates which cements aggregates together (Franzluebbers et al., 2000; Gupta and Germida, 1988; Tisdall and Oades, 1982). Slow-decomposing, organic compounds from decaying plants, fungi and arthropods, such as lignin and chitin, also may act as persistent binding agents (Schreiner and Bethlenfalvay, 1995). Aliphatic compounds (or long-chain hydrocarbons) that have been measured in high concentrations in organic matter are hydrophobic and promote water-stability in aggregates (Capriel et al., 1990; Caron et al., 1996; Chenu et al., 2000; Piccolo and Mbagwu, 1999).

Without the protection of hydrophobic coatings, soil aggregates may be disrupted by rainfall because of slaking, the differential swelling of clays and mechanical dispersion by the kinetic energy of raindrops (Piccolo and Mbagwu, 1999). Therefore, non-uniform distribution of organic matter which contains high

concentrations of aliphatic groups may increase aggregate stability and decrease the decomposition of more labile organic matter within aggregates (Piccolo and Mbagwu, 1999). These aliphatic, hydrophobic groups increase the contact angle for water penetration, which reduces slaking, lower wettability and increase the internal cohesion of aggregates (Caron et al., 1996; Chenu et al., 2000). Within aggregates, the formation of clay-metal-humic linkages (where O-containing hydrophilic groups in humic substances orient the hydrophobic moiety to the aggregate surface) creates a water-repellent coating on aggregates (Piccolo and Mbagwu, 1999).

Aggregates are separated into two large size classes: macroaggregates (>250 μm) and microaggregates (<250 μm). The hierarchical model for aggregate formation states that macroaggregates consist of smaller aggregates bound together by roots, hyphae and labile organic fractions (Bethlenfalvay et al., 1999; Miller et al., 1995; Tisdall and Oades, 1982). The objectives of this study were to determine which organic matter fractions were related to WSA and the distribution of organic carbon in each aggregate size class. Because fungal hyphae have an important functional role in the formation of macroaggregates, three sizes of macroaggregates (1-2, 0.5-1, and 0.25-0.5 mm) were separated from five native U.S. soils. The percentage of WSA was measured by wet-sieving and related to fractions of organic matter – Residual POM, glomalin, humic acid (HA), fulvic acid (FA), and humin.

Materials and Methods

Soils

Bulk soil samples (0-10 cm depth) were collected with a shovel from the following soil series: Baltimore (MD) (site b), Sampson and Haxtun (CO), and Pacolet

and Cecil (GA). (For more details on these soils, see Chapters 2 and 3.) Soils were freshly collected. All sites had native vegetation. Prior to aggregate separation and organic matter extraction, soils were air-dried and passed through a 2-mm screen.

Aggregate separation

Air-dried soil was dry sieved with the appropriate screen size to remove 1- to 2-mm (A), 0.5- to 1-mm (B) and 0.5- to 0.25-mm (C) aggregates. Aggregate stability was determined according to Kemper and Koch (1966). Briefly, 2-4 g of air-dried soil in each aggregate size classes (A, B and C) was placed individually onto screens ¼ of their size and capillary rewetted for 10 min. Aggregates were separated via mechanically wet sieving for 5 min using an apparatus described by Kemper and Koch (1966). Material collected on the sieve was washed gently into weigh boats, dried at 70°C and weighed. The coarse material was removed by adding 0.5% sodium hexametaphosphate and shaking periodically over a 5 min period to disrupt the aggregates. Coarse material was collected on a screen matching the aggregate size and subtracted from the amount of aggregates collected after wet sieving. Percentage water-stable aggregates (WSA) was calculated as:

$$\frac{(\text{weight of material left after wet sieving}) - (\text{weight of coarse material})}{(\text{original weight placed in screen prior to wet sieving})} \times 100 \quad (1)$$

Iron Analysis

Iron was extracted from soil by a modified Aqua Regia (McGrath and Cunliffe, 1985) procedure and quantified by Atomic Absorption (AA) at USDA-ARS. Briefly, concentrated HNO₃ was added to the sample and heated to 85-90°C (a temperature high enough to cause evaporation but not boiling) for 2 hrs. Next,

concentrated HCl (1:3 HNO₃:HCl) was added followed by incubation at 60°C for 1 hr. After hydrolysis, samples were decanted through a Whatman 1 filter into a volumetric flask and brought to volume with deionized water (dH₂O). Iron concentration was measured with a Varian Atomic Absorption Spectrometer (AA-400, Palo Alto, CA) with deuterium background correction.

Soil organic matter extractions

Density separation of particulate organic matter

Particulate organic matter was removed by flotation in a high-density solution (Wolf et al., 1994). Soil samples were covered with a NaCl solution (12%, w/v), vortexed, and allowed to settle. After the mineral fraction had settled, the solution was carefully decanted. Floating organic matter (i.e. the POM fraction) was collected on a 0.053 mm screen. This procedure was repeated four more times. The POM fraction collected on the screen was washed with distilled water to remove salt, rinsed from the screen into pre-weighed weigh boats and dried at 70°C. The mineral fraction (i.e. soil minus POM) was washed with distilled water, pelleted by centrifugation, rinsed into pre-weighed weigh boats and dried at 70°C.

Citrate extraction of glomalin

Samples (the POM fraction and soil minus POM) were extracted with 50 mM sodium citrate (Wright and Upadhyaya, 1999). (See Appendix A2 for a detailed description of the glomalin extraction method.) Extraction was repeated until the supernatant was straw-colored (up to three more times). Glomalin was purified by acid precipitation, solubilized in NaOH and dialyzed against deionized water (dH₂O). Dialyzed material was centrifuged and the supernatant was collected and freeze dried.

Sodium hydroxide extraction of humic and fulvic acids

A method from Swift (1996) recommended by the International Humic Substances Society was used to extract HA and FA. (See Appendix A3 for a detailed extraction method.) Modifications of the method were primarily in sample size (2 g instead of 50 g) and in the purification procedures. After a pre-incubation in HCl, HA and FA were co-extracted from subsamples using a multi-step NaOH extraction procedure: (i) neutralization with NaOH under N₂, (ii) extraction with NaOH overnight, (iii) acidification of the supernatant, and (iv) separation of HA (precipitate) from FA (supernatant) by centrifugation. The NaOH extraction followed by acidic separation was repeated two more times (until the solution was almost clear) to assure that all humic and fulvic acids were extracted.

Purification of the HA precipitate involved the removal of insoluble particles and ash material using the following procedures. Insoluble solid particles were removed from HA by: (i) re-dissolving in a minimum volume of KOH under N₂; (ii) adding KCl (until $[K^+] \geq 3 M$); (iii) centrifugation at 10844 x g to remove suspended solids; and (iv) precipitation with HCl. After settling overnight, samples were centrifuged again and the supernatant was discarded. To reduce ash content, the precipitated HA fraction was: (i) suspended in 0.1 N HCl and 0.3 N HF, (ii) incubated overnight, and (iii) centrifuged at 6850 x g with the supernatant discarded. The HCl/HF treatment was repeated twice. Residual acid was removed by repeated washing with dH₂O and centrifugation at 10844 x g for 3 min.

After removal of insoluble particles and ash material, the HA precipitate was re-dissolved in a known minimum volume of 0.1 N NaOH. A subsample (0.5 mL) was

removed for protein assays (see below). The remaining solution was acidified rapidly to precipitate HA. After precipitation, acid was removed by centrifugation at $10844 \times g$ and rinsing with water. The precipitate then was freeze-dried.

The fulvic acid fraction (i.e. acid soluble material) was purified by dialysis against water until the pH was neutral. Insoluble material was collected by centrifugation at $6850 \times g$ for 10 min and the supernatant was freeze dried.

Extraction sequence

In each aggregate size class (A, B and C), organic matter was extracted sequentially from five 2-g subsamples of dry-sieved aggregates in each soil. (See Appendix A for detailed descriptions of the extraction methods and Appendix B for a diagram of the extraction sequence.) The POM fraction was separated first followed by glomalin and humic and fulvic acids from both the POM and soil minus POM fractions. This extraction sequence yielded nine fractions: POM after extraction of glomalin, HA and FA (Residual POM); glomalin, HA and FA extracted from the particulate organic matter fraction (i.e. P. glomalin, P. HA and P. FA); glomalin; glomalin extracted after humic and fulvic acid extraction (i.e. R. glomalin); HA; FA; and unextractable carbon material left behind after all the extractions were complete (i.e. humin).

Organic matter quantification

Protein measurements

Total and immunoreactive protein concentrations were measured on subsamples of glomalin, HA, FA, and R. glomalin extracted from the POM fraction or the soil (See Appendix C for detailed description of the protein assays.) Assay values were extrapolated to $g\ kg^{-1}$ by correcting for the weight of soil and volume of extract

solution. For glomalin, R. glomalin and FA, the subsamples were collected prior to dialysis. For HA, the subsamples were collected from HA re-dissolved as discussed above.

A modified Bradford protein assay (Wright et al., 1996) was used to measure total protein (TP) concentration. Samples were diluted in PBS (phosphate buffered saline) and reacted with Bio-Rad® (Hercules, CA) Bradford protein dye reagent. Absorbance was read at A_{595} after 5 min. Protein concentration was determined by comparison with a bovine serum albumin (BSA) standard curve and reported as g protein kg^{-1} soil.

Immunoreactive protein (IRP) concentration was measured by 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 A_{405} after 15 min. Test samples were compared to a standard curve produced by dilutions of highly immunoreactive glomalin extracted from a temperate soil under native grasses. Immunoreactive protein concentrations were reported as g kg^{-1} soil. Percent immunoreactivity was calculated as amount of IRP divided by amount of TP times 100 and reported as the range and the mean for all soils.

Gravimetric weight measurements

Non-soluble materials (i.e. POM, Residual POM, and soil before (Initial) and after (Residual) extraction) were dried at 70°C and weighed. Freeze-dried, purified

soluble extracts (i.e. glomalin, HA and FA) were weighed. Sample weights were to the nearest 0.1 mg. Gravimetric weights were reported as g kg⁻¹ soil.

Carbon weight measurements

All samples were analyzed by combustion for percentage carbon using a Perkin-Elmer Series II 2400 CHNS/O Analyzer. Soil samples, POM and Residual POM were ground with mortar and pestle prior to CHN analysis. Carbon concentration was calculated based on gravimetric weight and reported as g kg⁻¹ soil.

Statistical analysis

Gravimetric and carbon weight values were corrected for subsamples removed for protein measurement. In each aggregate size class, means and SEs for WSA and gravimetric and carbon weights were calculated for all five soils combined. Gravimetric and carbon weight values for each organic matter fraction were compared within an aggregate size class. Water-stable aggregates were analyzed across all three aggregate size classes. All means comparisons were made at the $\alpha \leq 0.05$ level by ANOVA (Analysis of Variance) using REML (Restricted Maximum Likelihood) test after the residuals met the assumptions for normality and homogeneity of variance. When needed, the appropriate transformation was made to meet the assumptions.

Pearson product-moment correlation coefficients (r) were calculated for WSA, percentage Fe in soil and carbon weights in the bulk soil and all the organic matter fractions. Regression analysis was used to compare the percentage iron in the soil to the weights of carbon in each fraction and total carbon in the soil. All statistical analyses were performed using SAS software, ver. 8 (SAS Institute, 1999).

Results

Water-stable soil aggregates

The percentage WSA varied across soils and aggregate size classes (A, B and C) (Table 4A). In the Pacolet and Cecil soils, all three aggregate size classes had high water stability ($\geq 95\%$) and did not vary from A to C. The A sized aggregates in the Baltimore and Sampson series soils were about 50% water-stable, while both the B and C sized aggregates were over 90% stable in these soils. The Haxtun soil had a steady increase in aggregate stability with a decrease in aggregate size. For the five soils, the average amount of WSA was not significantly different among the aggregate size classes.

Organic matter quantification

The mean gravimetric weight in each fraction – Residual POM, P. glomalin, glomalin, R. glomalin, HA, and FA – was different in each of the three aggregate size classes (Table 4B). (See Appendix J for a table of weight values in each fraction in each soil.) The amounts of HA and FA extracted from the POM fraction in all three aggregate size classes were small (0.25 and 0.15 g kg⁻¹) and could not be measured in some soils. Therefore, these fractions (P. HA and P. FA) are not discussed further. The Residual POM and glomalin fractions were significantly higher than all the other fractions. However, the weight of Residual POM decreased with decreasing aggregate size, whereas glomalin increased. The recalcitrant glomalin fraction also increased with decreasing aggregate size along with HA and FA.

The protein assays confirmed the presence of glomalin in the P. glomalin, glomalin, R. glomalin, and HA fractions. For P. glomalin, the amount of TP accounted

for 80, 41 and 67% of the gravimetric weight in aggregates A, B and C. (See Appendix K for protein and percentage IRP values in the glomalin and HA fractions in each soil.) Lesser amounts of the weights were proteinaceous in the glomalin (33, 28 and 26%) and R. glomalin (30, 31 and 31%) fractions for the A, B and C aggregates. In HA, protein weight was 54, >100 and 99% of the gravimetric weight. Aggregate size A, B and C glomalin was 23, 21, and 16% immunoreactive and HA was 37, 35, and 26% immunoreactive. POM glomalin and R. glomalin ranged from 16 to 17% and 13 to 15% immunoreactive, respectively, for the three aggregate sizes.

Carbon weights in the seven organic matter fractions varied across aggregate size class and soil (Fig. 4A). The Baltimore soil contained very little Residual POM and P. glomalin. In the other four soils, especially the Sampson and Haxtun soils, the carbon contributions from Residual POM and P. glomalin declined from aggregate size B to C. The other fractions increased in aggregates B and C. For all of the soils, except the Haxtun soil, the total amount of carbon measured increased from aggregate size A to C. The total amount of carbon in glomalin (i.e. sum of P. glomalin, glomalin and R. glomalin) was 21 (± 5), 20 (± 4) and 26 (± 3) percent of the total carbon measured in these soils for the A, B and C aggregates (mean \pm SE). The humin fraction (or carbon remaining in the soil after all extractions were complete) contained significantly more carbon than all the other fractions, followed closely by Residual POM and glomalin.

Aggregate stability was weakly but significantly correlated with total carbon, carbon in R. glomalin and FA (Table 4C). The R. glomalin fraction was significantly correlated with total carbon and carbon in glomalin, HA, FA, humin. Strong, significant correlations also existed between Residual POM and P. glomalin.

Regression analysis showed that total soil carbon and the more recalcitrant carbon fractions (i.e. humin, HA, FA and R. glomalin) were functionally related to the percentage iron in the soil ($P < 0.01$) (Figs. 4B and 4C). The more labile Residual POM and potentially more labile P. glomalin and glomalin carbon fractions were not significantly related to iron ($P > 0.05$).

Discussion

The greatest amounts of aggregation are found in surface soils where plant roots and AM fungi are actively growing (Six et al., 2001). Aggregate stability is important for maintaining soil fertility, reducing erosion and sequestering carbon. Organic compounds that contribute to aggregate formation and stability should be effective in terms of flocculation, cementation, rewetting processes, and occlusion of pores. In this study, the percentage of water-stable aggregates in all five soils and all three aggregate size classes was high, especially when compared to tillage systems (17 to 38%) (Wright et al., 1999), and increased with decreasing aggregate size (Table 4A). As aggregate size decreased, the amount of persistent binding agents (i.e. organo-mineral complexes) compared to temporary binding agents (i.e. hyphae and roots) may increase (Tisdall and Oades, 1982). Roots and hyphae may be excluded from smaller aggregates due to the small interaggregate pore size (Jastrow et al., 1998; Tisdall and Oades, 1982). The carbon distribution in the organic matter fractions (Fig. 4A) showed that recalcitrant organic compounds (i.e. humic substances and R. glomalin) that may be in organo-mineral complexes increased with decreasing aggregate size while the more labile Residual POM and P. glomalin fractions decreased.

Regression analysis showed that these more stable fractions were related to the percentage of iron in the soil (Fig. 4B). This was a further indication that these organic fractions persist in soil by forming organo-mineral complexes that resist decomposition by making organic matter inaccessible to microbes and having a complex structure that resists enzymatic attack (Degens, 1997; Dinel and Nolan, 2000; Jastrow et al., 1996). These persistent binding agents form water-stable aggregates that physically protect organic matter, including the more labile organic matter fractions, against microbial decomposition (Piccolo and Mbagwu, 1999; Tisdall and Oades, 1982). In macroaggregates, turnover of organic carbon was 140 y (Jastrow et al., 1996). The persistence of labile fractions within stable aggregates may enhance the ability of soils to sequester carbon.

In these highly stable soils, only R. glomalin and FA organic carbon weights were significantly, but weakly, correlated with WSA (Table 4B). Correlations between organic carbon content and water-stable aggregates are not always good, especially in undisturbed soils, because: (1) only a fraction of the organic matter may be responsible for aggregation, (2) the disposition of organic matter may not favor stabilization, (3) some of the water stability in native soils is related to physical factors (Schreiner and Bethlenfalvay, 1995; Tisdall and Oades, 1982), and (4) there is a threshold (2%) at which the addition of organic matter does not increase stability (Kemper and Koch, 1966). Kemper and Koch (1966) found that the primary variables that contribute to aggregate stability are: free iron oxides, clay, organic matter and exchangeable sodium.

In this study, glomalin and R. glomalin are major fraction of the organic matter within aggregates. The iron (0.8 to 8.8 %, Wright and Upadhyaya, 1998) in glomalin

may help it form organo-mineral complexes that help to stabilize aggregates. When aggregate stability is high, aliphatic organic compounds improve water infiltration and water retention rates and internal cohesion to form larger stable aggregates (Caron et al., 1996; Capriel et al., 1990; Chenu et al., 2000; Piccolo and Mbagwu, 1999). The hydrophobins described by Wessels (1997) are hydrophobic, self-aggregating biomolecules. Glomalin, which has similar characteristics to hydrophobins, appears to contain high concentrations of hydrophobic groups or hydrophobic amino acids (Chapter 7) and to self-aggregate. On the surface of an aggregate, glomalin will self-aggregate into complexes that form a water stable lattice, such as the one described by Capriel et al. (1990). In this way, glomalin is an important component to the water-stability of aggregates.

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Table 4A. Percentage water-stable aggregates in three dry sieved aggregate size classes [1-2 (A), 0.5-1 (B), and 0.25-0.5 (C) mm] collected from five undisturbed U.S. soils.

Aggregate size class (mm)	Baltimore (MD)	Sampson (CO)	Haxtun (CO)	Pacolet (GA)	Cecil (GA)	Mean^{†‡}
A	56	52	62	95	97	72 ± 10a
B	92	93	78	95	97	91 ± 3a
C	94	97	82	96	96	93 ± 3a

[†] Mean ± SE.

[‡] Different letters in a column indicate significant differences according to REML (P = 0.2874).

Table 4B. Mean† gravimetric weights (g kg⁻¹ soil) for the seven organic matter fractions – Residual particulate organic matter (POM), glomalin extracted from POM (P. glomalin), glomalin, glomalin extracted after humic and fulvic acid extraction (R. glomalin), humic acid (HA), and fulvic acid (FA) – extracted from three aggregate size classes [1-2 (A), 0.5-1 (B), and 0.25-0.5 (C) mm] collected from five undisturbed U.S. soils.‡

Fraction	Aggregate size A	Aggregate size B	Aggregate size C
Residual POM	13.03 ± 4.03a	13.24 ± 4.39a	8.09 ± 2.33a
P. glomalin	1.29 ± 0.39b	1.82 ± 0.47b	0.82 ± 0.16c
Glomalin	5.92 ± 0.99a	5.85 ± 1.03a	7.55 ± 1.63a
R. glomalin	1.92 ± 0.34b	1.99 ± 0.31b	2.79 ± 0.83b
HA	0.62 ± 0.25b	0.58 ± 0.30c	0.97 ± 0.47c
FA	0.95 ± 0.45b	0.55 ± 0.23c	0.88 ± 0.39c

† Mean ± SE.

‡ Different letters in a column indicate significant differences according to REML (P <0.0001).

Table 4C. Correlation coefficients comparing water-stable aggregates (WSA), iron (Fe) concentration in the soil, and carbon concentrations in bulk soil (Total), Residual particulate organic matter (POM), glomalin extracted from POM (P. glomalin), glomalin, glomalin extracted after humic and fulvic acid extraction (R. glomalin), humic acid (HA), fulvic acid (FA), and unextractable material left behind after all the extractions were complete (humin).†

Variables	Bulk Soil Fe	Bulk Soil C	Residual POM	P. glomalin	Glomalin	R. glomalin	HA	FA	Humin
WSA	-0.3819	0.6080**	0.1157	-0.1256	0.4050	0.5579**	0.3424	0.6246*	0.1561
Fe		0.0383	-0.4250	-0.5198**	0.0317	-0.2602	0.1449	-0.2423	-0.0417
Total			-0.0927	-0.2388	0.3465	0.4716*	0.6086**	0.5318	0.0990
Residual POM				0.8270***	-0.0144	-0.1053	-0.3325	-0.4280	-0.3102
P. glomalin					-0.1800	-0.2654	-0.4644	-0.3798	-0.1824
Glomalin						0.6338**	0.4301	0.3100	0.6026**
R. glomalin							0.7767***	0.9199***	0.5716**
HA								0.7559**	0.5915**
FA									0.5026

† All values were tested for normal distribution and were transformed when appropriate.

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

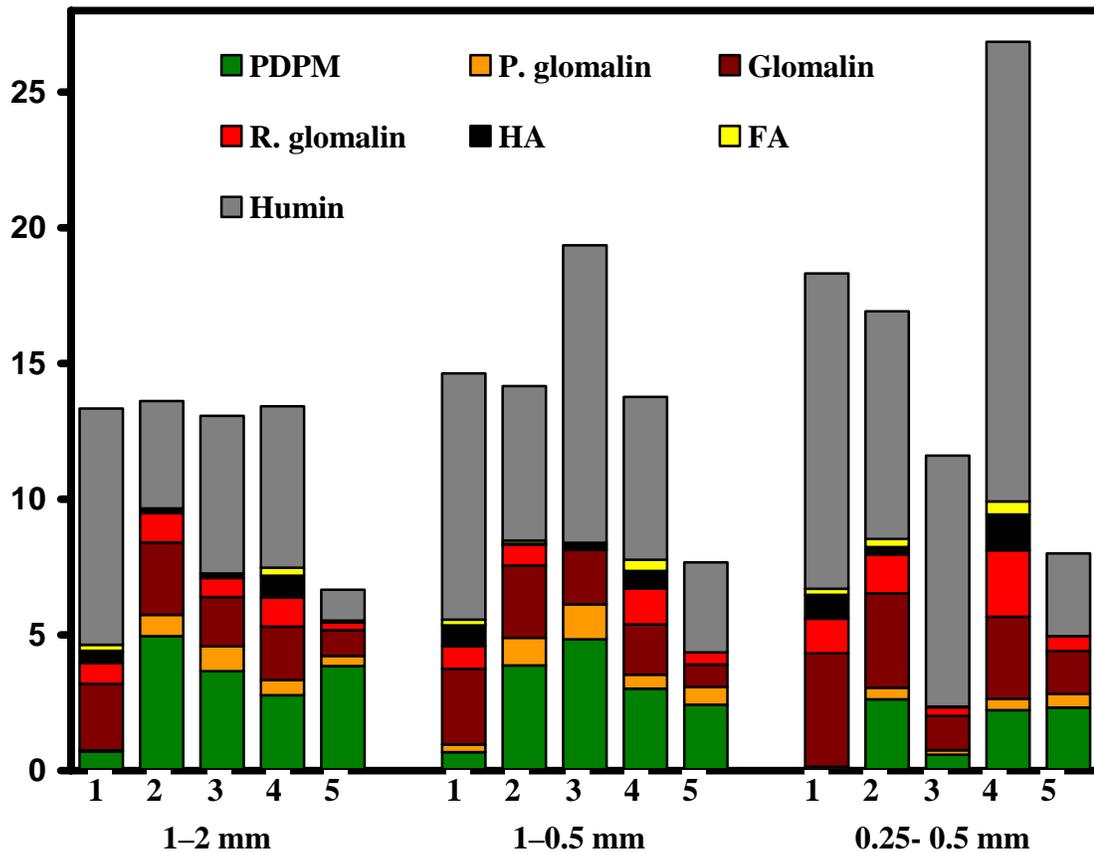


Figure 4A. Carbon concentrations (g C kg⁻¹ soil) in Residual particulate organic matter (POM), glomalin extracted from POM (P. glomalin), glomalin, glomalin extracted after humic and fulvic acid extraction (R. glomalin), humic acid (HA), fulvic acid (FA), and unextractable material remaining after all extractions were complete (humin) extracted from three aggregates size classes [1-2, 0.5-1 and 0.25-0.5 mm] collected from five undisturbed U.S. soils: Baltimore (1), Sampson (2), Haxtun (3), Pacolet (4) and Cecil (5).

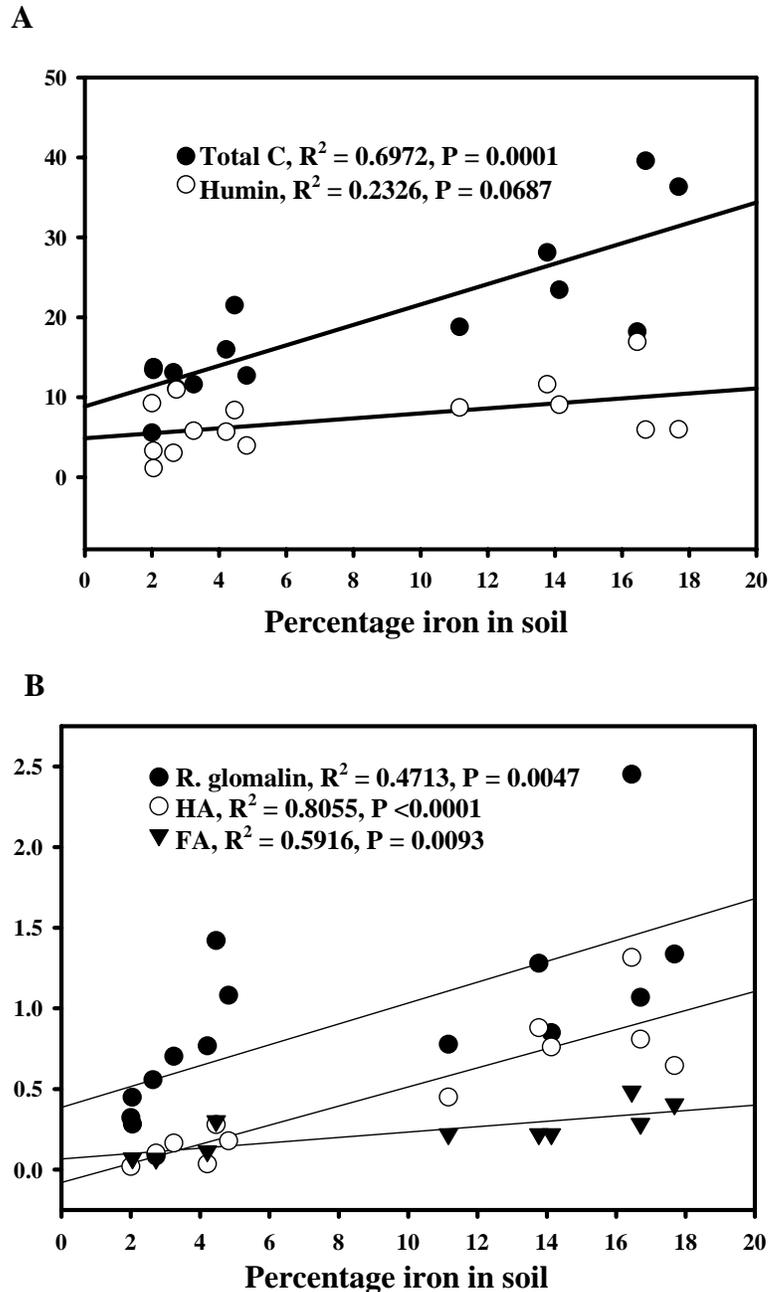


Figure 4B. Relationships of the percentage of iron in 15 samples [three aggregate size classes (1-2, 0.5-1 and 0.25-0.5 mm) collected from five U.S. soils] to the amount of carbon in A: the total carbon concentration in the soil and the amount of carbon in the unextractable material remaining after all the extractions were complete (humin) and B: glomalin extracted after humic and fulvic acid extraction (R. glomalin), humic acid (HA) and fulvic acid (FA).

CHAPTER 5

SOIL ORGANIC MATTER DISTRIBUTION IN FARMING SYSTEMS

Abstract

At the USDA-ARS Farming Systems Project (FSP) site in Beltsville, MD, nine regionally-specific farming systems were started in 1996. These systems were incorporated into three experimental comparisons: (1) Tillage Experiment in 2000 – Systems 1.1 [(Synthetic NT (no-till) C(corn)-W(wheat)-SB(soybean))], 2.1 [Synthetic CT (conventional till) C-W-SB] and 6.1 [Organic MT (minimum till) C-SB-W]; (2) Fertilizer Treatment Experiment in 2001– Systems 1.1, 2.1, 3A [Synthetic MT C-W-SB 2X raw manure], 3B [Synthetic MT C-W-SB 1X raw manure], 4A [Synthetic MT C-W-SB 2X composted manure], and 4B [Synthetic MT C-W-SB 1X composted manure]; and (3) Rotation Length Experiment in 2001 – Systems 1.1, 2.1, 51 [Organic MT C-SB], 6.3 [Organic MT C-SB-W], and 7.2 [Organic MT C-SB-W-H]. In each experiment, a Normalized Stability Index (NSI) was measured on four composite samples from each system. Using the appropriate extraction methodology, all five organic matter (OM) fractions – plant-derived particulate matter (Residual POM), glomalin, glomalin from particulate organic matter (P. glomalin), humic acid (HA), and fulvic acid (FA) – were extracted sequentially from soil in the Tillage Experiment. In the Fertilizer Treatment and Rotation Length Experiments, only particulate organic matter (POM) and glomalin were extracted sequentially. Systems that used sustainable agricultural practices (i.e. reduced tillage, organic inputs and greater crop diversity) were hypothesized to have larger NSIs and higher concentrations of glomalin and POM. In all three experiments, glomalin was present in significantly higher concentrations than the other OM fractions. The CT (2) and two-crop rotation (5) systems had the lowest NSI, while the NT (1) and organic three- (6) and four-crop (7) rotation systems

had the highest NSI. Labile OM (Residual POM) declined with tillage and with the addition of manure at a 1X (3B and 4B) compared to a 2X concentration (3A and 4A).

Introduction

Nearly one-third of the world's arable land has already been lost to erosion, and the current rate of loss is 10 million hectares per year (Pimentel et al., 1995). The formation of water-stable soil aggregates helps prevent topsoil loss by erosion (Degens, 1997; Tisdall and Oades, 1982). In surface layers of many agricultural soils, organic matter (OM) plays a major role in binding aggregates together (Degens, 1997; Tisdall and Oades, 1982). Decreases in OM below 1% cause large reductions in aggregate stability (Kemper and Koch, 1966). Organic matter content and aggregate stability decrease with cultivation due to increased microbial mineralization (Cambardella and Elliott, 1994; Chenu et al., 2000; Schreiner and Bethlenfalvay, 1999; Studdert and Echeverria, 2000).

Soil organic matter (SOM) is a key soil component of sustainable systems because it influences soil biological, physical and chemical properties that define soil fertility (Six et al., 2001; Studdert and Echeverria, 2000). The amount of SOM is a result of the balance between humification and mineralization rates. Increases in high biomass production, high C/N ratio crop residues, the use of organic manures, irrigation and/or reduced tillage will add SOM (Ding et al., 2002; Studdert and Echeverria, 2000). Changes in the size of the OM pool with intermediate turnover rates account for most of the differences in SOM content between management treatments after 20 y of cultivation (Cambardella and Elliott, 1994).

Conventional tillage affects carbon (C) dynamics in the soil through its effects on crop residue decomposition, soil aeration and facilitation of microbial attack on OM fractions released from protected sites within aggregates (Studdert and Echeverria, 2000). Frequent cultivation also leads to exposure of aggregates to physical disruption by rapid wetting and raindrop impact as well as to shearing by implements (Tisdall and Oades, 1982). Long-term conservation tillage creates an organic-rich surface zone promoting fungal growth, while tillage mixes light fraction material exposing it to more rapid bacterial decomposition (Ding et al., 2002; Franzluebbers et al., 1999). Fungal growth increases macroaggregation (aggregates >250 μm), while bacterial growth has a negative impact (Ding et al., 2002).

Macroaggregation is influenced most by tillage because it is related to root and hyphal growth and its binding agents include labile OM compounds (i.e. polysaccharides) (Cambardella and Elliott, 1992; Degens, 1997; Franzluebbers et al., 1999; Six et al., 2001; Tisdall and Oades, 1982). There are four primary ways for OM to contribute to aggregate formation and stability: (1) fungi form a framework to collect OM, (2) polysaccharides glue aggregates together, (3) organic polymers bind to clay minerals via polyvalent cations to increase stabilization, and (4) hydrophobic polymers coat the surface of aggregates to keep them water stable (Chenu et al., 2000; Degens, 1997; Miller and Jastrow, 1990; Piccolo and Mbagwu, 1999; Schreiner and Bethlenfalvay, 1999; Tisdall and Oades, 1982).

Arbuscular mycorrhizal (AM) fungi are the most abundant type of fungi in the soil and are important to aggregate formation and stability (Miller and Jastrow, 1990; Schreiner and Bethlenfalvay, 1999; Tisdall and Oades, 1982; Wright and Upadhyaya,

1996). In farming systems, tillage disrupts the hyphal network and fallow treatments or non-mycorrhizal plants diminish mycorrhizal production due to the lack of roots from a living host (Schreiner and Bethlenfalvay, 1999; Wright and Anderson, 2000). A diverse rotation system results in a variety of AM fungi, higher plant yields and tissue nutrients, while monoculture systems may select for inferior AM species (Douds and Millner, 1999; Paul and Clark, 1996; Schreiner and Bethlenfalvay, 1999). Glomalin is a glycoproteinaceous substance that coats AM fungal hyphae and is highly correlated with stability and organic C (Wright and Anderson, 2000; Wright and Upadhyaya, 1996; Wright et al., 1999). A path model developed by Rillig et al. (2002) shows that the water-stability of 1 to 2-mm aggregates is directly related to root length, soil glomalin, and percent cover and indirectly to hyphal length through root length and soil glomalin.

In this study, a normalized stability index (NSI) was used to measure aggregate stability of the soil from nine farming systems in three experimental comparisons: (1) Tillage Experiment in 2000 – Systems 1.1 [Synthetic NT (no-till) C(corn)-W(wheat)-SB(soybean)], 2.1 [Synthetic CT (conventional till) C-W-SB] and 6.1 [Organic MT (minimum till) C-SB-W]; (2) Fertilizer Treatment Experiment in 2001– Systems 1, 2, 3A [Synthetic MT C-W-SB 2X raw manure], 3B [Synthetic MT C-W-SB 1X raw manure], 4A [Synthetic MT C-W-SB 2X composted manure], and 4B [Synthetic MT C-W-SB 1X composted manure]; and (3) Rotation Length Experiment in 2001 – Systems 1.1, 2.1, 5.1 [Organic MT C-SB], 6.3 [Organic MT C-SB-W], and 7.2 [Organic MT C-SB-W-H] (Table 5A). The advantages of using NSI over other stability measurements are that it (1) combines both slaking and capillary rewetting measurements to

characterize whole soil stability and eliminate errors from rewetting and antecedent water content, (2) corrects for differences in sand size distribution among soils, aggregate size classes and pretreatments, and (3) uses a maximum level of disruption to normalize the level of disruption imposed by slaking (Six et al., 2000). The NSI is sensitive to changes in agricultural management especially at sites that have similar soil characteristics (Six et al., 2000). Generally, the NSI decreases with increasing cultivation and is highest at native sites (Six et al., 2000). The normalized stability index and OM concentrations, especially for glomalin and POM or Residual POM, should be higher in more sustainable systems (i.e. reduced tillage, greater crop diversity, and organic not synthetic inputs).

The objectives of this study were to: (1) measure differences in NSI in nine farming systems that differ in tillage practices, fertilizer amendments, and/or rotation length, and (2) compare the distribution of OM in POM or Residual POM, glomalin, humic acid (HA) and fulvic acid (FA) fractions to NSI. These SOM fractions include labile (POM or Residual POM), intermediate (glomalin and FA) and recalcitrant (glomalin and HA) fractions.

Materials and methods

Farming systems project site

The Farming System Project is a long-term farm management project that was established in 1993 at the USDA Beltsville Area Research Center in Beltsville, MD. After three years of site variability assessment in a no-till (NT) continuous corn field (See Appendix L), seven regionally-appropriate cropping systems – one no-till (NT), three low-input and three organic – were established (Table 5A). Two of the seven

systems were split with one receiving a 1X concentration of the amendment (i.e. raw or composted broiler litter) and the other a 2X concentration. To remove yearly season variability as a factor, all years in the rotation in each system were represented at the same time resulting in 2 to 4 subsystems (See Appendix L, Fig. L2). Each subsystem had four plots randomly distributed in blocks designed to eliminate the effects of drainage class, slope, soil series, and other soil characteristics (See Appendix L, Fig. L1 and L3).

Soil organic matter extractions

Density separation of particulate organic matter

Particulate organic matter (POM) was removed by flotation in a high-density NaCl solution (Wolf et al., 1994) and collected on a 0.053 mm screen. (See Appendix A1 for a detailed description of the extraction method.) The POM fraction was washed with distilled water, rinsed from the screen into pre-weighed weigh boats and dried at 70°C. The mineral fraction (soil minus POM) was washed with distilled water, pelleted by centrifugation, rinsed into pre-weighed weigh boats and dried at 70°C.

Citrate extraction of glomalin

Samples (POM and/or soil minus POM) were extracted for glomalin with 50 mM sodium citrate (Wright and Upadhyaya, 1998). (See Appendix A2 for a detailed description of the glomalin extraction method.) Extraction was repeated until the supernatant (citrate extract) was straw-colored (up to three more times). Glomalin was purified by centrifugation, precipitated in acid, solubilized in NaOH and dialyzed against deionized (dH₂O) water. Dialyzed material was centrifuged and the supernatant was freeze dried.

Sodium hydroxide extraction of humic and fulvic Acids

A method modified from Swift (1996) and recommended by the International Humic Substances Society was used to extract HA and FA. (See Appendix A3 for a detailed description of the extraction procedure.) Incubation conditions and solution concentrations used were described by Swift (1996). Modifications of the method were primarily in sample size (2 g instead of 50 g) and in the purification procedures. Briefly, after an acid pre-incubation, HA and FA were co-extracted from soil using a multi-step NaOH extraction procedure: (i) extraction under N₂ overnight, (ii) centrifugation and acidification of the supernatant to precipitate HA, and (iii) separation of HA (precipitate) from FA (supernatant) by centrifugation. The NaOH extraction was repeated two more times.

Purification of the HA precipitate involved the removal of insoluble particles and ash material. Insoluble solids were removed by: (i) re-dissolving in a minimum volume of KOH under N₂; (ii) adding KCl (until $[K^+] \geq 3 M$); (iii) centrifugation at 10844 x g to remove suspended solids; and (iv) precipitation with HCl. After settling overnight, samples were centrifuged again and the supernatant was discarded. To reduce ash content, the precipitated HA fraction was suspended in an HCl:HF solution, incubated overnight, and centrifuged at 6850 x g with the supernatant discarded. The HCl/HF treatment was repeated twice. Residual acid was removed by repeated washing with dH₂O and centrifugation at 10844 × g for 3 min.

After removal of insoluble particles and ash material, the HA precipitate was re-dissolved in a known minimum volume of 0.1 N NaOH. A subsample (0.5 mL) was removed for protein assays (see below). The remaining solution was acidified rapidly to

precipitate HA. After precipitation, acid was removed by centrifugation at $10844 \times g$ and rinsing with water. The precipitate was freeze-dried.

The fulvic acid fraction (i.e. acid soluble material) was purified by dialysis against water until the pH was neutral. Insoluble material was collected by centrifugation at $6850 \times g$ for 10 min and the supernatant was freeze dried.

Organic matter quantification

Protein measurements

Total and immunoreactive protein concentrations were measured on subsamples of glomalin, FA and HA. For glomalin and FA, the subsamples were taken prior to dialysis. For HA, the subsample was collected from re-dissolved HA precipitate as discussed above. (See Appendix C for detailed descriptions of the protein assay procedures.)

Briefly, a modified Bradford protein assay (Wright et al., 1996) was used to measure total protein (TP) concentration. Samples were diluted in PBS (phosphate buffered saline) and reacted with Bio-Rad® (Hercules, CA) Bradford protein dye reagent. Absorbance was read at A_{595} after 5 min. Protein concentration was determined by comparison with a bovine serum albumin (BSA) standard curve and reported as g protein kg^{-1} soil.

Immunoreactive protein (IRP) concentration was measured by 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 the color

developer, *p*-nitrophenyl phosphate in diethanolamine buffer (Wright, 1994), was added. Absorbance was read at A_{405} after 15 min. Test samples were compared to a standard curve produced by dilutions of highly immunoreactive glomalin extracted from a temperate soil under native grasses. Immunoreactive protein concentrations were reported as g kg^{-1} soil. Percent immunoreactivity was calculated as amount of IRP divided by amount of TP times 100 and reported as the range and the mean for all soils.

Gravimetric measurements

All non-soluble materials (i.e. POM and soil) were washed thoroughly with water to remove extract solution residue. For POM, this was done over a 53 μm screen. The soil was washed, vortexed and pelleted by centrifugation at $6850 \times g$ for 10 min. Washed POM and soil were rinsed into pre-weighed weigh boats, dried at 70°C and weighed. All purified soluble extracts (i.e. glomalin, HA and FA) were freeze-dried and weighed. Gravimetric weights were reported as g kg^{-1} soil.

Percentage C was measured by combustion on all freeze-dried OM fractions, initial soil, and residual soil remaining after extraction (i.e. soil containing the insoluble humin fraction) using a Perkin-Elmer Series II 2400 CHNS/O Analyzer. Both soil samples and POM were ground with mortar and pestle prior to C analysis. Carbon weight was calculated by dividing the percentage by 100 and multiplying by the gravimetric weight and reported as g C kg^{-1} soil.

Water-stable soil aggregates

Aggregates were separated and stability was measured according to Six et al. (2000). Soil was subjected to two pretreatments prior to wet sieving: (1) immersed immediately in water (slaked) or (2) capillary rewetted at 4°C overnight. The 50-g

samples were wet-sieved through three screens (2, 0.25, and 0.053 mm). The pretreated soil was submerged for 5 min atop the 2-mm screen. Aggregate stability was determined by manually moving the sieve submerged in a water column 3 cm up and down 50 times during a 2-min period. The >2-mm aggregates were collected. The soil <2-mm was rinsed onto the 0.25 mm sieve. Aggregates were again separated by manual wet-sieving. This procedure was repeated for the 0.053-mm screen. All aggregate size fractions were dried at 70°C, weighed, and corrected for coarse material after disrupting the aggregates with 0.5% sodium hexametaphosphate. A normalized stability index (NSI) was calculated using the equations found in Six et al. (2000). (See Appendix M.)

Experimental comparisons

Tillage Experiment – July, 2000

Immediately following the wheat harvest, a composite was made of 25 (10 cm depth x 1 cm width) cores collected randomly from four replicate plots in each of three systems: 1.1, 2.1 and 6.1. Soil was air-dried and passed through a 9.5-mm screen. Organic matter fractions were extracted from 10 g (five 2-g increments) of each replicate. The POM fraction was separated first followed by citrate extraction of glomalin and NaOH co-extraction of HA and FA from the POM and soil minus POM. (See Appendix B for a diagram of the extraction sequence.) The NSI was measured in unextracted soil from each plot.

Fertilizer Treatment and Rotation Length Experiments – April, 2001

Prior to spring planting, composite samples (0-10 cm depth) were collected from each plot in all nine systems (Table 5A). The nine systems were separated into two broad categories: Fertilizer Treatment (Systems 3A, 3B, 4A, and 4B) and Rotation

Length (Systems 5.1, 6.3 and 7.2). Systems 1.1 and 2.1 were positive and negative controls, respectively. In the statistical analysis of these systems, both of the controls were added to both experiments. Glomalin and POM were extracted from 10 g (2-g increments) of air-dried soil passed through a 9.5-mm screen. The NSI was calculated for unextracted soil from each plot.

Statistical analysis

Gravimetric and carbon weight values were corrected for subsamples removed for protein measurement. In each system, means and SEs were calculated for data from the four replicate plots, except for the C weight data in the Tillage Experiment. In the Tillage Experiment, the OM fraction samples from each of the four plots in a system were combined, which resulted in only one C weight value for each fraction per system.

All means comparisons were made at the $\alpha \leq 0.05$ level by ANOVA (Analysis of Variance) using REML (Restricted Maximum Likelihood) after the residuals met the assumptions for normality and homogeneity of variance. When needed, the appropriate transformation was made to meet the assumptions. In the Tillage Experiment, the gravimetric weight values for each OM fraction were compared within a system as well as for each fraction individually across the three systems. Means comparisons for the Fertilizer Treatment and Rotation Length Experiments were made for glomalin or POM across system in an experiment.

In each of the three experiments, Pearson product-moment correlation coefficients (r) were calculated for NSI and C weights in the bulk soil and all extracted OM fractions. All statistical analyses were performed using SAS software, ver. 8 (SAS Institute, 1999).

Results

Aggregate Stability

There were no significant differences in NSI for the different systems in July, 2000 and April, 2001 (Table 5B). Stability declined in the CT system from 2000 to 2001. The two-crop rotation system (System 5.1) had the lowest NSI, while the NT and four crop rotation systems had the highest NSI. Carbon weight in bulk soil or in any OM fraction was not significantly correlated with NSI.

Quantitative values

Protein values

In all three experiments, the TP and IRP assays confirmed the presence of glomalin in the citrate extract and the HA fraction. (See tables in Appendices N and O.) No measurable protein was present in FA fraction. Both the glomalin and HA fractions in the Tillage experiment (2000) were highly immunoreactive (97 and 91%, respectively). In the April, 2001 experiments, immunoreactivity of glomalin ranged from 45 to 48% and 40 to 48% for the Fertilizer Treatment and Rotation Length experiments, respectively.

Gravimetric and carbon weights from the Tillage Experiment – July, 2000

In all three systems, the amount of glomalin was significantly greater than amounts for all other OM fractions, and the P. glomalin fraction was the smallest (Table 5C). The weights of any of the five organic matter fractions did not vary significantly across all three systems ($P < 0.05$). The recalcitrant fractions (glomalin and HA) were higher in the CT (System 2.1) than the NT (System 1.1) system while the reverse was true for the labile (Residual POM) fraction.

Carbon weight in the labile Residual POM fraction from the MT system (System 6.1) was about twice that in the CT and NT systems (Fig. 5A). The C weight of the glomalin and HA fractions increased only slightly in the System 6.1, while the very recalcitrant, insoluble humin fraction did not change across the three systems.

Gravimetric and carbon weights from the Fertilizer Treatment and Rotation Length Experiments – April, 2001

There were no significant differences in glomalin or POM weight across systems within either experiment ($P > 0.10$) (Table 5D). In each of the nine systems, POM weights were significantly less than the glomalin weights ($P < 0.05$). Glomalin weights were highest in the NT (System 1.1), 2X raw manure MT (System 3A), and organic C-SB-W rotation MT (System 6.3) systems. The POM weights were highest in the 2X raw (System 3A) and 2X (System 4A) compost manure systems.

In the Fertilizer Treatment Experiment, there were no significant differences in either glomalin or POM across systems, but some trends were seen (Fig. 5B). The CT synthetic fertilizer (System 2.1) and MT 1X composted manure (System 4B) systems contained the lowest C weight in the glomalin fractions. The two systems without manure (Systems 1.1 and 2.1) had the lowest C weight in the POM fraction, while both the 2X manure systems (Systems 3A and 4A) contained the highest carbon weights in the POM fraction. Glomalin C weights were highest in the NT (System 1.1) and 2X manure systems (Systems 3A and 4A). There was significantly more glomalin-C (4 to 10 times) than POM-C in all of the systems ($P < 0.05$).

Glomalin also had significantly higher C weights (4 to 10 times) than POM in the Rotation Length Experiment (Fig. 5C). The NT C-W-SB (System 1.1) and MT

organic C-SB-W (System 6.3) systems had the highest C weights in the glomalin fraction, while the CT C-W-SB (System 2.1) system had the lowest. Carbon weight in POM was greatest in the MT organic C-SB (System 5.1) and C-SB-W-H (System 7.2) systems.

Discussion

At the Farming Systems Project site, there were no significant differences in NSI among the systems, but there were some interesting trends. In the CT system (System 2.1), NSI declined over the two years of sampling. This was expected, because tillage disrupts aggregates by physical disruption and increasing SOM mineralization (Cambardella and Elliott, 1994; Hu et al., 1995). Rotation length also appeared to affect stability with the two crop rotation system having the lowest stability index.

Monoculture systems have a negative impact on mycorrhizal fungi and aggregate stability by decreasing the number of AM species present (Cambardella and Elliott, 1992; Douds and Millner, 1999; Paul and Clark, 1996; Schreiner and Bethlenfalvay, 1999).

In System 6, the stability index was 0.80 in 2000 and 0.65 in 2001. This change may have been because different plots were sampled (i.e. 6.1 plots in 2000 and 6.3 plots in 2001). In order to remove yearly changes in climatic conditions as a variable, all crops in a rotation are present at the same time in different plots (which are indicated by the second number in the plot ID). (See Appendix L, Fig. L2, to determine where these plots were located in the field.) Timing of sampling (i.e. April, 200 and July, 2001) also may have resulted in differences in NSI. The July sampling occurred immediately after wheat harvest. The increase in C from crop residues may have stimulated a short-term

increase in aggregate stability via saprophytic fungal growth (Caesar-TonThat and Cochran, 2000; Degens, 1997; Hu et al., 1995) with plant debris acting as nucleation sites for aggregate formation (Cambardella and Elliott, 1992; Six et al., 2001). The April 2001 sampling occurred prior to planting and after the winter season when POM concentrations and microbial activity were low.

In reduced or NT systems, the stubble and mulch litter promote aggregate formation because fungal decomposition of OM produces gluing agents, such as polysaccharides and mucigels (Chaney and Swift, 1986; Caesar-TonThat and Cochran, 2002; Degens, 1997; Hu et al., 1995). Caesar-TonThat and Cochran (2002) found that ligninolytic basidiomycetes produce large quantities of polysaccharides, glycolipids or glycoproteins that bind to and stabilize soil particles in water-stable aggregates. However, many of the polysaccharides produced by microbial degradation will glue aggregates together quickly but are water-soluble and ephemeral and, therefore, do not contribute to the long-term stability of aggregates (Chaney and Swift, 1986; Caesar-TonThat and Cochran, 2002; Six et al., 2001).

No-till systems also promote hyphal growth of fungi. Frey et al. (1999) found that fungal hyphae length was about 2 to 2.5 times higher in NT than CT systems. Fungi are favored in NT systems because: (1) hyphal networks are maintained, (2) fungi may bridge the soil-residue interface and utilize spatially separated nutrients, especially C and N; and (3) fungi maintain activity, even in dry locations or across air-filled pores (Degens, 1997; Frey et al., 1999; Schreiner and Bethlenfalvay, 1999).

The humic fractions (i.e. HA, FA and humin) are long-term fractions (i.e. they resist decomposition over the long-term but also take a long time to form). Therefore,

they are not affected by tillage as much as POM, which requires the presence of living plants for production. Production of glomalin also is plant-dependent, but longevity is intermediate with an estimated turnover rate of 6 - 42 years (Rillig et al., 2001). The Tillage Experiment in July, 2000 showed very little change in the concentrations of the HA, FA or P. glomalin and these fractions accounted for very little of the total SOM. Therefore, further examination of these fractions was deemed unnecessary when sampling was expanded to include all nine systems in April, 2001.

Previous research shows that glomalin is a major fraction of OM in a variety of soils (Chapters 2, 3 and 4). All three experiments in this study also showed that glomalin was an abundant fraction. In fact, it was the most abundant fraction and accounted for a major part of the total carbon in the soils (ca. 19%). However, glomalin in the April, 2001 samples was two to three times more than glomalin in the July, 2000 samples. This possibly was due to the additional 1 h citrate extraction (i.e. four cycles instead of three) performed in 2001. In the Tillage Experiment (July, 2000), protein measurements showed that the HA fraction contained some co-extracted glomalin. In April, 2001, the additional high temperature extraction of glomalin may have solubilized some of the glomalin that was co-extracted with HA in 2000 and some of the glomalin that was bound clay mineral complexes and was normally insoluble (such as the recalcitrant glomalin that was discussed in Chapter 3). Finally, variation in glomalin values by sampling time may have reflected higher glomalin in the soil or greater solubility in the spring prior to planting than right at harvest. There currently is not sufficient information about this molecule to predict changes in solubility or how it builds up in the soil.

Previously, glomalin has been separated into two pools based on extraction technique: easily-extractable (30 min extraction in 20 mM citrate) and total (60 min extraction in 50 mM citrate) (Wright and Anderson, 2000; Wright et al., 1999; Wright and Upadhyaya, 1998). It has been hypothesized that the easily-extractable, highly immunoreactive fraction may be a more labile fraction that is related more closely to aggregation and changes in tillage management while the more recalcitrant fraction may be a resistant fraction important in the stability of aggregates (Wright and Upadhyaya, 1998). Significant differences in glomalin and aggregate stability measurements occurred within 2 to 3 y after initiation of NT treatments (Wright et al., 1999). Soil organic C and N concentration, carbohydrates and amino acids were higher in NT rather than CT systems (Hu et al., 1995) further indicating the presence of glomalin in NT systems. Therefore, glomalin could be an indicator of ecosystem recovery/restoration success due to the strong positive correlation with soil aggregate water stability and the response of this compound to land-use changes (Rillig et al., 2003; Wright et al., 1999).

The systems used in this study at the FSP site did not show the differences in aggregate stability and glomalin concentration that were expected and had been seen at other sites (i.e. higher values with NT and 3 to 4 y rotation systems). In a similar study by Wander and Traina (1996), crop yields declined while SOM concentrations and microbial biomass increased during the first five years of management transition from conventional to organic. Low (1955) stated that improvement in the structure of a degraded soil to that of a grassland soil may take from 5 to 50 y depending upon soil texture. The FSP systems had been in place for four years prior to sampling (three of which were drought years), and time was probably a major factor in the results from this

study. However, trends in the data do indicate that differences should be apparent after a few more years.

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Table 5A. Descriptions of systems at the Farming Systems Project site in Beltsville, MD.

System†	Tillage	Crop Rotation	Length of Rotation	Nutrient Management	Weed Management‡	Cover Crop§
1.1	No-till (NT)	Corn-Wheat-Soybean (C-W-SB)	2	Synthetic fertilizer	Herbicides	None
2.1	Conventional till (CT)	Corn-Wheat-Soybean (C-W-SB)	2	Synthetic fertilizer	Banded herbicides + cultivation	None
3A	Minimum till (MT)	Corn-Wheat-Soybean (C-W-SB)	2	Synthetic fertilizer + Raw broiler litter (2X)	Banded herbicides + cultivation	None
3B	Minimum till (MT)	Corn-Wheat-Soybean (C-W-SB)	2	Synthetic fertilizer + Raw broiler litter (1X)	Banded herbicides + cultivation	None
4A	Minimum till (MT)	Corn-Wheat-Soybean (C-W-SB)	2	Synthetic fertilizer + Composted broiler litter (2X)	Banded herbicides + cultivation	None
4B	Minimum till (MT)	Corn-Wheat-Soybean (C-W-SB)	2	Synthetic fertilizer + Composted broiler litter (1X)	Banded herbicides + cultivation	None
5.1	Minimum till (MT)	Corn-Soybean (C-SB)	2	Legume + Organic fertilizer	Cover crop + cultivation	Rye, Overseeded crimson clover
6.1, 6.3	Minimum till (MT)	Corn-Soybean-Wheat (C-SB-W)	3	Legume + Broiler litter	Cover crop + cultivation	Rye, Drilled crimson clover
7.2	Minimum till (MT)	Corn-Soybean-Wheat-Hay (C-SB-W-H)	4	Legume + Broiler litter	Cover crop + cultivation	Rye

† Systems are labeled by subsystem – system plus another number that identifies where it was in the rotation. For example, a plot identified as 6.3 would be in the third year of the rotation with wheat as the crop in System 6.

‡ A no-till high residue cultivator was used to kill weeds between rows and cover weeds within a row.

§ Cover crops are crushed with a stalk chopper prior to planting the main crop.

Table 5B. The normalized stability index (NSI) for the management systems at the 16 ha Farming Systems Project site sampled in July, 2000 and April, 2001. †

Experiment	System/Plot Sampled	NSI‡
July, 2000 Tillage	1.1 – Synthetic NT C-W-SB	0.72 ± 0.06
	2.1 – Synthetic CT C-W-SB	0.65 ± 0.05
	6.1 – Organic MT C-SB-W	0.80 ± 0.01
April, 2001 Controls§	1.1 – Synthetic NT C-W-SB	0.77 ± 0.04
	2.1 – Synthetic CT C-W-SB	0.53 ± 0.14
April, 2001 Fertilizer Treatment	3A – Synthetic MT C-W-SB, 2X raw manure	0.62 ± 0.08
	3B – Synthetic MT C-W-SB, 1X raw manure	0.63 ± 0.07
	4A – Synthetic MT C-W-SB, 2X composted manure	0.69 ± 0.07
	4B – Synthetic MT C-W-SB, 2X composted manure	0.62 ± 0.06
April, 2001 Rotation Length	5.1 – Organic MT C-SB	0.41 ± 0.12
	6.3 – Organic MT C-SB-W	0.65 ± 0.10
	7.2 – Organic MT C-SB-W-H	0.74 ± 0.05

† In the April 2001, the experiments were divided into the Fertilizer Treatments experiment plus controls and the Rotation Length experiment plus controls. There were no significant differences between the values in any experiment ($P > 0.09$).

‡ Mean ± SE.

§ These were the positive (no-till) and negative (conventional tillage) systems that were used as controls the statistics for both April, 2001 experiments.

Table 5C. Mean† gravimetric weights (g kg⁻¹ soil) for the five organic matter fractions – Residual particulate organic matter (POM), glomalin extracted from POM (P. glomalin), glomalin, humic acid (HA), and fulvic acid (FA) – extracted from three farming systems (System 1.1 – Synthetic NT C-W-SB, System 2.1 – Synthetic CT C-W-SB, and System 6.1 – Organic MT C-SB-W) at the Farming Systems Project site in Beltsville, MD in July, 2000.‡

Fraction	System 1.1	System 2.1	System 6.1
Residual POM	1.06 ± 0.27bc	0.89 ± 0.23c	1.93 ± 0.46b
P. glomalin	0.21 ± 0.05d	0.17 ± 0.04c	0.35 ± 0.06c
Glomalin	3.77 ± 0.15a	4.28 ± 0.23a	4.08 ± 0.30a
HA	1.59 ± 0.10b	1.80 ± 0.55b	2.12 ± 0.32b
FA	0.87 ± 0.30c	0.60 ± 0.23c	0.87 ± 0.22c

† Mean ± SE.

‡ Different letters in a column indicate significant differences according to REML (P <0.0001).

Table 5D. Mean† gravimetric weights (g kg⁻¹ soil) for two organic matter fractions – glomalin and particulate organic matter (POM)– extracted from nine systems that included no-till (NT), conventional till (CT) and minimum till (MT) with raw or composted broiler litter using corn (C), soybean (SB), wheat (W), and hay (H) crops.

Experiment	System	Glomalin	POM
April, 2000 Controls‡	1.1	16.20 ± 0.96	2.12 ± 0.67
	2.1	12.53 ± 1.76	1.63 ± 0.16
April, 2001 Fertilizer Treatment	3A	15.33 ± 0.71	3.40 ± 1.18
	3B	12.99 ± 3.11	1.72 ± 0.25
	4A	12.64 ± 1.37	3.43 ± 1.08
	4B	10.72 ± 2.58	2.59 ± 0.39
April, 2001 Rotation Length	5.1	13.53 ± 3.11	2.36 ± 0.17
	6.1	14.69 ± 1.37	2.68 ± 0.56
	7.2	13.66 ± 0.65	2.62 ± 0.42

† Mean ± SE.

‡ These were the positive (no-till) and negative (conventional tillage) systems that were used as controls the statistics for both April, 2001 experiments.

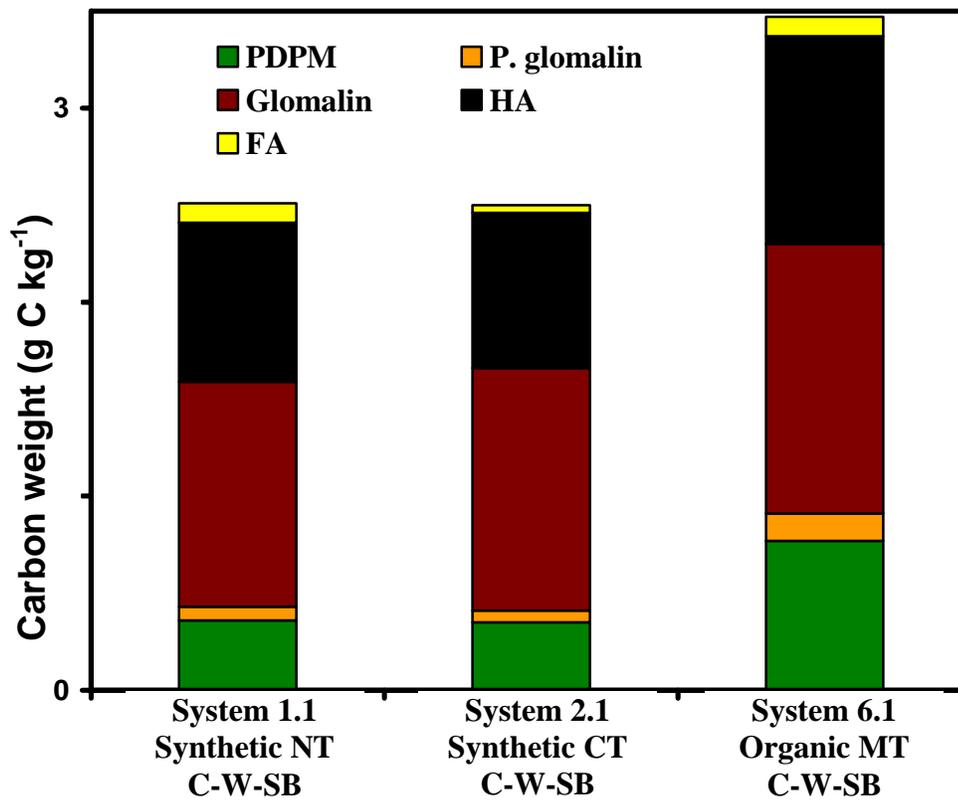


Figure 5A. Carbon weights in Residual particulate organic matter (POM), glomalin extracted from POM (*P. glomalin*), glomalin, humic acid (HA), and fulvic acid (FA) extracted from three farming systems.

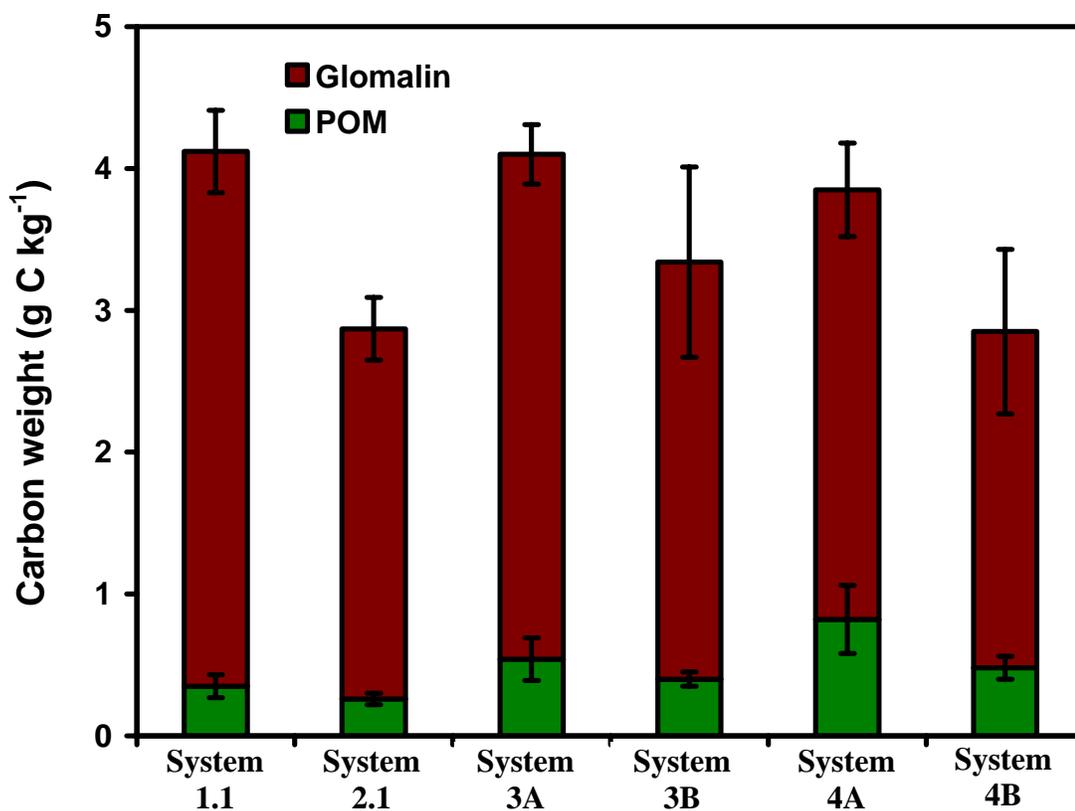


Figure 5B. Carbon weights in glomalin and particulate organic matter (POM) extracted from six systems: System 1.1 (Synthetic NT C-W-SB), System 2.1 (Synthetic CT C-W-SB), System 3A (Synthetic MT 2X raw manure C-W-SB), System 3B (Synthetic MT 1X raw manure C-W-SB), System 4A (Synthetic MT 2X composted manure C-W-SB) and System 4B (Synthetic MT 1X composted manure C-W-SB).

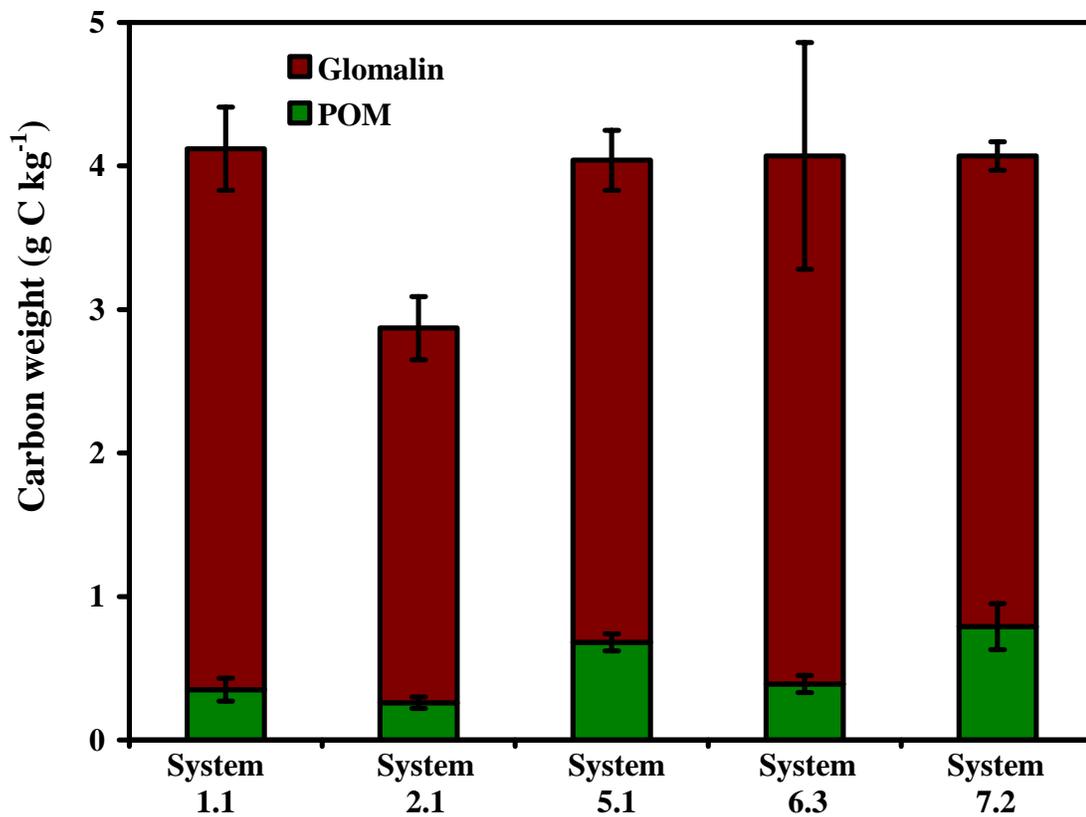


Figure 5C. Carbon weights in glomalin and particulate organic matter (POM) extracted from six systems: System 1.1 (Synthetic NT C-W-SB), System 2.1 (Synthetic CT C-W-SB), System 5.1 (Organic MT C-SB), System 6.3 (Organic MT C-SB-W), and System 7.2 (Organic MT C-SB-W-H).

CHAPTER 6

GLOMALIN ACCUMULATION IN SINGLE-SPECIES POT CULTURES

Abstract

Glomalin, a recalcitrant glycoprotein produced by arbuscular mycorrhizal (AM) fungi, is found in temperate soils in concentrations ranging from 2 to 15 mg g⁻¹. The hypothesis that glomalin from active cultures accumulates over time was tested in a soilless pot culture system. *Zea mays* roots were enclosed in the center of the pot (root chamber) by a 38- μ m nylon fabric. Hyphae penetrated the mesh openings and grew into the media surrounding the root chamber, forming a separate hyphal chamber. After each of three consecutive 14-week culture periods, glomalin was measured in different sections of pots: (1) on AM colonized roots and hyphae in the root chamber, (2) on AM fungal hyphae and associated debris in the hyphal chamber, (3) on sand:coal potting media in the hyphal chamber and (4) on horticultural mesh placed in the hyphal chamber. In this study, glomalin levels in the hyphal chamber did not increase over consecutive culture periods. Plant growth, hyphal weight and total glomalin accumulation in the whole pot appeared to be affected mostly by irradiance with greater production under high light conditions. Immunofluorescence assays showed that glomalin was present on hyphae and arbuscules of AM fungi and that an abundant amount of hyphae had accumulated over the three 14-week culture periods. The high flow-through watering system and the large pore size and low surface charge of the sand:coal media used in this study probably allowed much of the freshly-produced glomalin to be lost through the bottom of the pot.

Introduction

A symbiotic relationship exists between arbuscular mycorrhizal (AM) fungi and 80% of all vascular plants (Smith and Read, 1997). This mutualistic association has

existed for more than 400 million years or since plants first moved from an aquatic to terrestrial environment (Morton, 1990; Simon et al., 1993; Taylor et al., 1995). In this symbiosis, plants benefit from uptake of immobile nutrients (Jakobsen et al., 1994) and improved soil structure (Wright and Upadhyaya, 1999), while the fungus receives photosynthetic carbon and other essential nutrients from the host plant (Douds and Millner, 1999; Millner and Wright, 2002; Tinker et al., 1994). About 12 to 30% of plant photosynthetic carbon is translocated belowground in the form of sugars that support fungal growth and development (Tinker et al., 1994). These sugars are rapidly converted into sugar alcohols to maintain C flow to the fungus (Tinker et al., 1994). Carbon cost to the plant is balanced by access to a greater volume of soil through fungal hyphae. Hyphae have a much larger surface area to volume ratio than root hairs and fan out up to 8 cm beyond nutrient depletion zones around roots (Douds and Millner, 1999; Millner and Wright, 2002).

Arbuscular mycorrhizal fungi must be grown in a plant-fungus pot culture system to provide a living root system for AM fungal growth. Plant roots may be separated from fungal hyphae by using a nylon fabric barrier that is penetrable by hyphae, but not by roots (Wright and Upadhyaya, 1999). Hyphae may be collected from the rootless hyphal chamber by wet-sieving and floatation. In pot culture experiments, soilless (coarse sand) amended with nutrients in solution or soil diluted with sand or vermiculite may be used. Soilless media reduces AM colonization and sporulation, possibly due to inadequate maintenance of moisture (Biermann and Linderman, 1983). Millner and Kitt (1992) designed a watering system that maintains continuously moist conditions and significantly increases AM hyphal growth and sporulation. In addition,

the use of MES [2-(N-morpholino)-ethane sulfonic acid] buffer in the nutrient media increases the length of external hyphae and the density of propagules (Medeiros et al., 1993; Vilariño et al., 1997). The buffer solution helps to stabilize rhizosphere pH to maintain conditions for adequate nutrient availability.

At high pH levels, P availability to plants decreases. Medeiros et al. (1993) speculated that plant host dependence on AM fungi would increase with a corresponding increase in hyphal growth. Vilariño et al. (1997) found that with a Tris [Tris(hydroxymethyl)aminomethane] buffer solution they did not get the same results as with MES buffer. Therefore, rather than increasing host plant dependence on AM fungi, Vilariño et al. (1997) hypothesized that MES increased the activity of soil microorganisms (possibly through the liberation of sulfur from the MES molecule) whose 'metabolites favor external hypha growth'.

Large amounts of isolated hyphae for glomalin extraction may be produced by using a pot culture system that incorporates root and hyphal chambers, a watering system that keeps media continuously moist, and a low P nutrient solution containing MES buffer (Wright et al., 1996; Wright and Upadhyaya, 1996). Immunofluorescence assays show that glomalin attaches to any solid matrix in the hyphal chamber — horticultural mesh, nylon fabric, plant roots, arbuscules in the root cells, soil aggregates, and glass beads (Wright, 2000; Wright et al., 1996; Wright and Upadhyaya, 1996; 1999; S.F. Wright, personal communication). Glomalin may be extracted from hyphae and solid matrices in an alkaline citrate solution at 121°C and quantified by using protein and immunoassays (Wright et al, 1996; Wright and Upadhyaya, 1999).

In this study, the accumulation of glomalin in pot cultures of *Zea mays* was

examined using two isolates of AM fungi grown for three consecutive 14-week culture periods. After each culture period, glomalin was extracted from the root chamber and components of the hyphal chamber: potting media, horticultural mesh inserted in the media, and hyphae isolated by floatation and wet sieving. The objectives were to: (1) measure total glomalin concentration after each culture period and (2) identify where glomalin accumulated – roots, hyphae, mesh, or potting media.

Materials and methods

Potting media

Coarse sand (# 4 quartz sand Jim's Air, Tuxedo, MD; a local sandblasting supply company) was pre-extracted to remove glomalin by using 50 mM citrate, pH 8.0, at 121°C for 1 h. Extract was poured off and the sand was rinsed thoroughly (until colorless) to remove any residual glomalin reducing background glomalin levels to near zero. Extracted sand was mixed (1:1) with crushed coal (medium "Black Beauty" coal from Jim's Air, Tuxedo, MD). Pre-extraction of crushed coal was unnecessary, because previous extractions indicated that glomalin was not present (unpublished data). The mixture was sterilized at 121°C for 45 min and kept covered until placed in pots.

Potting design

Standard 15-cm diameter, 1300 mL volume azalea pots were sterilized in 10% sodium hypochlorite along with 12-in circles of 38 µm nylon fabric (Sefar America, Inc., Depew, NY) and horticultural mesh strips (WeedBlock®, Easy Gardner, Inc., Waco, TX) (ca. 3 x 6 cm) and squares (ca. 7 cm²). The mesh squares were placed in the bottom of the pots to cover 4 of the 8 holes (the other 4 holes were taped over) and allow for adequate drainage while retaining the sand media. Potting media (ca. 350 cm³)

was placed in a 1-L graduated cylinder. The nylon fabric was placed over the top of the cylinder and secured with a rubber band. The cylinder was then inverted and placed in the middle of the pot. Horticultural mesh strips were placed vertically along the inside wall of the pot and secured with clamps. The area around the cylinder was filled with potting media. The rubber band and cylinder were gently removed leaving behind the nylon fabric root chamber (Fig. 6A). Irrigation was through a ring (6 mm I.D.) made of drip tubing placed outside of the root chamber. The ring was suspended above the media with plastic electrical ties taped to the outside of the pot (Fig. 6A).

Planting

Corn (*Zea mays*) seeds were rinsed thoroughly to remove fungicide, surface sterilized with 10% (v/v) sodium hypochlorite for 10 min, rinsed, and pre-germinated on moist paper towels for 5 to 7 days. Seedlings were planted (3 per pot) in the root chamber and inoculated with spores and hyphae – 18 pots with *Gigaspora (Gi.) rosea* (Nicholson and Schenck) [INVAM (InterNational culture collection of Vesicular Arbuscular Mycorrhizal fungi) number (FL224)] and 18 pots with *Glomus (G.) etunicatum* (Becker and Gerdemann) (BR220) – collected from previous pot culture experiments in our lab and stored at 4°C. Inoculum was applied directly to roots, which then were covered with media and watered with ca. 100 cm³ of distilled water.

Culture

Pots were placed in the greenhouse and watered with a reduced phosphorus (P) (40 µM) half-strength Hoagland's solution automatically 4 times daily (Millner and Kitt, 1992). Sodium vapor lights for auxiliary lighting in the greenhouse were set on a 12-h day:night cycle. After 4 weeks, the P concentration in the Hoagland's solution was

reduced to 20 μM . Plants and fungi were allowed to grow for three 14-wk increments. Ambient irradiance (W m^{-2}) was measured outside of the greenhouse every 15 min throughout most of this period. The culture periods for *G. etunicatum* were from January 14 to April 22, April 22 to July 29 and July 29 to November 4, 2002 and for *Gi. rosea* from May 21 to August 27, August 27 to December 4 and December 8 to March 15, 2002 to 2003. (During the four days between December 4 and 8 while seeds were germinating, the watering regimen was continued to maintain moist conditions.)

Harvesting

After each 14-week increment, six of the pots were harvested completely. The remaining pots were replanted. Replanting consisted of removal of the old root chamber and placing a new root chamber (nylon fabric ‘bag’) in the hole vacated by the old ‘bag’. The new root chamber was filled with potting media and planted with uninoculated, pre-germinated corn seedlings.

Complete harvesting consisted of processing the root and hyphal chambers separately. The nylon fabric was removed and the root chamber was placed in a beaker and extracted for glomalin. A multi-step procedure was used to separate components in the hyphal chamber:

1. Horticultural mesh strips and mesh square from the bottom of the pot were carefully removed and extracted for glomalin along with the sand that adhered to the mesh via hyphae and glomalin. Following glomalin extraction, mesh area was measured.
2. The media was rinsed repeatedly with forced water to free hyphae. Released hyphae were collected with forceps and by pouring the wash over a 53- μm

screen. When no hyphae were visible, the sand was washed onto a 250- μm screen atop a 53- μm screen and rinsed thoroughly with forced water. (This was done to facilitate the release of any remaining hyphae.) Potting medium on the 250- μm screen was rinsed into autoclavable containers and extracted for glomalin.

3. Hyphae and fine particles from the 53 μm screen and hyphae released from the potting medium by forced water were combined (forming the hyphae fraction) and extracted for glomalin. After glomalin extraction, hyphae were dried at 70°C and weighed to get an estimate of yield – glomalin per amount of hyphae. (See Appendix P for a detailed discussion of a mini-experiment conducted on these samples to measure glomalin yield from hyphae.)

Glomalin extraction

All glomalin extractions were in 50 mM sodium citrate at 121°C for 1 h (Wright and Upadhyaya, 1999), and samples were submerged in the citrate solution. The supernatant was collected by centrifugation. Extraction was repeated for the hyphae fraction until the extract solution was straw-colored. Glomalin was purified by centrifugation, acid precipitation and dialysis against deionized water. (See Appendix A2 for a detailed description of the glomalin extraction and purification procedure.) Dialyzed material was centrifuged and the supernatant was collected and freeze dried.

Glomalin measurement

In each of the extracted fractions – roots, mesh, potting media and hyphae fraction – glomalin concentration was measured by the total protein (TP) assay and ELISA. (See Appendix C for detailed descriptions of the protein assays.) Values, except

for those from glomalin extracted from mesh, were corrected for the total volume of the extract solution and reported as mg protein. Concentration of glomalin extracted from mesh was extrapolated to mg cm^{-2} by correcting for total area of mesh extracted and for the volume of extract.

A modified Bradford protein assay (Wright et al., 1996) was used to measure TP concentration. Samples were diluted in PBS (phosphate buffered saline) and reacted with Bio-Rad® (Hercules, CA) Bradford protein dye reagent. Absorbance was read at A_{595} after 5 min. Protein concentration was determined by comparison with a bovine serum albumin (BSA) standard curve.

Immunoreactive protein (IRP) concentration was measured by ELISA as described by Wright and Upadhyaya (1998) with modifications in the enzyme and color developer. ExtrAvidin® (Sigma-Aldrich, Inc., St. Louis, MO) phosphatase was used instead of peroxidase. Wells were rinsed with Tris buffered saline with Tween 20 (polyoxyethylenesorbitan monolaurate) before adding the color developer (*p*-nitrophenyl phosphate in diethanolamine buffer) (Wright, 1994). Absorbance was read at A_{405} after 15 min. Test samples were compared to a standard curve produced by serial dilutions of highly immunoreactive glomalin extracted from a temperate soil under native grasses. Percentage immunoreactivity (%IRP) was calculated as amount of IRP divided by amount of TP times 100.

Immunofluorescence

Prior to extraction for glomalin, small samples were taken from the horticultural mesh strips (ca. 1 cm^2), roots (4 to 6 1-cm fragments), and potting media (>2-mm aggregates) to examine for the presence of glomalin using an immunofluorescence

assay (Wright, 2000). (See Appendix Q for a detailed description of the immunofluorescence assay procedure.) Briefly, samples were placed in wells of a 12-well plate and incubated sequentially with milk, monoclonal antibody 3211 (antibody specifically against glomalin) and goat anti-mouse IgM tagged with fluorescein isothiocyanate (FITC). Samples were mounted with VectaShield® (Vector Laboratories, Burlingame, CA) mounting medium. Immunofluorescence was examined using an epi-fluorescence microscope.

Statistical analysis

Glomalin protein values for each part of the pot (i.e. root chamber, mesh, potting media or hyphae) are means and SEs calculated from values in each replicate pot (up to 6 replicate pots per culture period). In rare cases, fine roots were able to penetrate the nylon fabric bag or to grow over the top of the nylon bag and into the hyphal chamber. Values obtained for these pots were not included in the analysis.

Protein values were compared for *G. etunicatum* and *Gi. rosea*, individually, across culture periods using ANOVA (Analysis of Variance) after meeting the assumptions for normality and homogeneity of variance (using the appropriate log or sine transformation when needed). Significant differences were made at the $P < 0.05$ level according to REML (Restricted Maximum Likelihood).

Ambient irradiance measurements (obtained every 15 min) were averaged for each h. These averages were totaled for each week. If more than 2 data points were missing in a 1 h period that week's totals were not calculated. For *G. etunicatum*, values were not obtained for the first culture period and during the first 5 weeks of the second culture period.

Results

Irradiance

Irradiance had a dramatic affect on plants grown in the greenhouse (Fig. 6B and Appendix R) with the plants directly under supplemental lighting being much taller than plants not directly under the lights. During each of the 14-week culture periods for both *G. etunicatum* and *Gi. rosea*, the ambient irradiance varied by season (Fig. 6C).

Protein values

Glomus etunicatum in the second culture period (April 22 to July 29) had significantly higher TP concentrations than the other two periods (Fig. 6D.A). This culture period also had the highest irradiance values (Fig. 6C.A). The third period had high irradiance for 9 weeks but these declined rapidly in the last 5 weeks of growth. The largest difference in glomalin levels between these culture periods was in the root chamber. Total protein values for glomalin extracted from the horticultural mesh were ca. 0.02 mg cm^{-2} at all three harvests.

The percentage of IRP (%IRP) in glomalin extracted from *G. etunicatum* hyphae was highly variable in different sections isolated from the pots (Table 6A). All of the glomalin fractions showed increases in % IRP as the culture time increased, except the horticultural mesh. For glomalin extracted from the horticultural mesh, the % IRP was greater than 100% in the first culture period, but decreased in periods 2 and 3. In the first culture period % IRP in the hyphae fraction was almost zero and from the root and hyphal chambers was much lower than in subsequent culture periods.

Gigaspora rosea had the highest amount of glomalin in all fractions at the end of the first culture period (Fig. 6D.B). However, glomalin collected from the hyphae

fraction after the third culture period was significantly higher than in the first period ($P = 0.0283$). Irradiance values during the first culture period were consistently $>200 \text{ W m}^{-2}$ (Fig. 6C.B). During the second culture period, irradiance measurements were above 150 W m^{-2} for the first half of the culture period, but they dropped rapidly to ca. 75 W m^{-2} during the latter half. During the third period, irradiance values were low (50 to 100 W m^{-2}) until the last two weeks. *Gigaspora rosea* cultures did not show wide variation in % IRP among different sections of the pot cultures examined (Table 6A). The second culture period had the highest % IRP in the four fractions.

Hyphal amounts

Large amounts of hyphae were produced (Fig. 6E and 5F) with estimated weight of 4.2 and 3.0 g for *G. etunicatum* periods 2 and 3, respectively, and 24.3, 4.9 and 3.1 g for *Gi. rosea* in periods 1, 2 and 3, respectively. Because fine sand and crushed coal grains adhered to hyphae, it was not possible to obtain 'pure' hyphae that would accurately reflect the total amount of hyphae in a pot. (Attempts to purify hyphae are described in Appendix P.) Although large amounts of hyphae were collected, there was no direct relationship between hyphal amounts and glomalin amounts. For example, in *Gi. rosea*, the amount of hyphae from period 1 was about 5 times the amount from period 2 but there was no significant difference in the amount of glomalin measured.

Immunofluorescence

Immunofluorescence was used to indicate the presence of glomalin on fungal hyphae (Fig. 6E and 5F), potting media (Fig. 6G), horticultural mesh (Fig. 6F), colonized roots and arbuscules (Fig. 6H). One sample of horticultural mesh had many strands of coiled hyphae that formed a 'rope' with accumulation of glomalin in discrete

areas along the various strands (Fig. 6E). A similar hyphal ‘rope’ in soil would protect hyphae crossing air-filled pores and/or entrap organic matter and soil particles to initiate aggregates formation. Other samples of mesh appeared to be covered with hyphae (Fig. 6F.A) and plaque-like spots of glomalin (Fig. 6F.B) reflecting an increase in hyphal production and glomalin accumulation with the repeated cultures (Fig. 6F). Sufficient amounts of glomalin and hyphae were produced during the 294 days of culturing to form pseudo-aggregates of sand and coal (Fig. 6G). Although spots of glomalin may be found on plant roots, AM fungal spores, intraradical hyphae and arbuscules were coated with glomalin (Fig. 6H).

Discussion

Glomalin production and hyphal growth are dependent upon photosynthetic C. Under low light, C is the limiting nutrient (Whitbeck, 2001; Tinker et al., 1994). Since a new root chamber was added at the start of each cultivation period, glomalin accumulation in this chamber does not reflect accumulation over time (Fig. 6D). Rather values in the root chamber directly reflect C inputs from the plant. During periods with the highest irradiance values – Period 2 for *G. etunicatum* and Period 1 for *Gi. rosea* – glomalin concentrations in the root chamber and hyphal weights were the highest of the three growth periods for both species. The amounts of glomalin in the sand and hyphae fractions increased over time, especially for the *Gi. rosea* cultures (Fig. 6D), indicating that glomalin may be accumulating slowly. However, these values did not double or triple in successive culture periods which indicates that more glomalin was being lost than was retained.

Glomalin resists enzymatic and chemical decomposition in the soil and laboratory (Wright and Upadhyaya, 1998; Steinberg and Rillig, 2003; Rillig et al., 2001). Therefore, it was unlikely that rapid turnover influenced glomalin accumulation. Some of the glomalin will absorb to roots, sand/coal particles and mesh, but under these high flow-through watering conditions, it was likely that glomalin was lost through the bottom of the pot. Visual observations and immunofluorescence assays on the horticultural mesh square placed in the bottom of the pot indicated that glomalin is deposited there, often forming plaque-like films (Fig. 6F). In addition, immunofluorescence assays on material that precipitated in the wastewater from a previous pot culture study indicated the presence of glomalin (data not shown). Also, glomalin is in colloidal suspension in water from shallow wells in an area where AM fungi colonize natural vegetation growing on sand in Florida (S. F. Wright, personal communication).

The automatic watering system used in this study maintains constantly moist conditions in pots. Under these conditions, most of the pore space would be filled with water, roots or fungal hyphae (especially following periods of high hyphal production). If glomalin has the same characteristics as hydrophobins produced by other fungi, it would not self-aggregate into rodlet-like complexes without an air-water interface (Wessels, 1997). Aggregated, floating glomalin (scum-like material) was noticed in this study when forced water was used to release hyphae from sand. This action probably released unbound glomalin that aggregated and accumulated at the air-water interface. A similar reaction is seen for hydrophobins that form a hydrophobic foam, similar to soap bubbles, when air or nitrogen is bubbled through the growth media (Askolin et al.,

2001; Wessels, 1997). Therefore, when glomalin sloughs from hyphae but does not self-aggregate, it may be lost from the pot with the wastewater.

In this study container volume and removal of the root chamber may have affected carbon allocation and growth patterns in these fungi. Container volume limits hyphal growth (Bethlenfalvay et al., 1999) and fungal and plant growth under different nutrient conditions (Biermann and Linderman, 1983). Removal of the root chamber prevented the plants from becoming root-bound but disrupted hyphal networks. Colonization of the new host plant was successful in all pots. However, it is unknown how disruption of the hyphal network affected glomalin production or fungal growth.

Other growing conditions, such as nutrient availability and moisture, and signals from the plant, cause differential fungal growth patterns. In some cases, intraradical structures (such as arbuscules, hyphal coils and hyphae) are favored, whereas extraradical hyphal growth increases under other conditions (Rillig et al., 2001; Whitbeck, 2001). Bethlenfalvay and Ames (1987) found that the fragility, type (i.e. function), and distribution of extraradical hyphae may be a function of age, nutrition, soil texture, host compatibility, or other environmental conditions. By the same token, the formation of biomolecules, such as glomalin, also depends on carbon allocation and other not well-understood signals.

Immunofluorescence assays showed that an abundance of hyphae was produced, but hyphae were not completely coated with glomalin (Figs. 5E and 5F). The spotty distribution on fungal hyphae indicated that glomalin was not an integral part of the hyphal wall but rather something attached to it. This could account for the lack of correspondence between glomalin values and hyphal weight. Like hydrophobins

(Wessels, 1997), glomalin may have a signal sequence for secretion and be funneled to the surface of the hyphal wall. On the surface of hyphae, hydrophobins, and possibly glomalin, self-assemble into rodlets or amphipathic membranes. Rodlets of aggregated hydrophobins perform a variety of functions, notably protecting hyphae from moisture changes and facilitating hyphal attachment to surfaces (Askolin et al., 2001; Wessels, 1997). Glomalin produced similar aggregated or plaque-like structures on horticultural mesh (Fig. 6G.B).

The bright and patchy distribution of immunofluorescence due to the presence of glomalin was readily distinguished from auto-fluorescence in plant roots (Fig. 6H). Arbuscules produced by AM fungi were immunofluorescent while the root cell was not. This was further proof that the antibody against glomalin was against something produced by AM fungi, because only AM fungi produce arbuscules.

Although glomalin concentrations did not increase with successive culture periods, this does not mean that plants were not colonized by AM fungi or that glomalin was not produced in subsequent periods. Rather glomalin amounts in these pots were influenced by three factors (other than the length of culture): (1) reduction in photosynthetic activity and C allocation to AM fungi because of low light, (2) loss of glomalin through the bottom of the pot, and (3) disruption of hyphal networks by removing the root chamber. To eliminate or reduce the effects of these other variables, future research comparing glomalin production and accumulation should include: (1) growing plants only during the summer season or in a growth chamber, (2) additional supplemental lighting, (3) examining wastewater from pots to quantify glomalin concentrations, (4) using a different nutrient delivery system that does not keep the

media constantly moist, (5) using different potting media to retain more glomalin within the pot, or (6) using larger pots and keeping the root chamber in the pot.

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Table 6A. Percentage immunoreactive protein for glomalin extracted from different sections or section-components of single species pot cultures of *Glomus etunicatum* and *Gigaspora rosea* after one, two and three consecutive 14-week culture periods. ‡

Species	Section and components	Number of culture periods		
		1	2	3
<i>Glomus etunicatum</i>	Hyphae chamber:			
	Hyphae	0.3 ± 0.2a	44.5 ± 1.1a	58.1 ± 10.4a
	Mesh	311.2 ± 73.5a	58.2 ± 3.8a	40.6 ± 2.9a
	Potting medium	26.9 ± 3.4b	51.0 ± 8.2a	54.0 ± 9.1a
	Root chamber	2.2 ± 0.5a	36.9 ± 18.4a	53.6 ± 3.5a
<i>Gigaspora gigantea</i>	Hyphae chamber:			
	Hyphae	25.5 ± 2.3a	51.2 ± 8.0b	23.4 ± 1.3a
	Mesh	22.0 ± 1.5a	77.0 ± 30.2b	26.0 ± 2.2a
	Potting medium	42.6 ± 16.0a	47.4 ± 11.9a	25.4 ± 1.4a
	Root chamber	37.5 ± 14.1a	55.0 ± 28.3a	47.3 ± 2.7a

† Mean ± SE.

‡ Different letters in a row indicate significant differences according to REML (P < 0.05).

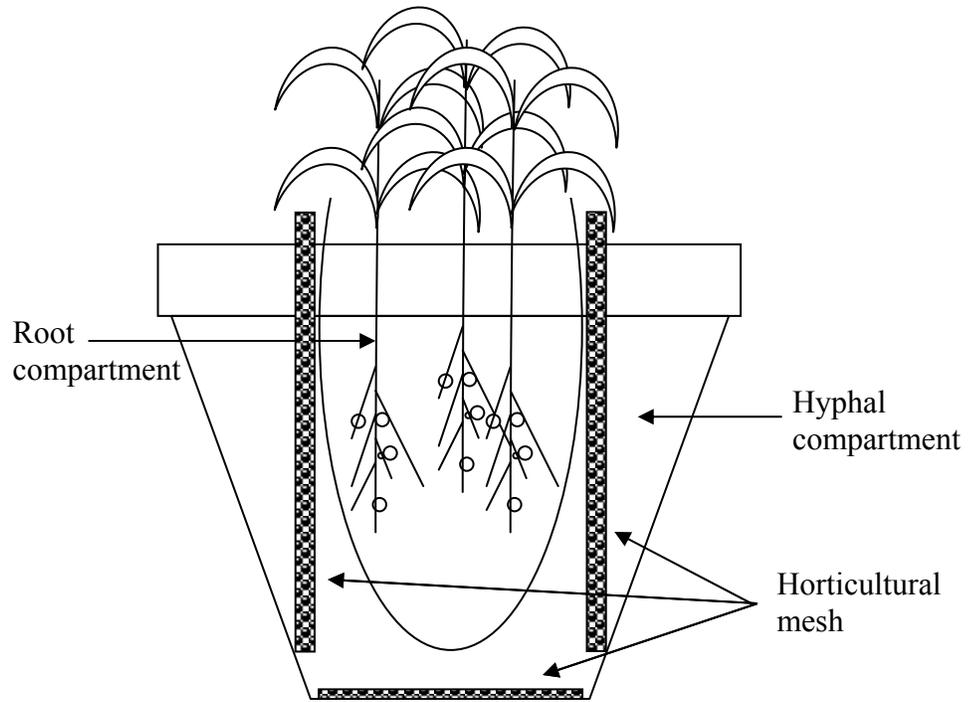


Figure 6A. Schematic representation of corn pot cultures inoculated with arbuscular mycorrhizal fungi grown in a root compartment that is kept separate from a hyphal compartment by a 38 um nylon fabric bag. Horticultural mesh is inserted in the sides and at the bottom of the pot to measure glomalin accumulation on solid surfaces.



Figure 6B. Corn plants inoculated with *Gigaspora rosea* in the greenhouse at the end of the third culture period. The taller plants on the far left were directly under a sodium vapor lamp, while those on the right were between lamps.

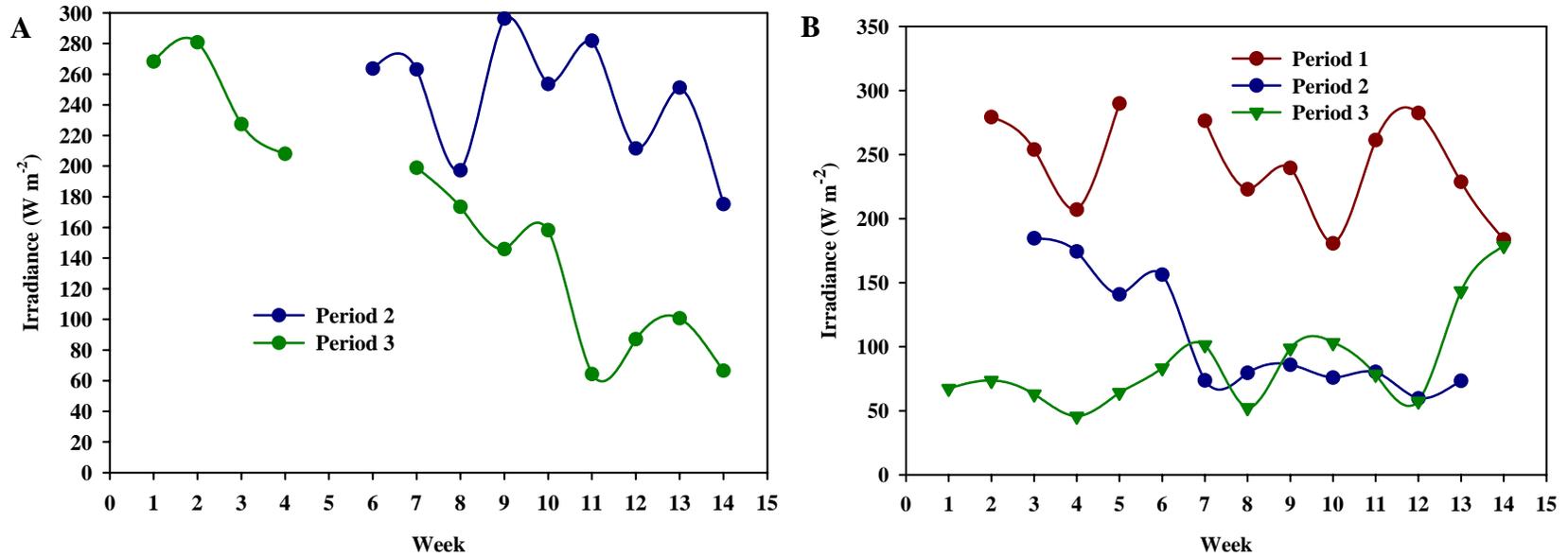


Figure 6C. Irradiance measurements from outside the greenhouse during three 14-week culture periods (2002 to 2003) – Period 1 (Jan. 14 to April 22 and May 21 to Aug. 27), Period 2 (April 22 to July 29 and Aug. 27 to Dec. 4), and Period 3 (July 29 to Nov. 4 and Dec. 8 to March 15) – for corn plants inoculated with *Glomus etunicatum* (A) and *Gigaspora rosea* (B) respectively. Equipment problems resulted in several missing data points.

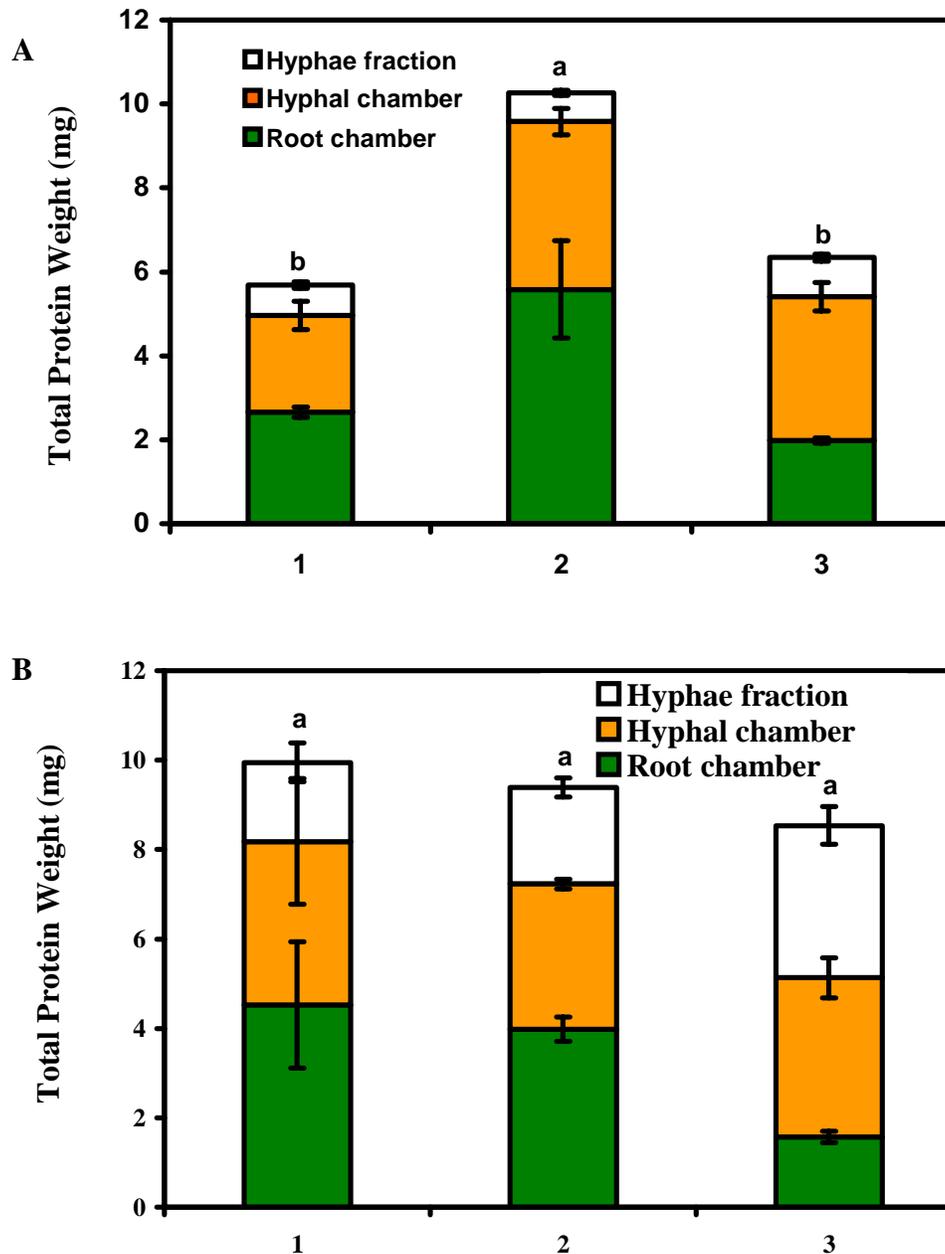


Figure 6D. Total protein weight of glomalin isolated from different sections (hyphae fraction and hyphae and root chambers) separated from single species pot cultures of *Glomus etunicatum* (A) and *Gigaspora rosea* (B) after one, two and three 14-week culture periods. Different letters indicate significant differences according to REML ($P < 0.05$).

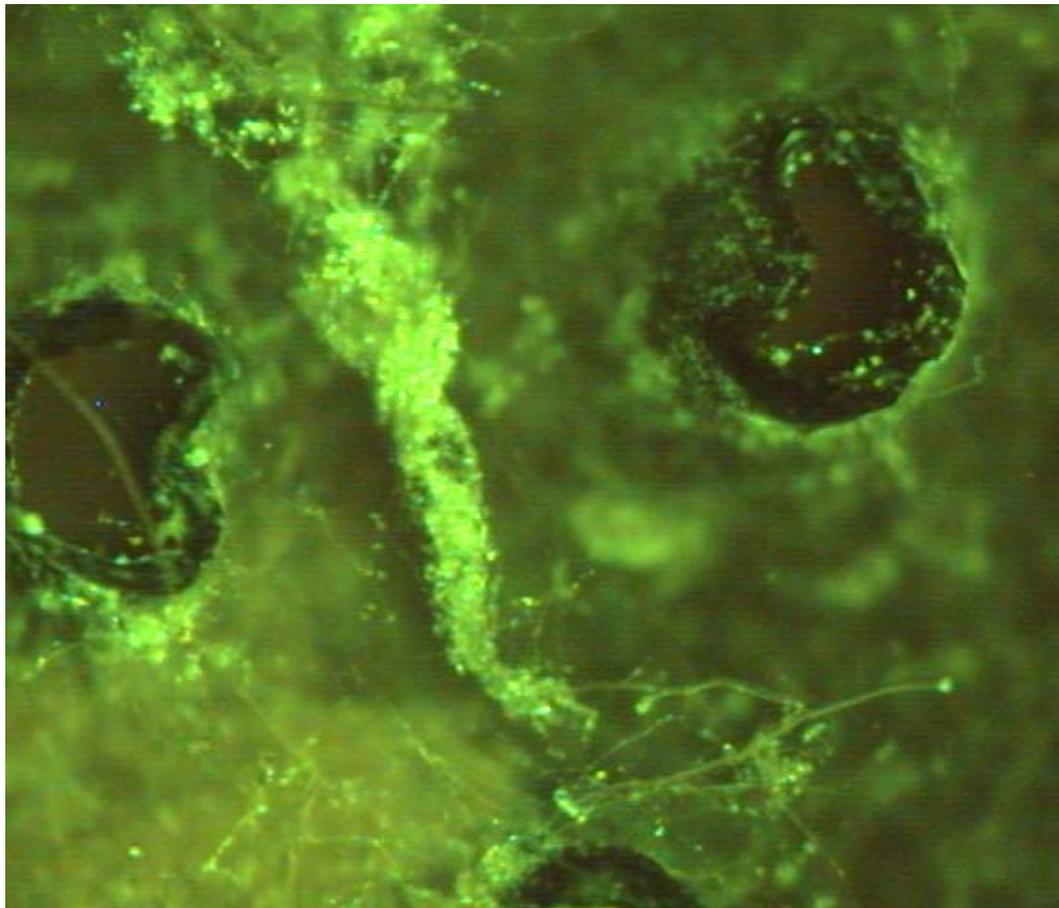
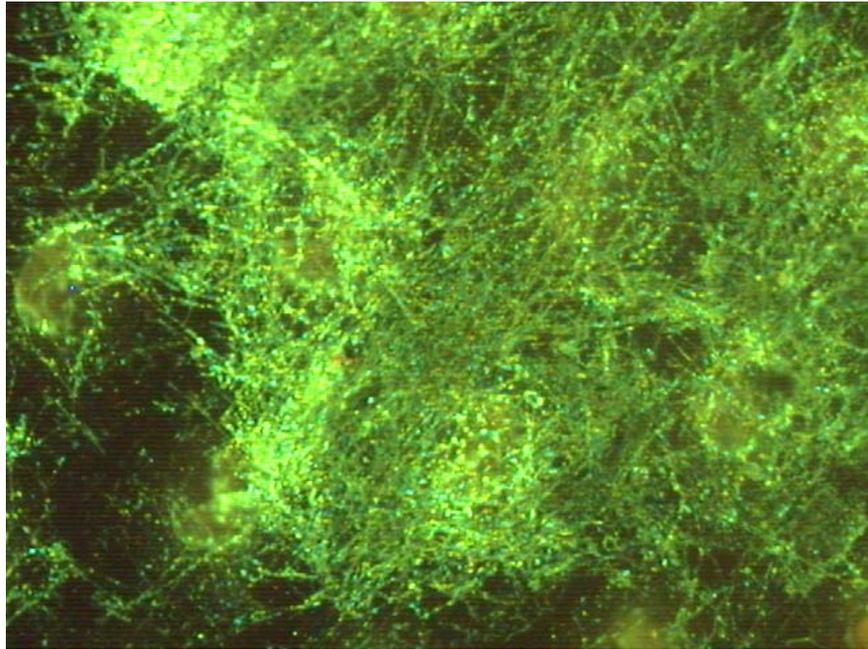


Figure 6E. Glomalin present on a rope of *Gigaspora rosea* hyphae bridging two spatially separated holes in the mesh. Bright green spots indicate the presence of glomalin.

A



B

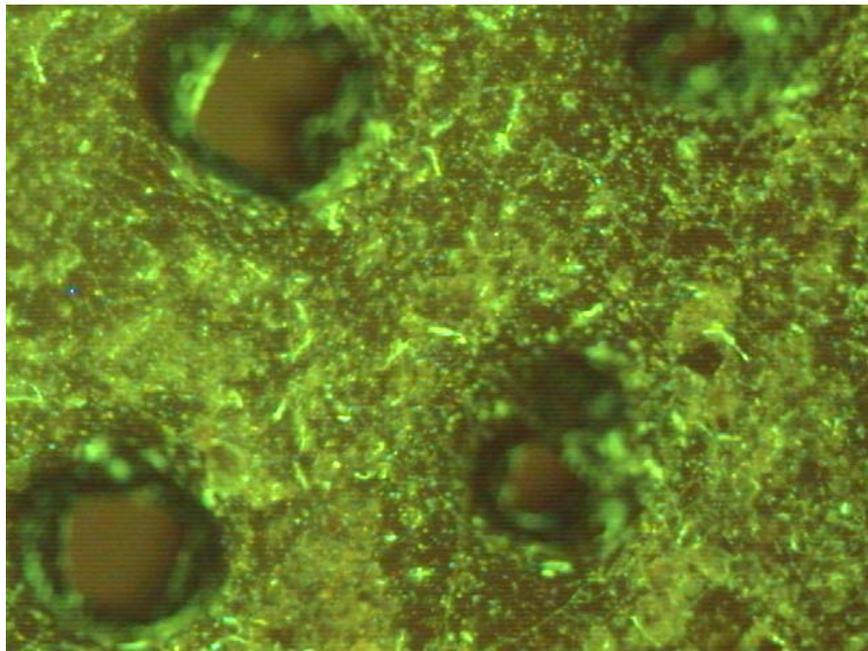


Figure 6F. After three 14-week culture periods, glomalin was present on a massive network of *Gigaspora rosea* hyphae and deposited on horticultural mesh placed in the hyphal chamber (A) or at the bottom of the pot (B). Bright green spots indicate the presence of glomalin.

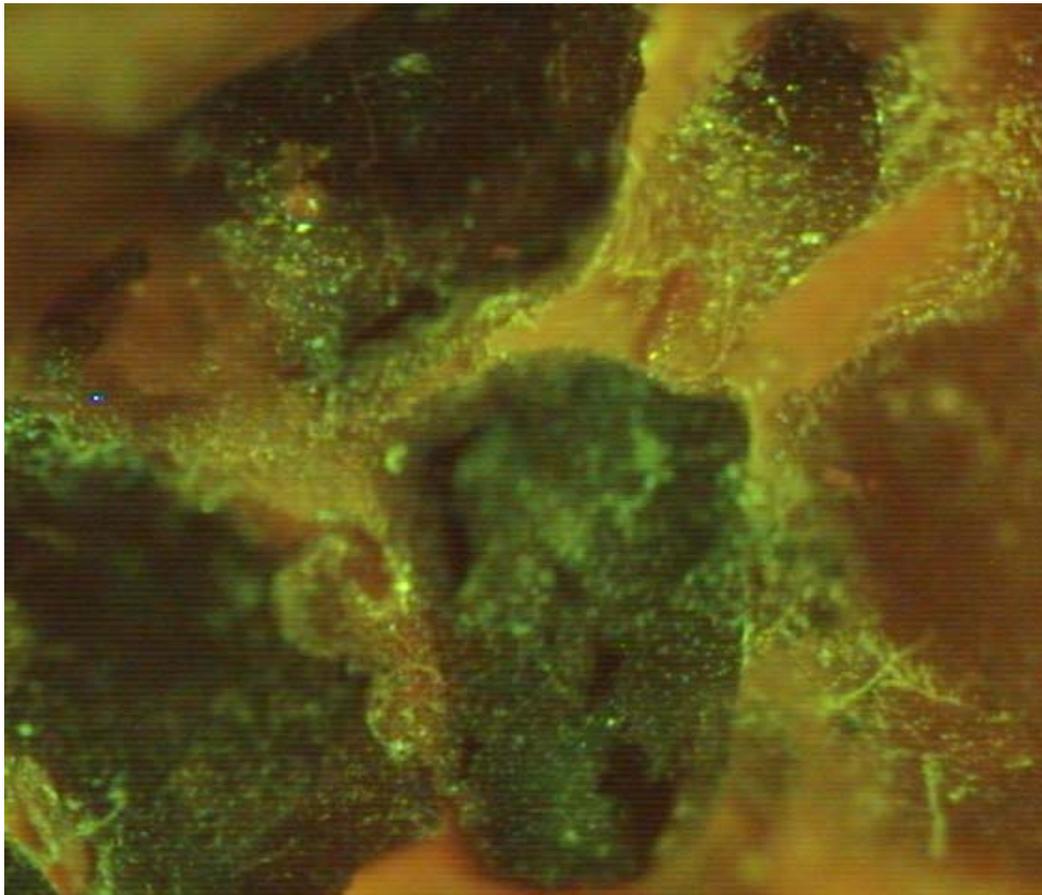


Figure 6G. After three 14-week culture periods, some of the sand grains and crushed coal had formed a >2-mm aggregate-like structure with glomalin on the surface gluing these particles together. Bright green spots indicate the presence of glomalin.



Figure 6H. Glomalin found on a colonized corn root, a *Glomus etunicatum* spore (A) and intraradical fungal structures, including arbuscules (B). Bright green spots indicate the presence of glomalin.

CHAPTER 7

COMPOSITIONAL ANALYSIS OF GLOMALIN

Abstract

Glomalin is a complex macromolecule produced by arbuscular mycorrhizal (AM) fungi and is classified as a glycoprotein. In addition to structural oligosaccharides and amino acids, glomalin binds iron and organic matter. Glomalin is difficult to hydrolyze with acid or proteases for analysis of functional groups. The incorporation of iron as an apparent integral component and as a tightly bound shell increases molecular stability. High heat (121⁰C) used to solubilized glomalin apparently ‘fused’ bound iron and organic matter to the molecular structure and prevented removal following extraction. To remove iron, which causes interference in spectroscopic and colorimetric assays, competitive chelation and deglycosylation were attempted. Results indicated that pre-treatment of the soil with HCl to remove iron or with NaOH to disrupt organo-mineral complexes and extraction with sodium pyrophosphate may improve analytical results. In addition, purification and separation of the oligosaccharide and proteinaceous components of glomalin was accomplished. The procedures defined herein will aid in future work to elucidate the structural units of glomalin. Elucidating the structure of glomalin will help to understand the roles of this molecule in plant health and soil quality.

Introduction

Proteins are the most complex naturally occurring macromolecules. Although all proteins have the same peptide backbone, the 20 different amino acid side chains may be combined in chains of various lengths with a number of different folding patterns. Therefore, an infinite number of molecules may form. Considering the diverse biological functions for proteins, a vast array of molecules is expected. The formation

of glyconjugates or glycoproteins creates even more complex molecules because of the branching and stereochemistry of the added oligosaccharides.

Carbohydrate modifications to proteins are a key factor in their structure and function (Bahl, 1992). Glycoproteins act in enzyme catalysis, hormonal control, immunology, ion transport, structural support, cell adhesion, and cell recognition (Bahl, 1992; Varki, 1993). Carbohydrates affect viscosity, thermal stability, solubility, and resistance to proteolysis (Bahl, 1992; Iyer and Lonnerdal, 1993).

Glomalin is produced by arbuscular mycorrhizal (AM) fungi and is resistant to trypsin and chemical (acid) hydrolysis (Wright et al., 1996). Lectin-binding capability and high performance capillary electrophoresis (HPCE) indicate that glomalin is a glycoprotein with one major asparagine-linked (N-linked) chain of carbohydrates (Wright et al., 1998). The procedures used to denature and deglycosylate glomalin for HPCE are not fully successful. Protein that remained intact precipitated from solution and was separated by centrifugation prior to analysis. These results indicate that some fractions of glomalin may be more resistant to denaturation and deglycosylation than others.

In its native state, glomalin is insoluble in aqueous solutions. High heat (121°C) treatment in 1 h intervals is used to solubilize glomalin. This treatment would denature heat-labile proteins that may be co-extracted from the soil (Wright et al., 1996). These denatured proteins and other small molecules are lost during the primary purification process of acid precipitation, re-dissolution in an alkaline solution and dialysis.

Because glomalin is so resistant to decomposition, it is a fraction of organic matter (OM) that may be present in both the transient and persistent pools with a

turnover time of at least a decade (Rillig et al., 2001; Steinberg and Rillig, 2003).

Molecular stability comes from chemical characteristics, such as hydrophobicity and iron binding. Hydrophobicity makes glomalin water-insoluble, prevents microbial access to the molecule, and helps it bind to surfaces. Iron-binding prevents microbial decomposition and bridges glomalin to clay minerals and other types of organic matter.

Iron causes interference with a number of techniques used to examine molecular structure, such as NMR and some colorimetric assays, and adds structural stability that may help glomalin resist chemical and enzymatic treatment used for analysis. Iron may also act as a bridge between clay minerals and glomalin. This process would bind inorganic molecules such as clay silicates to glomalin which would account for the high gravimetric weight compared to protein weight seen in other studies (Chapters 2 and 3) and would account for the high silica values measured previously in our lab using ICP (inductively-coupled plasma).

Purification of proteins with hydrophobic characteristics with phenyl column-HPLC (high-pressure liquid chromatography) is commonly used. In a phenyl column, ammonium sulfate salt increases surface tension in the buffer solution which brings hydrophobic groups such as hydrophobic amino acids (leucine, isoleucine, alanine, valine, phenylalanine, tyrosine, and tryptophan) and aliphatics to the surface of the protein. The hydrophobic environment of a phenyl column is so strong that proteins will absorb to the column and not to each other. Upon lowering salt concentration in the buffer solution, the surface tension will decrease and the protein will desorb from the column.

Lectin affinity chromatography is used to purify hydrophilic glycoproteins or oligosaccharide in solutions. Lectin chromatography with Con A-Sepharose may be used to isolate glycoproteins or oligosaccharides from mixtures of substances. Con A recognizes the core sugars and their substitution pattern including the three major classes of asparagine-linked (N-linked) sugar chains: (1) high mannose chains, (2) biantennary complex oligosaccharides, and (3) branched tri- and tetra-antennary complex-type oligosaccharides (Varki, 1993).

In this study, procedures were developed to examine the compositional groups in glomalin from hyphae and soil extracts. This work will provide a context for future experiments to determine the structure of glomalin and its importance in global climate change and soil fertility issues.

Materials and methods

Glomalin samples

In this study, freeze-dried glomalin extracted from soil and hyphae samples in previous experiments was used. Hyphae samples [*Glomus (G.) intraradices* Schenck & Smith (WV795) and *Gigaspora (Gi.) rosea* Nicolson and Schenck FL224 (INVAM)] were collected from the hyphal chamber in pot culture experiments. (See Chapter 6 for detailed culturing methods). Soil samples were from a variety of other experiments in our laboratory: (1) a Weld silt loam from Colorado (CO) (samples from field and native grass plots) (Wright and Anderson, 2000), (2) soils from the Eastern Shore of Maryland (MD) (samples from plots in fields or woods at two sites –R. Reily and Ed Q), (3) the A and B horizons of Gilpin soil from Maryland, and (4) from soils used Chapters 2 and 3.

Each of the glomalin samples was extracted with citrate at 121°C. (See Appendix A2 for a detailed description of the glomalin extraction and purification methods.)

Treatments to remove iron

Competitive chelation experiments

EDDHA incubation

Glomalin samples (2.5 mg) - extracted from horizons A and B of a Gilpin soil - were reconstituted in 10 mM borate, pH 8.0, and placed in dialysis capsules in a 100 mM EDDHA [ethylenediamine di-(*o*-hydroxyphenylacetic) acid] and 10 mM borate, pH 9.2, solution. After mixing for 2.5 weeks, samples were removed and protein concentration was measured (see below).

8-hydroxyquinoline incubation

Glomalin samples (~20-100 mg) from Baltimore series soil (Chapters 2 and 3) and from hyphae of *G. intraradices* and *Gi. rosea* were reconstituted in 20 mM MES [2-(N-morpholino)-ethane sulfonic acid] buffer at pH 5.5. Chloroform (2 to 5 mL) was added followed by 8-hydroxyquinoline (1-5%). Samples were covered tightly and incubated for about 12 h. During this time, the chloroform phase was replaced after it turned a green-black color. This process was repeated until the chloroform phase was nearly colorless. After treatment, samples were washed using a centrifugal filter device with a 50 kD cut-off (Centricon 50, Millipore). Other samples were washed in progressively smaller Centricon tubes (50>30>10>3 kD). Retentate and filtrate were collected and assayed for protein (see below).

Tiron treatment

Freeze-dried glomalin samples (Chapters 1 and 2) from the Baltimore site *a* (MD) and Pacolet (GA) soils were reconstituted with a 0.1 *N* NaOH solution containing 0.15 *M* 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron®, Sigma Aldrich, St. Louis, MO) and H₂O (Simpson, 2001; Fan et al., 2000). Samples were incubated overnight, centrifuged at 6850 x *g* for 10 min, and dialyzed extensively against water. Glomalin samples were analyzed for carbohydrate concentration using the carbohydrate estimation kit (see below) and compared to glomalin that was not treated with Tiron.

Acid hydrolysis and iron oxide extraction experiments

Hydrolysis in acid under vacuum

Glomalin samples (1 – 10 mg) from the Weld, Eastern Shore, and Gilpin soils and from *Gi. rosea* hyphae were placed into vacuum hydrolysis tubes and 1 mL of constant boiling point 6 *N* HCl was added. Tubes were capped loosely and subjected to 3 cycles of evacuation and injection of N₂ gas followed by a final evacuation. Tubes were capped tightly and incubated at 100 – 115 °C overnight. After incubation, samples were centrifuged to pellet the precipitate. Both the supernatant and precipitate were tested for iron concentration using the colorimetric iron assay (see below).

Sodium dithionate extraction of iron oxides

Glomalin was extracted from the NE (Wymore and Pawnee) and CO (Sampson and Haxtun) soils (Chapter 3) before or after treatment with HCl and NaOH co-extraction of HA and FA. Iron oxides were extracted by heating glomalin dissolved in a 0.3 *M* sodium citrate and 1 *M* sodium bicarbonate solution to 75 to 80°C (Loeppert and Inskeep, 1996). When the solution reached temperature, sodium dithionate powder

(equivalent to 20% of the weight of the sample) was added followed by continuous stirring for 1 min and intermittent stirring for 5 min. Another, similar-sized portion of dithionate was added followed by intermittent stirring for 10 min. The samples were removed from the heat and saturated NaCl was added to promote flocculation. Glomalin was pelleted by centrifugation at $10844 \times g$. The supernatant was decanted through a Whatman #1 filter into a volumetric flask or graduated tube and brought to volume with deionized (dH_2O). Iron concentration was measured in each sample by the colorimetric assay and/or AA (see below). The pellet of treated glomalin was a red-brown color, which indicates the presence of iron. These samples were then subjected to a follow-up digestion using the modified Aqua Regia procedure.

Modified Aqua Regia

The modified Aqua Regia procedure (McGrath and Cunliffe, 1985) consisted of adding concentrated HNO_3 to the sample and heating to $85-90^\circ C$ (a temperature high enough to cause evaporation but not boiling) for 2 hrs. Next, concentrated HCl was added followed by incubation at $60^\circ C$ for 1 hr. Samples were centrifuged at $6850 \times g$ for 10 min. The supernatant was decanted through a Whatman #1 filter into a volumetric flask or graduated tube and brought to volume with dH_2O . Iron concentration was measured in each sample by the colorimetric assay and/or AA (see below). In a few glomalin samples, most, but not all, of the sample was hydrolyzed. The remaining material was collected by centrifugation at $10844 \times g$, rinsed with dH_2O , lyophilized, weighed and subtracted from the original sample weight.

Microwave digestion

Glomalin from the Weld and Eastern Shore soils (ca. 10 mg) were placed in Teflon tubes. The samples were treated with 1 mL dH₂O water and 3 mL of Ultra Pure concentrated nitric acid (HNO₃). The caps were screwed on until just tight, and samples were placed into a microwave and connected to a pressure regulator tube. Microwave digestion was run with the following program: 6.9 x 10⁵ pascal (Pa) for 10 min; 4.83 x 10⁵ Pa for 15 min; and 3.45 x 10⁵ Pa for 10 min. After the pressure in the chamber returned to zero, the tubes were removed from the microwave and the caps were unscrewed slowly. Condensation in the tops was rinsed into the tubes. Samples were filtered through Whatman #40 filter paper to remove particulate matter and brought up to 25 mL with dH₂O. Iron concentration was measured in each sample by atomic adsorption (AA) and the colorimetric assay.

Column purification of glomalin

Phenyl column

Samples (12) from soil (Chapter 3) and hyphae extracts were processed through a phenyl column using a HPLC (High-Pressure Liquid Chromatography) pump (Waters 600E). Before adding samples, the column was washed with ca. 250 mL of filtered (0.25 µm filter) dH₂O. About 200 mL of filtered (0.25 µm filter) 1 M ammonium sulfate in 25 mM phosphate buffer, pH 7.0, was used to equilibrate the column.

Glomalin was dissolved in 30 mL of 25 mM phosphate buffer, pH 7.0 and centrifuged at 6850 x g to remove any insoluble material. The supernatant was transferred to a new tube and ammonium sulfate was added to a concentration of 1 M. In some samples, a large amount of the material precipitated. These samples were

centrifuged again at 6850 x *g*. The supernatant was collected and sampled for total (TP) and immunoreactive protein (IRP) assays (see below).

When a large amount of glomalin precipitated with 1 *M* ammonium sulfate, the samples were centrifuged at 6850 x *g*. The supernatant was loaded onto the column and the precipitate was redissolved in the phosphate buffer with ammonium sulfate at 0.25 *M*. Prior to loading, samples were centrifuged again at 6850 x *g*.

Samples were loaded onto the column at the rate of 3 mL min⁻¹. When all of the sample volume was on the column, the column was washed with 150 to 200 mL of buffer before running the gradient and collecting the fractions. In a select number of samples, this wash was collected and examined further. A linear gradient was used to decrease the concentration of 1 *M* ammonium sulfate in 25 mM phosphate buffer, pH 7.0 to dH₂O over 1 hr at 5 mL min⁻¹. The column was washed with dH₂O for another 20 min. Fractions were collected in 1-min intervals while the gradient was running and for the 20 min after the end of the gradient (giving a total of 80 fractions). The three or four tubes containing the highest amounts of protein were pooled for TP and IRP assays (see below). The remaining volume was dialyzed extensively against water (dialysis tubing with a MWCO (molecular weight cut-off) = 6-8,000 D), freeze-dried and weighed.

Lectin affinity column

Concanavalin A (Con A)-Sepharose (Sigma Aldrich, St. Louis, MO) columns were prepared by transferring a well-mixed suspension into a 10 mL column to the 1 mL mark. The column was equilibrated by washing with 10 column volumes of TBS buffer [(0.01 *M* Tris (Tris(hydroxymethyl)aminomethane), 0.15 *M* NaCl, 1 mM CaCl₂ and 1 mM MgCl₂), pH 8.0]. The final wash was drained to the top of the column bed.

Samples were loaded onto the column, washed, and eluted according to protocols of Fattman et al. (2000) and Sanjay et al. (1997). Briefly, a sample was reconstituted in TBS buffer (5 mL) and loaded onto the column in 1-mL increments. The column was washed with TBS (8 mL). When the last wash was at the top of the bed, 10 mL of the eluent 10 mM μ -methyl-D-glucopyranoside in TBS buffer was added. When the μ -methyl-D-glucopyranoside elution was complete, the column was eluted with 10 mL of warmed (60°C) 100 mM μ -methyl-D-manno-pyranoside in TBS buffer. The column retained the red-brown color of glomalin making a third eluent - 3% acetic acid - necessary. Fractions were collected from the wash and each eluent separately and analyzed for total protein (TP) concentration (see below). Fractions with the highest protein values were pooled, dialyzed against water in dialysis tubing with a MWCO = 6-8,000, and freeze-dried.

Separation of oligosaccharides from a glycoprotein

Samples that had only been through the phenyl column or had been through both the phenyl and lectin affinity columns were reconstituted in 200 μ l of 50 mM sodium phosphate buffer, pH 7.5 containing 0.5% (w/v) SDS (sodium dodecyl sulfate) and 50 mM 2-mercaptoethanol. Samples were denatured at 100°C in boiling water bath for 5 min. After the samples had cooled, the SDS was diluted out with 270 μ l 50 mM sodium phosphate buffer, pH 7.5. Concentration of SDS at this point was 0.2%. A non-ionic detergent (NP-40) was added to protect the enzyme from denaturation by the residual SDS. The final concentration of NP-40 was 1.5% (v/v) providing a greater than 7-fold excess over the concentration of SDS. The samples were treated with 10 units of PNGase F (peptide-N-glycosidase F; Sigma Aldrich, St. Louis, MO) and incubated at

37 °C in a water bath for ~18 h. The reaction was terminated by boiling in a water (100°C) bath for 5 min.

Released oligosaccharides were separated from the peptide using a Sep-Pak® C₁₈ (Waters Associates, Milford, MA) column. The column was cleaned by washing with 3 mL methanol and was equilibrated by washing six or seven times with 3 mL dH₂O. The sample was loaded and washed three times with 1 mL dH₂O.

Oligosaccharides were collected in 3 – 5 mL 5% (v/v) acetic acid. The protein fraction was then collected with 3-5 mL a 60% propanol and 40% acetic acid (5%) solution.

Each fraction (i.e. oligosaccharide and protein) was evaporated to dryness. Samples were dissolved in pyridine-acetate solution and quantitative measurements were made for TP, IRP and neutral hexose concentrations (see below). (The pyridine-acetate solution was added to the blanks used in the TP, IRP, and neutral hexose assays to check of interference.)

NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was conducted on two sets of samples. One set contained carbohydrate and protein fractions of glomalin extracted from *Glomus etunicatum* hyphae and Cecil soil and separated as described above after purification through phenyl and Con A columns. The second set contained a citrate alone control and three glomalin samples extracted in 1 h intervals from the Sampson series soil with (1) 50 mM citrate, pH 8.0, at 121°C, (2) 100 mM sodium pyrophosphate, pH 9.0, at 121°, and (3) 50 mM citrate, pH 8.0, at 121°C, following a sequential extraction of glomalin with pyrophosphate and HA with NaOH solution at RT (see Chapter 3). (See Appendix G for details on similar extractions from hyphae.)

All samples (10 to 20 mg) were reconstituted with 95% D₂O and placed in NMR sample tubes. One-dimensional (1D) ¹H-NMR experiments were conducted using a 300 MHz Bruker QE Plus NMR spectrometer with Tecmag Aquarius ver. 5-6 operating system. Spectral width was 3000 Hz and chemical shifts were referenced relative to the HDO peak at 4.8 ppm. The HDO signal was minimized using a pre-saturation pulse sequence (Braun et al., 1998). Baseline correction was applied.

Quantitative measurements

Protein assays

Protein measurements were made on glomalin samples that were dissolved in the appropriate solution from the experiments above. A modified Bradford protein assay (Wright et al., 1996) was used to measure TP concentration. Samples were diluted in PBS (phosphate buffered saline) and reacted with Bio-Rad® (Hercules, CA) Bradford protein dye reagent. Absorbance was read at A₅₉₅ after 5 min. Protein concentration was determined by comparison with a bovine serum albumin (BSA) standard curve.

Immunoreactive protein concentration was measured by ELISA as described by Wright and Upadhyaya (1998) with modifications in the enzyme and color developer. ExtrAvidin® (Sigma Aldrich, St. Louis, MO) phosphatase was used instead of peroxidase. Wells were rinsed with Tris buffered saline with Tween 20 (polyoxyethylenesorbitan monolaurate) before the color developer (*p*-nitrophenyl phosphate in diethanolamine buffer) was added (Wright, 1994). Absorbance was read at A₄₀₅ after 15 min. Test samples were compared to a standard curve produced by dilutions of highly immunoreactive glomalin extracted from a temperate soil under native grasses.

Iron concentration measurements

Colorimetric iron assay

An aliquot of sample was placed in a 15 mL screw cap centrifuge tube. If the sample was in a strong alkaline solution, 1 mL of 6 N HCl was added. Two mL of 10% (w/v) hydroxylamine HCl was added to each tube plus 1 mL of 1.5% (w/v) o-phenanthroline in 95% ethanol. Tubes were filled to 10 mL with dH₂O and shaken. Samples were read at 490 nm after at least 30 min and concentration was determined by comparison to a standard curve with a range of 0-60 µg. Percentage iron in glomalin was calculated by converting the µg values into µg mg⁻¹ values using the assay volumes and the weights of the glomalin samples and multiplying by 0.1.

Atomic adsorption

An aliquot of sample was injected into a Varian Atomic Absorption Spectrometer (AA-400, Palo Alto, CA) with deuterium background correction and heated until the elements were burned off. Concentration was determined by comparison to a standard curve with a range of 0-30 µg L⁻¹. Percentage iron was calculated by converting the µg L⁻¹ values into µg mg⁻¹ values using the sample volumes and the weights of the glomalin samples and multiplying by 0.1.

Carbohydrate concentration measurements

Glycoprotein detection

Glomalin samples (0.5 mg) were dissolved in 1mL of a commercial Glycoprotein Assay Buffer (Pierce Glycoprotein Carbohydrate Estimation Kit, Rockford, IL). A solution (0.1 mL) containing 10 mM sodium meta-periodate in the assay buffer was added. After vortexing, samples were incubated for 10 min at room

temperature. A 1 N NaOH solution containing 0.5% (w/v) aldehyde detection reagent (Pierce Glycoprotein Carbohydrate Estimation Kit) was added. Samples were again vortexed and then incubated at room temperature for 1 hr. Samples were centrifuged at $10844 \times g$ to remove the precipitate. The supernatant was transferred to cuvettes and read at 550 nm. Glycoprotein concentration was determined by comparison to a standard curve made from glycoproteins containing known concentrations of glycosyl groups and were treated the same as the samples.

Modifications to periodate treatment

Precipitation of glomalin during the deglycosylation step of the carbohydrate assay suggested that not all of the material in the glomalin samples was deglycosylated. The procedure described above was performed again with the following modifications: incubating with periodate for longer times (1, 1.5 and 2 h), using a higher periodate concentration (30 mM), and keeping samples either exposed to air or sealed to create anaerobic conditions.

Neutral hexose assay procedure

A sample (200 μ l) from the carbohydrate and protein fractions of the deglycosylated samples in pyridine-acetate buffer was placed in a test tube. Phenol (200 μ l) was added followed by vortexing. Concentrated sulfuric acid (1 mL) was added rapidly to the samples, which were vortexed and allowed to cool. Absorbance was read at 492 nm.

Results

Competitive chelation experiments

None of the competitive chelators was successful in removing all of the iron from glomalin as evidenced by lack of total removal of the red-brown color considered to be indicative of iron. In addition, there were only small changes in the carbohydrate and IRP concentrations before and after treatment with Tiron and dialysis (Table 7A). The results of the carbohydrate analysis showed that when glomalin was extracted before NaOH extraction of HA and FA (Experiment 1), the percentage of carbohydrates was higher than when glomalin was extracted after HA and FA (Experiment 2). Following incubation in 8-hydroxyquinoline, proteinaceous material was collected in all size fractions separated with the Centricon 50, 30, 10 and 3 tubes (Table 7B). However, the Centricon 3 filtrate was not immunoreactive.

Acid hydrolysis and iron oxide extraction experiments

The percentage iron was measured on samples subjected to acid hydrolysis under vacuum, microwave digestion, dithionate extraction, and modified Aqua Regia hydrolysis. In all of these methods, except the microwave digestion, a portion of glomalin remained insoluble. Percentage iron values were lower in glomalin extracted from hyphae (*G. intraradices* and *Gi. rosea*), from the native grass Weld soil (CO) plots, and from the Eastern Shore soils (MD) than in glomalin from the Gilpin soil (MD) (Table 7C) and the soils from Chapters 2 and 3 (Table 7D). The two methods used to measure percentage iron (AA and colorimetric assay) gave different values for the samples that were digested in the microwave with the colorimetric assay values being lower (Table 7C). Values in samples where hydrolysis was under vacuum and

iron was measured by the colorimetric assay were similar to values from samples that were digested in the microwave and iron was measured by AA (Table 7C).

Extraction of iron using dithionate was compared to extraction using a modified Aqua Regia method on glomalin samples collected before and after treating the soil with HCl (Table 7D). The dithionate treatment did not extract all of the iron from glomalin. Glomalin from soil that was pre-treated with HCl had less iron than glomalin from soil that was not treated with HCl.

Carbohydrate measurements – Periodate treatment

Periodate treatment to release carbohydrates from glomalin resulted in the formation of a precipitate regardless of the length of incubation, concentration of periodate, or whether the sample was covered or uncovered. Therefore, values obtained by the assay used herein may not be an accurate assessment of carbohydrate concentration in glomalin.

Analysis of glomalin purified by column chromatography

Glomalin was purified using both phenyl and lectin column chromatography. For the phenyl column, colored, proteinaceous material was collected immediately after the ammonium sulfate gradient had finished and the column was being washed with water (Fig. 7A). Glomalin was not eluted from the lectin affinity column as easily – three eluent solutions were required. Fractions, collected from each of the three eluent solutions, contained proteinaceous material (Fig. 7B). All the protein-containing fractions were combined for analysis by protein assays and NMR.

After purification, glomalin samples were deglycosylated to give oligosaccharide and protein fractions. The samples were examined using ^1H NMR (Fig.

7C). Both samples had similar peaks in the two fractions. The oligosaccharide fraction had several peaks in the 3-4 ppm range which is characteristic of carbohydrates and a major peak at 2 ppm, which would represent aliphatic methines and some methylenes (e.g. CH-N , $\text{CH}_2\text{-N}$, CH-C=O , $\text{CH}_2\text{-C=O}$) (Cavanagh et al., 1996). The protein fractions had major peaks in the 0.5 to 2.5 ppm region that are typical of protons in the methyl-type groups: (1) methyls (CH_3) at <1.0 ppm; (2) methylenes (CH_2) between 1.0 to 2.0 ppm; and (3) aliphatic methines and methylenes between 2.0 and 2.5 (Cavanagh et al., 1996; Wüthrich, 1986). The peak at 0.8 indicated the presence of amino acids: valine, isoleucine, and leucine (Cavanagh et al., 1996). There were no major corresponding peaks between the oligosaccharide and protein fractions, which indicated that two different fractions were isolated by this procedure and that the protein fraction contained more aliphatic protons.

When citrate was used to extract glomalin, some of the citrate apparently was bound to the glomalin molecule and was removed by acid precipitation and dialysis. The ^1H NMR spectra showed that the four peaks at 2.5 to 3.0 ppm in the glomalin extracted with citrate are from citrate (Fig. 7D). When glomalin was extracted with pyrophosphate, it did not contain these citrate peaks. However, when citrate was used to extract the recalcitrant glomalin fraction (i.e. glomalin extracted after NaOH treatment for humic acid), the citrate peaks returned.

Discussion

Several iron chelators with increasing strength (EDDHA < 8-hydroxyquinoline < Tiron) were used to attempt to remove iron from glomalin. The competitive chelation treatments and other attempts to remove iron by chemical treatment, such as

deglycosylation, were only partially successful. In addition to the methods used here, others have tried using ion exchange columns, including a Chelex 100 resin column, without success (J. Gander and A. Simpson, personal communications). It seems that some iron was loosely associated with glomalin and may be removed by these methods or with dithionate, but the majority of iron can be removed only by complete hydrolysis of the protein (Table 7D). When the soil was pre-treated with acid, as was done in Chapter 3 for the glomalin extracted after NaOH extraction, the concentration of iron in glomalin was significantly lower than when it was not pre-treated (Table 7D; Chapter 3-Table 3E).

The hydrolysis methods (microwave digestion and acid hydrolysis under vacuum) used in this study and the methods used to measure percentage iron (AA and colorimetric assay) gave inconsistent results for the same glomalin samples. The incomplete hydrolysis of glomalin treated under vacuum would account for some variability in the hydrolysis methods. Changing the matrix solution for the colorimetric assay may account for some variability in the measurement methods. However, the data indicate that methodology must be carefully examined before making any definitive conclusions.

The smaller-sized proteinaceous fragments collected with the Centricon tubes after treatment with 8-hydroxyquinoline indicated that glomalin extracted from soil and hyphae could be a multimeric complex of aggregated monomers formed by the interactions of iron. Iron may be bridging glomalin monomers together or may be inducing a folding pattern that increases hydrophobic interactions among monomers.

Release of iron from glomalin was suggested by observations made during phenyl column chromatography. When the phenyl column was washed after the glomalin sample was loaded and prior to running the gradient, some red-brown material was washed off as well. This material was not highly proteinaceous but probably contained iron. When hydrophobic groups were brought to the surface by the ammonium sulfate solution for glomalin to bind to the phenyl column, glomalin underwent conformational changes that may have caused some iron to be released.

Because glomalin was purified by both hydrophobic (phenyl column) and hydrophilic (Con-A column) interactions, it was unlikely that there was much contamination in the polypeptide sample that was deglycosylated and examined by NMR spectroscopy (Fig. 7C-B and D). The NMR spectra showed that the protein fraction contained aliphatic peaks and the oligosaccharide fraction (Fig 7C-A and C) contained peaks in the carbohydrate region. Further analysis will be required to identify functional groups.

Glomalin shares many characteristics with hydrophobins (a class of hydrophobic proteins found on the surface of AM fungal hyphae) (Wessels, 1997), transferrins (a class of iron-binding glycoproteins) (Iyer and Lonnerdal, 1993) and humic substances (Hayes and Clapp, 2001). Like all of these molecules, glomalin is structurally complex and its native or original structure may not be the same as the structure after deposition and incubation in soil or, more importantly, after extraction to obtain a soluble form of the molecule. Changes occur as the result of chemical reactions in the soil, such as binding metals or other organic matter and microbial degradation. Chemical reactions occur during extraction, such as hydrolysis and oxidation (Burdon, 2001; Hayes and

Clapp, 2001; Iyer and Lonnerdal, 1993). Glomalin extracted from hyphae typically has a much lower iron concentration than glomalin from soil (Table 7C). The NMR spectra in this study showed that the extraction method tightly binds citrate to glomalin (Fig. 7C). This indicated that glomalin was capable of binding organic substances and may bind organic matter in the soil to assist in aggregate formation. A number of other, as yet unidentified, changes may be occurring in glomalin in the soil and during extraction. In the soil, many of these changes may help in the stability of glomalin and impact its role in soil quality. The methods used in this study demonstrated many of the unique characteristics of glomalin and defined procedures that provide a context for future work.

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Table 7A. Percentage carbohydrates and immunoreactive protein (IRP) in glomalin extracted from the Baltimore site *a* (MD) and Pacolet (GA) soils from Extraction Sequences 1 – glomalin extraction before HA and FA and 2 – glomalin extraction after HA and FA and treated or untreated with 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron) to remove iron.†

Site	Experiment	Tiron-treated	% carbohydrates	IRP (µg)
Baltimore site <i>a</i> (MD)	1	Yes	6.6	0.0295
	2	Yes	5.9	0.0168
	1	No	6.3	0.0221
	2	No	5.6	0.0188
Pacolet (GA)	1	Yes	5.6	0.0169
	2	Yes	2.8	0.0139
	1	No	5.9	0.0192
	2	No	3.2	0.0124

† Detailed descriptions of Extraction Sequences 1 and 2 are in Chapter 3.

Table 7B. Total and immunoreactive protein concentrations of glomalin extracted from soil or mycorrhizal hyphae stripped of iron with 8-hydroxyquinoline and fractionated with centrifugal filter devices that retained 3, 10, 30, and 50 kD molecules.

Sample	Sample ID	Molecular weight fraction	Total protein (µg)	Immunoreactive protein (µg)†
Baltimore soil	1	>3 kD	50.55	ND
		<3 kD	5.21	ND
	2	30-50 kD	27.80	1.10
		3-10 kD	13.87	0.50
	3	>3 kD	174.95	0.00
		30-50 kD	34.99	1.80
Glomus intraradices hyphae	1	>3 kD	186.55	ND
		<3 kD	18.66	ND
	2	30-50 kD	30.93	0.60
		<3 kD	69.00	0.00
Gigaspora rosea hyphae	1	>3 kD	158.80	ND
		<3 kD	14.53	ND
	2	30-50 kD	322.70	0.70
		3-10 kD	22.97	0.04
		>3 kD	48.31	3.80
	<3 kD	218.20	0.00	

† ND = not detected (i.e. below the detection limit)

Table 7C. Percentage iron in of glomalin samples digested in the microwave compared with acid hydrolysis under vacuum and measured by atomic adsorption or the colorimetric assay.

Glomalin extracted from	Soil	Percentage iron		
		Atomic adsorption Microwave digestion	Microwave digestion†	Colorimetric assay Acid hydrolysis†
Gilpin soil (MD)	Bench mark	1.47	0.25	1.27
<i>G. intraradices</i> hyphae	Hyphae	0.48	0.19	NA
<i>Gi. rosea</i> hyphae	Hyphae	0.52	0.23	0.55
Weld (CO)	Field plots	1.09	0.08	2.45
Weld (CO)	Native grass plots	0.49	0.13	1.18
R. Reily (Eastern shore –MD)	Woods	0.23	NA	0.11
R. Reily (Eastern shore –MD)	Field	0.20	NA	0.25
Ed Q (Eastern shore –MD)	Woods	0.59	NA	0.57
Ed Q (Eastern shore –MD)	Field	0.29	NA	0.14

† NA = not available (i.e. analysis was not conducted)

Table 7D. Percentage iron in glomalin extracted before or after treating a soil with HCl. Sodium dithionate extraction is compared to hydrolysis by Aqua Regia.

Values were obtained using atomic adsorption spectroscopy. †‡

Iron Extraction Method	Glomalin Extraction Method	Wymore soil	Pawnee soil	Sampson soil	Haxtun soil
Dithionate	Before	1.85	ND	ND	0.65
	After	NA	ND	NA	0.24
Aqua Regia after Dithionate	Before	3.82	2.06	0.28	0.63
	After	NA	0.04	NA	0.09
Aqua Regia alone	Before	6.00	4.98	0.65	1.72
	After	NA	0.29	0.17	0.27

† NA = not available (i.e. analysis was not conducted)

‡ ND = not detected (i.e. values were below the detection limit)

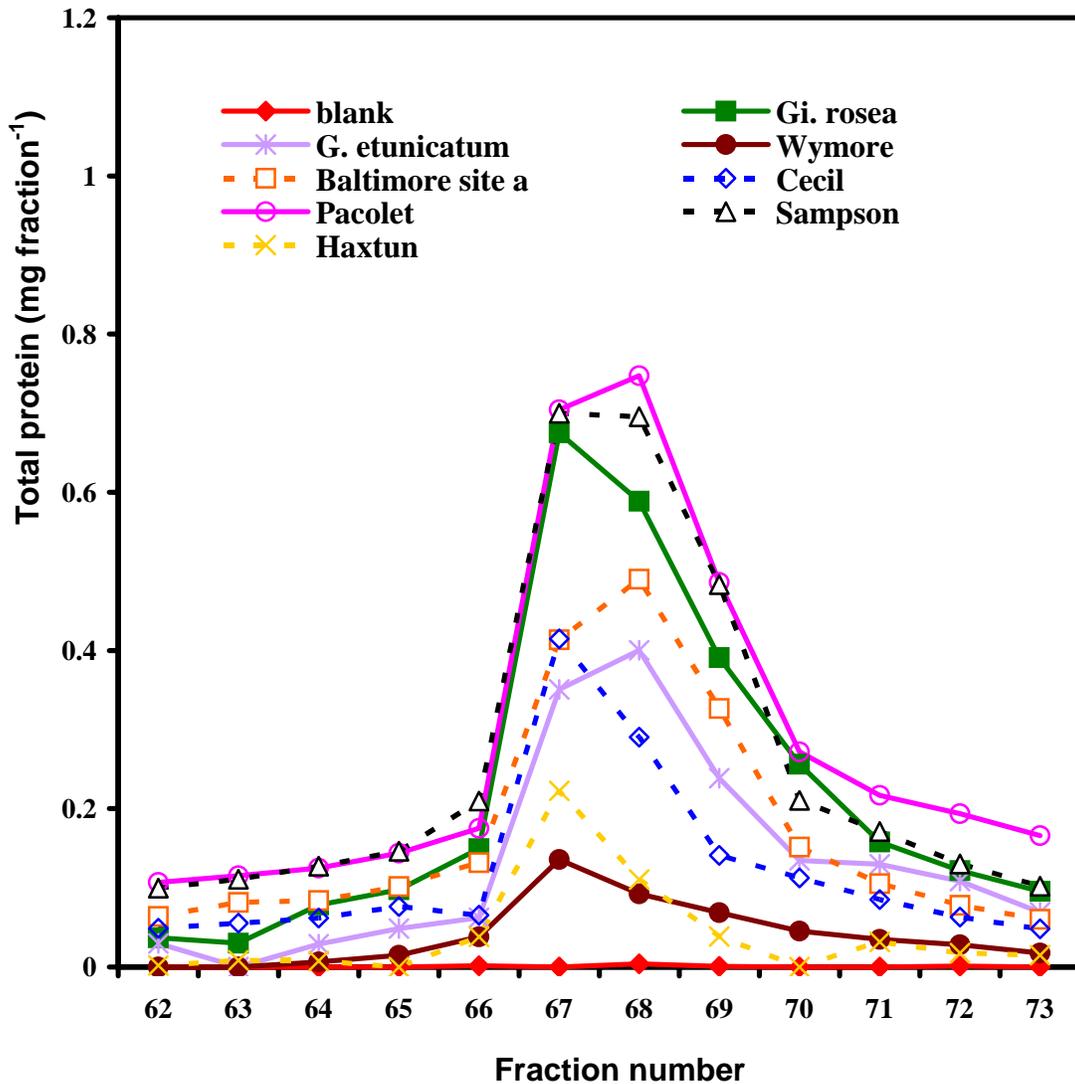


Figure 7A. Total protein values in fractions of glomalin purified on a phenyl column. The gradient of ammonium sulfate solution ended at fraction 60 and had completely evacuated the column at fraction 67.

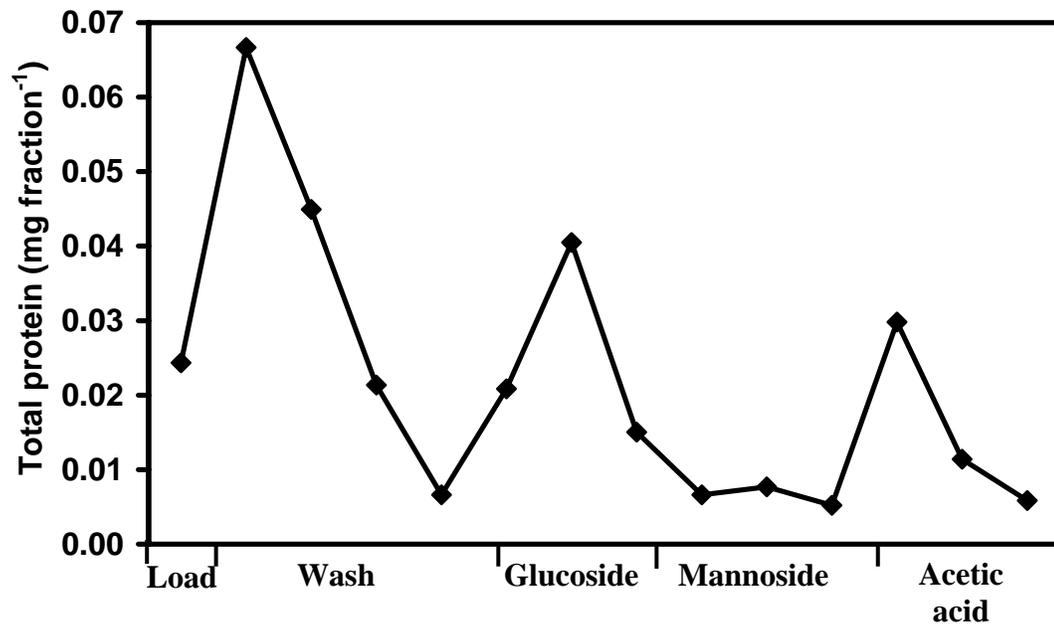


Figure 7B. Total protein values in fractions collected from the Concanavalin-A Sepharose lectin affinity column sequentially eluted with: (1) μ -methyl-D-glucopyranoside in TBS buffer (glucoside), (2) μ -methyl-D-mannopyranoside in TBS buffer (mannoside), and (3) acetic acid. Fractions also were collected when the sample was being loaded onto the column (load) and when the column was washed with TBS buffer solution (wash).

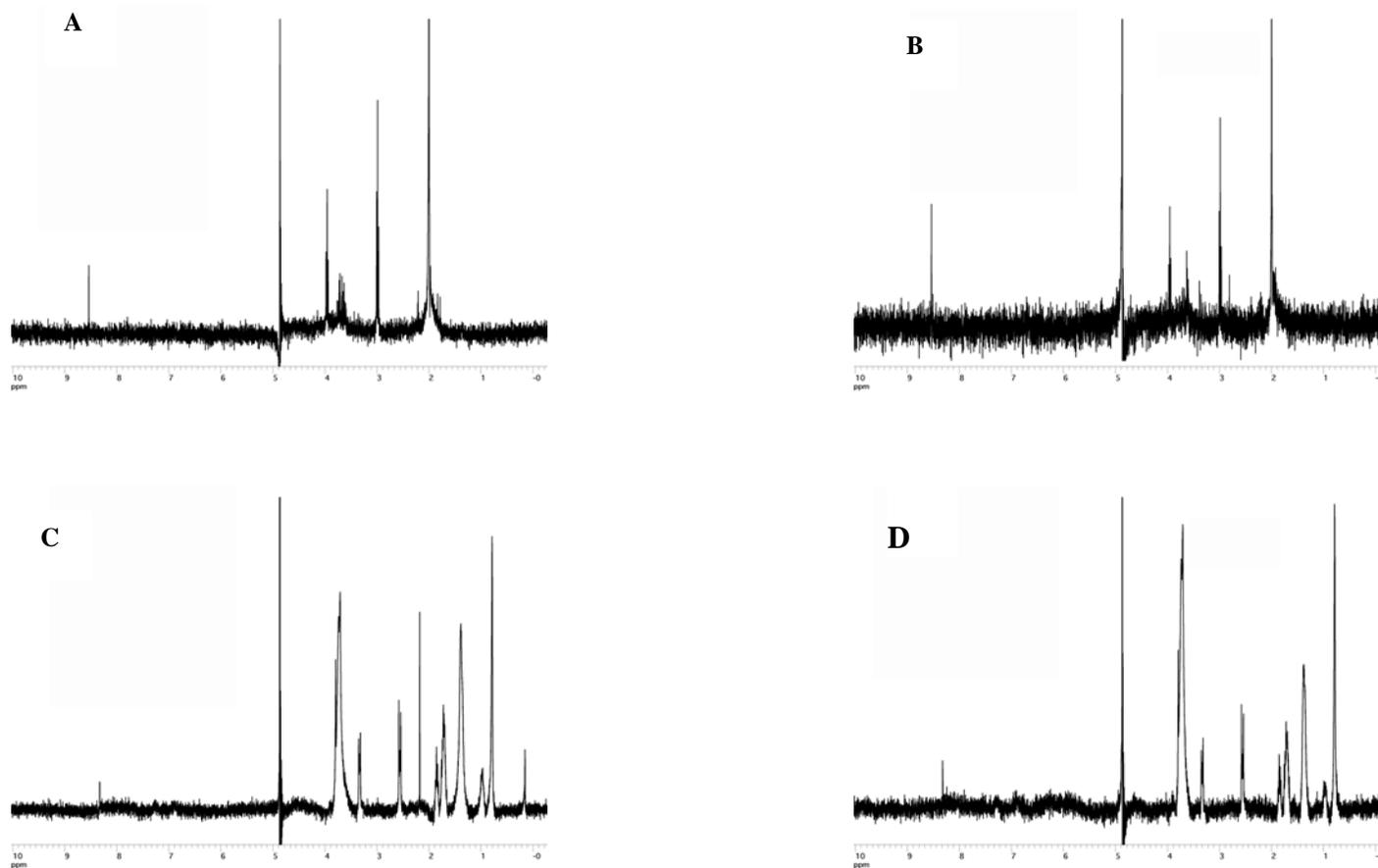


Figure 7C. ^1H NMR spectra of two glomalin samples extracted from hyphae of *Glomus etunicatum* (A and C) and Cecil soil (B and D) that purified using both phenyl and lectin columns, deglycosylated, and separated into oligosaccharide (A and B) and protein (C and D) fractions.

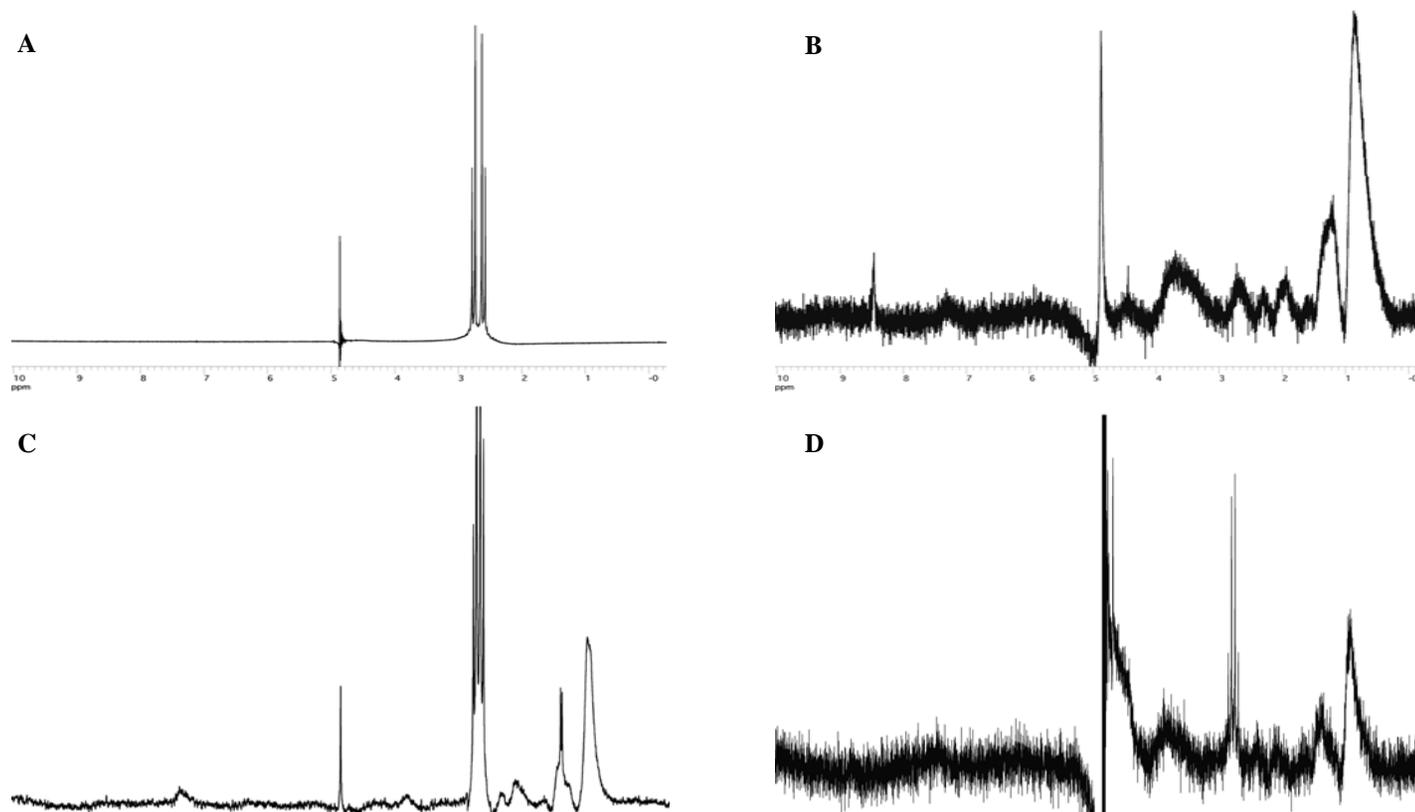


Figure 7D. ^1H NMR spectra of citrate buffer alone (control sample) (A) and glomalin extracted from Sampson series (CO) soil with pyrophosphate (B), citrate (C), or citrate after pyrophosphate extraction of glomalin followed by NaOH treatment to extract humic acid (D).

CHAPTER 8

CHARACTERIZATION OF GLOMALIN

Background

Glomalin is a complex macromolecule produced by arbuscular mycorrhizal (AM) fungi. This molecule is a hydrophobic, iron-binding glycoproteinaceous substance that is a major fraction of organic matter and is important in the long-term structure of soil. It shares many similarities with other biomolecules, such as hydrophobins (Wessels, 1997), transferrins (Iyer and Lonnerdal, 1993) and humic substances (Hayes and Clapp, 2001). Immunofluorescence assays show that glomalin is present on AM fungal structures in single-species pot cultures, while cultures of other soil fungi were not immunoreactive (Wright et al., 1996). Although glomalin is produced only by AM fungi, copious amounts (up to 60 mg g^{-1}) have been measured in the soil (Wright and Upadhyaya, 1996). High glomalin amounts primarily are due to an abundance of hyphae in the soil (lengths equaling $>100 \text{ m cm}^{-3}$) (Miller et al., 1995) and the slow decomposition rate of glomalin (7 to 42 years) (Rillig et al., 2001; Steinberg and Rillig, 2003).

Analysis (i.e. protein and compositional) of glomalin has been performed only on samples extracted at high temperature (121°C) in a buffer solution (typically a chelator). Under these conditions, glomalin may have undergone chemical reactions that changed its composition from its native state. Previous studies showed that the extraction procedure reduces immunoreactivity by inducing conformational changes and non-glomalin substances, such as iron and the citrate extraction buffer, to become 'fixed' to glomalin in such a way that they may not be removed by chemical treatment. Hydrolysis and oxidation reactions are also possible under the extraction conditions.

In addition, when glomalin is extracted, other substances may be co-extracted. Some of these contaminants may be removed during the precipitation and dialysis procedure. However, since gravimetric, total protein (TP) and immunoreactive protein (IRP) weights are not equivalent, it was impossible to prove that all of the material that is referred to as glomalin is actually glomalin. Immunofluorescence assays show that immunoreactive glomalin is present on fungal structures, including hyphae, spores and arbuscules, colonized roots and rhizosphere and mycorrhizosphere particles that are associated exclusively with AM fungi (Wright et al., 1996; Wright, 2000). Immunoreactive glomalin extracted from single-species pot cultures is equivalent to glomalin extracted from soil according to protein banding on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoreactive protein assays, glycoprotein assays, C, N and H concentrations and nuclear magnetic resonance (NMR) spectra (Wright and Upadhyaya, 1996; Wright et al., 1998; Rillig et al, 2001; unpublished data). Research results presented in this dissertation characterized glomalin from ecological (by comparison with other types of organic matter), developmental (pot culture accumulation), and structural (molecular composition) perspectives.

Glomalin – A unique and major pool of soil organic matter

Each extractable organic matter fraction – glomalin, Residual POM, humic acid (HA), and fulvic acid (FA) – is operationally defined by extraction technique and quantified by gravimetric and C weights (Chapters 2 and 3). Protein assay values were used to indicate the presence of glomalin in the extract solution. Only glomalin and HA contained immunoreactive proteinaceous material.

The extract-defined glomalin fraction is a major fraction of soil organic matter (SOM) (Fig. 8A). Only Residual POM was present in amounts that were equivalent to or more than glomalin. The extraction and purification techniques and C, N and H values have shown that glomalin was a different fraction from Residual POM, HA and FA. Percentages of C, N and H in each fraction showed that the HA fraction contained material with a high C concentration while the glomalin fraction had a higher C and/or N concentration than the Residual POM and FA fractions.

Glomalin is either present in the traditional HA extraction or remains unextracted in the soil as part of the humin fraction. [Recalcitrant glomalin (R. glomalin) was extracted from the humin fraction.] The presence of glomalin in the HA extract would account for the proteinaceous and aliphatic contaminants measured in HA by other laboratories (Hayes and Clapp, 2001; MacCarthy, 2001). Recent analysis using ^{15}N NMR and ^{13}C NMR has shown that HA contains high concentrations of amino acids, possibly in the peptide form, and aliphatic groups (hydrocarbon chains) (Simpson, 2001; Hatcher et al., 2001). These proteins and long chain hydrocarbon, fatty acid and ester structures are of plant and microbial origins and are merely associated with true humic molecules (Hayes and Clapp, 2001; MacCarthy, 2001; Simpson, 2001).

In the past, HA has been described as a high molecular weight compound (Schulten and Schnitzer, 1997). Burdon (2001) argues that degradation products from plant and microbial debris (i.e. humic substances) cannot be of higher molecular weight than the original material and cannot contain substances that would not be formed as degradation products. Glomalin binds to Fe (Chapter 3), organic matter (Chapter 7) and

clay minerals. Similar mechanisms may cause glomalin to bind to HA and account for the high molecular weight of HA.

Glomalin in dry sieved aggregates

The role of mycorrhizal fungi in aggregation and thus water penetration appears to be as important as, if not more so than, their role in nutrient adsorption (Bethlenfalvay et al., 1999; Degens, 1997). Hyphae and roots act like a “sticky string bag” that physically trap organic matter and soil particles in aggregates, while biomolecules, like glomalin, bind these aggregates together (Jastrow et al., 1998). Several researchers have identified an amorphous substance on hyphae as a binding agent for clay minerals and organic matter (Gupta and Germida, 1988; Schreiner and Bethlenfalvay, 1995; Tisdall and Oades, 1979). It is likely that this substance is glomalin (Rillig et al., 2002; Wright et al., 1996; Wright and Upadhyaya, 1996; 1998).

Glomalin is resistant to decomposition (Wright et al., 1996; Rillig et al., 2001) and may stabilize aggregates and organic matter in aggregates (Wright and Upadhyaya, 1998; Rillig et al., 1999). Hydrophobic (aliphatic) compounds form a water-insoluble lattice around soil aggregates to help keep these aggregates water-stable (Capriel et al., 1990). For humic substances, aliphatics have been implicated in their ability to stabilize soil aggregates. However, these aliphatics are not part of the humic molecule but are co-extracted with HA (Burdon, 2001; Hayes and Clapp, 2001). Many types of fungi produce hydrophobins – proteins with spans of hydrophobic (aliphatic) amino acids – that protect hyphae from changes in moisture and help hyphae adhere to surfaces (Wessels, 1997). Glomalin may be the hydrophobin equivalent for AM fungi that not only protect fungal hyphae but also helps to form and stabilize aggregates.

Plant-derived particulate matter, glomalin, HA and FA were extracted from dry sieved aggregates in three aggregate size classes (1-2, 0.5-1, and 0.25-0.5 mm).

Research herein showed that the labile and intermediate turnover fractions (Residual POM and glomalin) were present in high concentrations in the 1-2 mm aggregates but declined with aggregate size. On the other hand, the recalcitrant fractions (HA and R. glomalin) increased with decreasing aggregate size. Glomalin also was related to aggregate stability and to persistent binding agents, such as iron and clay minerals.

Glomalin in managed agricultural systems

Increased mineralization, organic matter content and, as a result, aggregation are reduced in agroecosystems. Below 1%, organic matter causes a rapid decline in aggregate stability (Kemper and Koch, 1966). Above 2%, organic matter may be less important in the binding and stability of aggregates (Kemper and Koch, 1966; Tisdall and Oades, 1982). Under high organic matter contents, certain fractions, such as the recalcitrant glomalin, HA and humin fractions, and soil clay and iron content may help to form aggregates.

Conventional agroecosystems are generally more productive but far less diverse than natural systems and are far from self-sustaining (Gliessman, 2001). Their productivity can be maintained only with large additional inputs of energy and material from external, human sources. When converting to sustainable practices, a range of processes and relationships are transformed, beginning with aspects of basic soil structure, organic matter content, and diversity and activity of soil biota (Gliessman, 2001). Therefore, changes in the physical and biological components of agroecosystems

may be difficult to assess over the short term. Long-term study sites, such as the Farming Systems Project site (Chapter 5) will be used to measure these changes.

At the Farming Systems Project site, systems varied by tillage (no-, conventional, or minimum till), crop rotation length (2, 3 or 4 y), and amendments (raw or composted at 1X or 2X concentrations). Aggregate stability was measured using a method that corrects for problems with both the slaking and capillary re-wetting methods (i.e. the Normalized Stability Index) (Six et al., 2000). In all nine systems, the labile and intermediate turnover rate fractions (Residual POM and glomalin) were extracted over the recalcitrant fractions (HA and R. glomalin), because labile fractions are more apt to show changes between management systems in a shorter period of time. Trends in data indicated differences in aggregation and the labile POM and glomalin fractions with the conventional system having lower values. The data presented here was collected after only 3 y of management and will be used as baseline for future comparisons.

Glomalin accumulation in a pot culture system

A soilless, single-species pot culture system was used measure glomalin accumulation at 14-week intervals in a 294-day experiment. Protein concentrations (measured by the total and immunoreactive protein assays) showed that glomalin production was primarily influenced by irradiance and, therefore, photosynthetic C production. Arbuscular mycorrhizal fungi are obligate biotrophs that require photosynthetic carbon to grow. Distribution of carbon by AM fungi to fungal structures (i.e. hyphae or arbuscules) and production of biomolecules (such as glomalin) are not

very well understood. Glomalin production and accumulation levels and the processes controlling them may impact aggregation, soil fertility and carbon sequestration.

Composition of the glomalin molecule

Methods were developed to analyze the composition of the glomalin molecule, including removing iron, measuring iron concentration, purifying by column chromatography, and separating amino acids and carbohydrates. Results showed that iron and organic matter might bind to glomalin in the soil and/or during glomalin extraction. The stability and complexity of the glomalin molecule increases with iron and organic matter binding, which makes it difficult to analyze glomalin structure. This study has identified a number of methods that will be useful in future studies to measure types and concentrations of functional groups in glomalin and relate them to aggregate stability.

Summary and conclusions

Organic matter within aggregates, even the labile POM, has a slower decomposition rate than free organic matter (Six et al., 2001). Typically, the majority of surface and root-derived residues are respired by microbes in a matter of a few years, with almost all residue carbon lost in two decades (Gale and Cambardella, 2000; Six et al., 2001). Only a very small fraction of POM remains for centuries or millennia as humified matter. To increase the concentration of SOM, improve soil quality, and sequester C, labile organic matter must be stabilized increasing the amount of organic matter with an intermediate turnover rate. Carbon sequestration in the soil may lower atmospheric carbon concentrations and mitigate the Greenhouse Effect. Rising CO₂ levels increase belowground carbon allocation to plant roots and arbuscular mycorrhizal

hyphal length (Rillig et al., 1999). When hyphal length increases and more carbon is allocated belowground, glomalin production is favored (Rillig et al., 1999).

Although HA contains 24% more C than glomalin, glomalin is ca. 50% of the extracted organic C (Chapter 2). Humic acid has a turnover time of centuries to millennia but only ca. 8% of the total soil carbon (Fig. 8A). Whereas glomalin, which is about 20% of the total carbon, has mean residence time of 7 to 42 yr with certain fractions potentially having a century turnover time (Fig. 8A) (Steinberg and Rillig, 2003; Rillig et al., 2001). Data herein showed that R. glomalin might be the fraction of glomalin with a century turnover time.

Results from these studies demonstrated that glomalin: (1) is a large and important component of SOM, (2) may help to form and stabilize soil aggregates along with persistent binding agents such as clay minerals and iron, (3) may indicate changes in organic matter concentrations and aggregate stability with differences in management, (4) may increase under conditions of high irradiance and photosynthetic activity, and (5) has a complex structure with tightly-bound iron and organic matter along with amino acid and carbohydrate groups. Future work will use this information to better understand the structure of the glomalin molecule and its roles in C sequestration and soil health.

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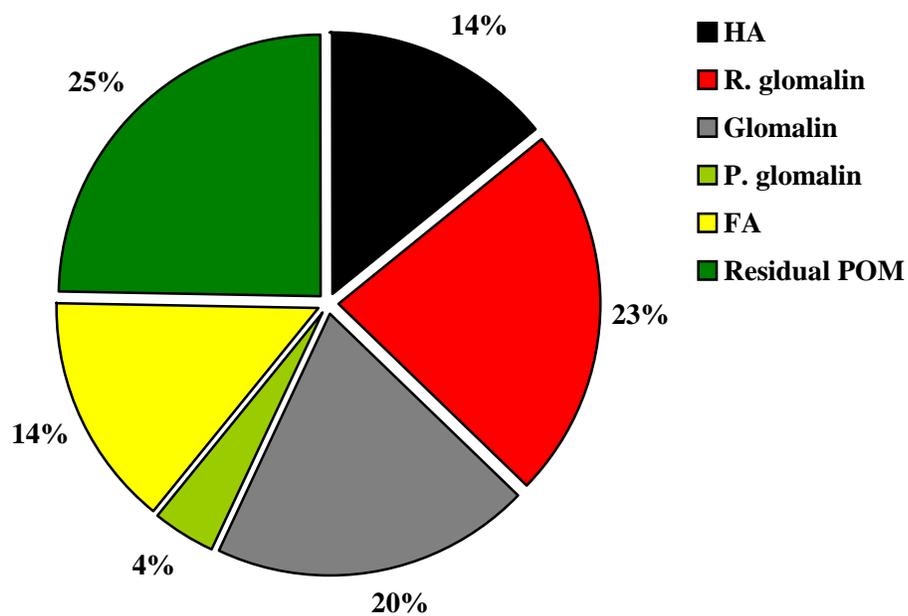


Figure 8A. Distribution of organic C in six organic matter fractions – humic acid (HA), recalcitrant glomalin (R. glomalin), glomalin, glomalin extracted from the particulate organic matter fraction (POM) (P. glomalin), fulvic acid (FA), and Residual POM – extracted from 6 soils (two each from Maryland, Georgia and Colorado). Data from Chapters 2 and 3.

APPENDICES

Appendix A. Detailed methods for organic matter extraction

Appendix A1. Physical separation of POM

Method

1. Weigh out five 2-g samples (10 g total) into autoclavable centrifuge tubes.
2. Add 10 mL of 12% (w/v) NaCl to each tube.
3. Vortex briefly. Let settle at RT for 30 min.
4. Decant carefully over a 53 μm screen. Collect organic debris from each of the five tubes in one soil to have a bulk POM fraction from each soil. Keep the soil in each tube separate.
5. Add 5 mL of 12% NaCl to each tube.
6. Repeat steps 3-5 for a total of 5 extractions.
7. Fill tube about 1/4 full with distilled water.
8. Centrifuge at $6850 \times g$ for 10 min and decant rapidly over screen.
9. If more POM is visible in the sample, use forceps to remove or repeat steps 7-8.
10. Rinse POM collected on screen thoroughly with distilled water to remove salt.
11. Rinse POM off screen into a pre-weighed weight boat.
12. Dry material in weigh boats on slide warmer at 70°C and record dry weight.
13. If the remaining soil will be used for additional analyses, wash the soil repeatedly using distilled water and centrifugation. Next, wash the soil into weigh boats, dry at 70°C and weigh.

To extract other fractions of organic matter (i.e. glomalin, HA and FA) from POM or soil minus POM:

1. Carefully, rinse or scrape material from weigh boats into autoclavable centrifuge tubes with a minimal amount of water.
2. Use centrifugation, if necessary, to remove the wash water. Excess water will dilute extraction solutions.
3. Extract glomalin or HA and FA using the appropriate procedure – Appendices A2 and A3 respectively.

Appendix A2. Chemical extraction of glomalin

Method

1. Add 8 mL of 50 mM citrate, pH 8.0, to each sample.
2. Autoclave for 1 hr.
3. Centrifuge at $6850 \times g$ for 10 min and decant.
4. Repeat steps 1-3 until solution color lightens (i.e. straw-colored), combine all repeated extracts but keep extracts from each sample separate.
5. Centrifuge at $6850 \times g$ for 10 min, measure volume and transfer to a new tube.
6. Remove a 1 mL sample for protein assays and set aside.
7. Precipitate glomalin from the extract with a minimum volume of 1 N HCl by drop wise addition until the pH is 2.5. Keep the solution on ice for 45 min to 1 h.
8. Centrifuge at $6850 \times g$ and dispose of supernatant.
9. Quickly re-dissolve the precipitate with a minimum volume of 0.1 N NaOH.
10. Place the solution in dialysis tubing with MWCO = 8,000 to 12,000 D.
11. Dialyze against de-ionized water with at least five, and up to ten, changes of water (depending on the number and size of samples and size of dialysis container).
12. Transfer the dialyzed sample to a centrifuge tube and centrifuge at $6850 \times g$ for 10 min.
13. Carefully decant the supernatant, freeze dry and weigh freeze-dried material.

Appendix A3. Chemical Extraction of Humic and Fulvic acid

Method

1. Equilibrate sample to pH 1-2 with 1 mL 1 *N* HCl at room temp.
2. Add 10 mL 0.1 *N* HCl .
3. Shake 1 h.
4. Centrifuge at $6850 \times g$ for 10 min, decant and save supernatant as part of fulvic acid fraction.
5. Neutralize the precipitate with 1 mL 1 *N* NaOH under N_2 .
6. Add 10 mL 0.1 *N* NaOH under N_2 .
7. Extract for 4 h with intermittent shaking under N_2 .
8. Allow to settle overnight under N_2 .
9. Centrifuge at $6850 \times g$ for 10 min and collect supernatant as part of fulvic acid fraction.
10. Repeat steps 5-9, and continue adding the supernatant to acidified (fulvic acid) supernatant.
11. Acidify supernatant with 6 *N* HCl to pH 1.0.
12. Allow to stand for 12-16 h.
13. Centrifuge at $6850 \times g$ for 10 min and separate humic acid (precip) and fulvic acid (supernatant).
14. Add fulvic acid supernatant to supernatant collected at step 1.

To purify the humic fraction:

1. Re-dissolve the precipitate in a minimum volume (~5 mL) of 0.1 *N* KOH under N_2 .
2. Add solid KCl to attain a concentration of 3 M [K⁺].

3. Centrifuge at $10844 \times g$ for 10 min to remove suspended solids.
4. Re-precipitate with 6 N HCl pH 1.0.
5. Allow to stand for 12-16 h.
6. Centrifuge at $6850 \times g$ for 10 min and dispose of supernatant.
7. Suspend the precipitate in ~ 5 mL (depending on amount of material) 0.1 N HCl/0.3 N HF.
8. Shake overnight at room temp.
9. Centrifuge at $6850 \times g$ for 10 min and carefully decant.
10. Repeat steps 7-9 twice.
11. Add de-ionized water to rinse away acid, centrifuge $10844 \times g$ for 3 min and decant and discard the supernatant.
12. Re-suspend the precipitate in ~ 5 mL 0.1M KOH.
13. Remove a 0.5-1 mL sample for protein assays and set aside.
14. Precipitate with 6 N HCl.
15. Let settle overnight, centrifuge and decant carefully and discard the supernatant.
16. Add de-ionized water, centrifuge and decant carefully and discard the supernatant.
17. Freeze-dry and weigh freeze-dried material.

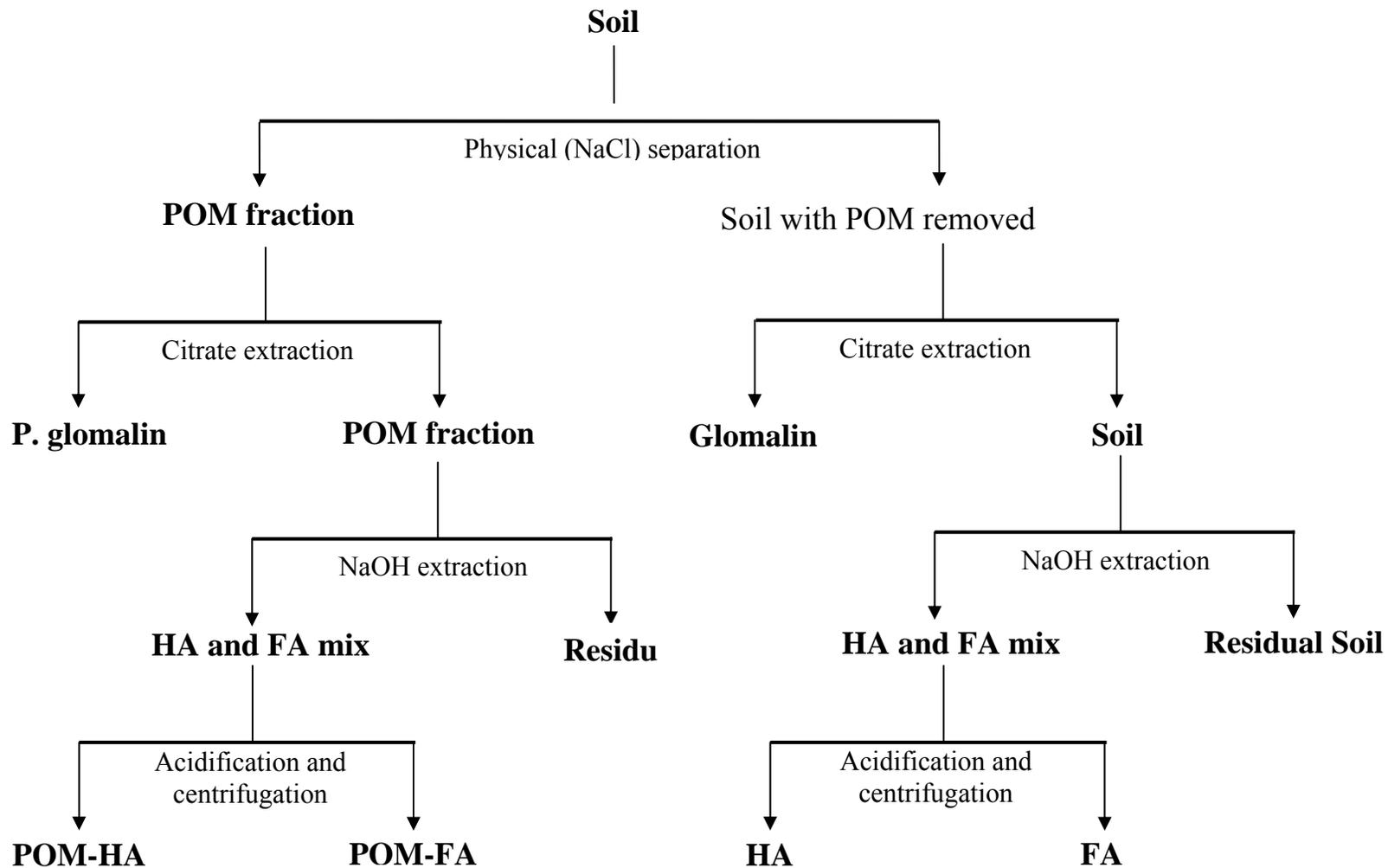
To purify fulvic acid:

1. Remove 1 mL from composited supernatants and set aside for further analysis.
2. Transfer the remaining supernatant to dialysis tubing (MWCO = 8,000 to 12,000 D) and dialyze against de-ionized water. This pore size dialysis tubing does not retain

all of the low molecular weight FAs but was used in this work to determine whether glomalin was co-extracted with FA.

3. When pH is neutral, freeze dry.
4. Weigh freeze-dried material.

Appendix B. Diagram of the sequential extraction of organic matter from six U.S. soils



Appendix C. Protein measurements

C1. Bradford total protein assay

Introduction

This procedure is used to measure protein concentration in samples that were extracted for glomalin, after either the total or easily extractable extraction procedure. This assay does not give the most accurate glomalin concentration, because it is not specific for glomalin, but it will measure any protein that has survived the extraction procedure at a concentration greater than 1.25 µg/100 µl sample. Bradford protein values are usually higher than the values calculated from the ELISA procedure, but values from this assay determine the dilution factor needed to perform the ELISA procedure and are used to compare samples based on the percentage of total protein that is immunoreactive.

Methods

1. Prepare standard curve, using BSA (bovine serum albumin) as outlined below.
2. Add 200 µl of PBS (phosphate buffered saline) minus the volume of extract to each well in a 96 well plate. (For example, if you put 5 µl of sample in the well, you will need 195 µl of PBS.) Extract volume will typically be 10µl, 5µl, or 2µl depending on color. (Follow color chart to determine amount of sample to add.)
3. Start 5 min on timer. Carefully add 50 µl of Bio-Rad (Hercules, CA) protein dye reagent to each well. **Mix quickly and well with a pipette.**
4. Pop bubbles with needle (clean needle between samples) and read at 595 nm after 5 min has expired. The Bradford reagent is acidic and glomalin will eventually precipitate. If a precipitate forms within 5 min, use less sample.

5. Use BSA standard curve to calculate g protein kg⁻¹ material extracted.

BSA standard curve preparation

1. Make 1 mL stock solutions of 5 µg BSA/200µl PBS (25 µg/1 mL) and freeze, until needed.
2. Thaw and dilute with PBS as outlined below:

Well designation	µg/well	BSA stock solution(µl)	PBS (µl)
Blank	0	0	200
Standard 1	0	0	200
Standard 2	1.25	50	150
Standard 3	2.5	100	100
Standard 4	3.75	150	50
Standard 5	5	200	0

Bradford color chart

The color of the extract can help you determine the right amount of sample to use for the reading to be somewhere within the values of the standard curve.

Sample color	µl sample/well
Golden	50+
Golden-brown	25-50
Brown	10-25
Reddish brown	5-10
Reddish black	1-5

C2. Indirect ELISA with biotinylated secondary antibody

Introduction

This assay allows you to determine the concentration of glomalin reactive with a monoclonal antibody. It uses an indirect assay that employs a biotinylated secondary antibody with a long spacer arm that apparently overcomes steric hindrance in the reaction between the antigen, the monoclonal antibody and the secondary antibody. Enzyme-linked streptavidin is the third reagent, and the reaction between biotin protruding from the site of reaction with the monoclonal antibody and streptavidin is covalent. This procedure allows detection of low concentrations of immunoreactive glomalin due to amplification of the signal. It is important to note that U-shaped polyvinyl chloride microtiter plates should be used. The shape of the well and type of plastic make a difference in the attachment of the antigen. The amount of sample needed is determined by using the Bradford total protein assay and adding an amount equal to 0.02 μg protein/well.

Methods

1. Add sample (amount determined from Bradford total protein assay to equal 0.02 μg per well) plus enough PBS to equal a total of 50 μl or 50 μl of pre-diluted sample to a well (Dynex 96 well microtiter plate). Prepare standard curve using glomalin from a highly immunoreactive soil as outlined below.
2. Let it dry over night (make sure that it is completely dry).
3. Add 250 μl /well of freshly prepared 2% non-fat milk to wells and incubate on shaker for 15 min. Flip plate into sink to remove milk and blot (via inverting and hard taps) on an absorbent paper towel.

4. Add 50 μl /well of diluted MAb 32B11 antibody (monoclonal antibody against glomalin) and incubate on shaker for 1 hr. Flip plate to remove and blot with paper towel. Wash with PBST (PBS with 0.2 mL/L Tween 20) 3x, blotting between washes.
5. Add 50 μl /well of biotinylated IgM antibody, diluted in 1% BSA, and incubate on shaker for 1 hour. Flip plate to remove and blot with paper towel. Wash with PBST 3x, blotting between washes.
6. Dilute the ExtrAvidin® Alkaline Phosphatase (Sigma Aldrich, St. Louis, MO) in 1% BSA and add 50 μl of the diluted solution to each well. Incubate for 1 hr.
7. After incubation, flip plate to remove and blot with paper towel. Wash with PBST 3x, blotting between washes.
8. The fourth wash should be done with TBST [Tris Buffered Saline (250 mM NaCl, 10 mM Tris(hydroxymethyl)aminomethane, and 0.2 mL/L of Tween 20)] at pH 7.4, because PBST will react with the phosphatase enzyme.
9. Dissolve one tablet of Sigma 104® Phosphatase Substrate (disodium 4-nitrophenyl phosphate hexahydrate) (Sigma Aldrich, St. Louis, MO) (5mg tablets) in 5 mL of DEA buffer (Mix 97 mL of diethanolamine buffer, 10%, with 1L of 0.01% MgCl_2 solution, and adjust the pH to 9.8 with 1 N HCl. The solution must be kept sterile and stored covered at room temperature).
10. Add 50 μl to each well and incubate for 30 min. Read plate at 405 or 410 nm.
11. Use standard curve to calculate g protein kg^{-1} material extracted.

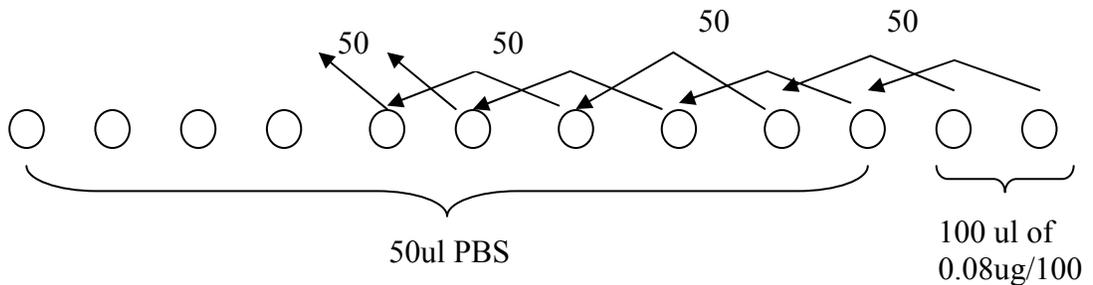
Standard curve preparation – Serial dilution

1. Put 100 μl of the 0.08 μg protein/100 μl of PBS in 2 of the wells and 50 μl PBS in the other 10 wells.
2. Transfer 50 μl of the 0.08 μg sample to a neighboring well that has 50 μl PBS.
3. Mix 3-4 times with the micropipet by pulling the sample up and down.
4. Remove 50 μl from these 2 wells and transfer to 2 neighboring wells. Mix 3-4x.
5. Repeat step 4 for a 3rd dilution.
6. After the 3rd dilution, remove 50 μl from the 2 wells that have 100 μl and dispose of it.

Wells will have the concentrations as outlined below:

Well #	Protein concentration ($\mu\text{g}/50 \mu\text{l}$)
1	0
2	0
3	0
4	0
5	0.005
6	0.005
7	0.01
8	0.01
9	0.02
10	0.02
11	0.04
12	0.04

Serial dilution on ELISA plate:



Appendix D. Immunoreactive protein (g protein kg⁻¹ soil) in glomalin extracted from the particulate organic matter (POM) fraction (P. glomalin) and glomalin and humic acid (HA) extracted from soil minus POM. †‡

Fraction	Baltimore a	Baltimore b	Sampson	Haxtun	Pacolet§	Cecil§	Mean¶
P. glomalin	0.01b	0.01b	0.32b	0.11b	0.04b	0.09ab	0.10±0.05b
Glomalin	0.75±0.05a	0.57±0.03a	0.76±0.07a	0.28±0.01a	0.30±0.00a	0.15±0.01a	0.47±0.11a
HA	0.00±0.00b	0.01±0.00b	0.18±0.00b	0.01±0.00c	0.04±NDb	0.03±NDb	0.04±0.03b
P	0.0001	<0.0001	0.0042	0.0001	<0.0001	0.0367	<0.0001

† Significant differences (P=0.05) were determined according to REML.

‡ Mean ± SE were determined for fractions extracted from soil but not for fractions extracted from POM that was combined before extraction.

§ ND = not determined.

¶ Mean ± SE for all six soils.

Appendix E. Gravimetric weights (g kg⁻¹ soil) of organic matter fractions – glomalin, humic acid (HA) and fulvic acid (FA) – extracted from soil and particulate organic matter (POM) and of soil and POM before (Initial) and after (Residual) extraction for six U.S. soils.

Fraction		Baltimore a	Baltimore b	Sampson	Haxtun	Pacolet	Cecil	Mean†
	Soil – Initial	990.59	993.90	982.70	994.37	982.66	976.39	984.47±5.27
POM fraction	POM – Initial	9.41	6.10	17.30	5.63	17.34	23.61	15.53±5.27
	P. glomalin§	0.70	0.05	1.89	0.68	1.45	2.24	1.46±0.45b
	HA	0.01	0.00	0.13	0.00	0.36	0.32	0.23±0.11
	FA	0.00	0.00	0.09	0.00	0.07	0.00	0.02±0.02
	Residual POM	3.15	2.63	16.65	3.49	6.65	7.05	5.73±1.13a
Soil minus POM	Glomalin	17.76	9.13	5.54	6.28	7.40	6.38	6.68±0.36a
	HA	1.62	0.80	0.54	0.17	2.41	0.38	0.99±0.72b
	FA	0.76	0.85	1.94	0.30	1.02	0.29	0.53±0.24b
	Residual soil	929.30	934.72	943.10	971.32	902.42	937.30	937.01±19.89

† Significant differences ($P < 0.0001$) were determined for the five major extractable organic matter fractions according to REML.

‡ Mean ± SE for all six soils.

§ P. glomalin = glomalin extracted from the POM fraction

Appendix F. Carbon weights[†] (g kg⁻¹ soil) of organic matter fractions – glomalin, humic acid (HA) and fulvic acid (FA) – extracted from soil and particulate organic matter (POM) and of soil and POM before and after extraction for six U.S. soils.^{‡§}

	Fraction	Baltimore a	Baltimore b	Sampson	Haxtun	Pacolet	Cecil	Mean[¶]
	Soil – Initial	29.12	21.57	15.90	7.76	33.51	15.13	20.50±3.90
	POM – Initial	29.22	21.07	24.79	21.93	18.06	23.63	23.12±1.54
POM fraction	P. glomalin[‡]	0.26	0.02	0.73	0.24	0.61	1.01	0.48±0.15c
	HA	NA	NA	0.06	NA	0.18	NA	0.12±0.06
	FA	NA	NA	0.04	NA	0.03	NA	0.03±0.00
	Residual POM	1.27	0.87	3.24	0.63	2.03	1.93	1.66±0.39b
	Soil minus POM	Glomalin	5.27	2.68	2.32	1.95	2.87	2.33
	HA	0.88	0.43	0.28	0.00	1.28	0.20	0.51±0.20c
	FA	0.20	0.22	0.26	0.09	0.36	0.08	0.20±0.04c
	Residual soil	13.75	11.93	10.75	3.01	18.68	10.31	11.41±2.09

[†] Carbon weight = gravimetric weight x (percentage C ÷ 100)

[‡] Significant differences (P < 0.0001) were determined for the five major extractable organic matter fractions according to REML.

[§] NA = not enough material available to assay

[¶] Mean ± SE for all six soils.

[‡] P. glomalin = glomalin extracted from the POM fraction

Appendix G. Extraction of glomalin from hyphae with different buffer solutions

Introduction

Glomalin is typically extracted from soil and hyphae using a citrate buffer solution at high heat (Wright et al., 1996; Wright and Upadhyaya, 1996). Analysis with ^1H NMR (Chapter 7) showed that when extracted with citrate, the high temperature conditions caused some citrate to bind or ‘fuse’ to glomalin such that it could not be removed even after treatment with a strong acid (HCl) or a strong base (NaOH), such as were used in the purification procedures (Appendix A2). Other organic solutions had been used to extract glomalin, such as malate and citrate at different concentrations, pH levels, temperatures, and times, but 50 mM citrate at pH 8.0 extracted the most glomalin (Wright and Upadhyaya, 1996). This experiment was done to determine if glomalin could be extracted in other buffered solutions (such as borate, Tris and pyrophosphate).

Materials and methods

Four arbuscular mycorrhizal (AM) species – *Gigaspora (Gi.) rosea* (FL224), *Acaulospora (A.) morrowiae* (CL551), *Glomus (G.) etunicatum* (BR220), and *G. intraradices* (WV964)] were grown on corn (*Zea mays*) in sterile, single-species pot cultures. After 14 weeks, pots were harvested: (1) shoots were removed and disposed of, (2) nylon mesh bag was removed, and (3) sand outside of nylon mesh bag was rinsed with water to separate hyphae. (Complete details of the pot culturing and harvesting procedures are in Chapter 6.)

Hyphae for each species were divided into four different tubes. Glomalin was extracted from hyphae using one of four buffer solutions: Tris (Trizma® base, 2-Amino-2-(hydroxymethyl)-1,3-propanediol), sodium citrate, sodium borate, and sodium

pyrophosphate. Extractions were made at 121°C in 100 mM solutions, pH 9.0, for 1 hr. Extract solution was collected after centrifugation at $10844 \times g$ for 3 min. Hyphae was rinsed into weigh boats, dried at 70°C and weighed. Glomalin concentration (mg g^{-1} hyphae) was measured in each extract solution using total and immunoreactive protein assays. (For detailed descriptions of the protein assays, see Appendix C.)

Results and Discussion

The buffers were chosen for the following reasons: (1) citrate was the positive control, (2) Tris is frequently used in protein assays, (3) borate is a common buffer (frequently used in dialysis of proteins) and is relatively inert, and (4) pyrophosphate has been used to extract humic substances from the soil (Clapp and Hayes, 1999; Stevenson, 1994).

Glomalin was successfully extracted from hyphae using each of the extraction buffers (Fig. E1). The Tris buffer extracted the least amount of glomalin, while the pyrophosphate and borate extracted the most. Borate was the most inert buffer and would not contribute an organic contaminant to the glomalin fraction. Pyrophosphate may not add more organic material to the glomalin fraction, but with the amount of iron that is typically found in glomalin (0.2 to 6.0%) (Chapter 7) some phosphate may bind to glomalin.

In soil, sodium pyrophosphate releases humic material from organo-mineral complexes (where Fe- and Al-(hydr)oxides bridge organic matter and clay minerals together) with divalent cations via isomorphic substitution (a monovalent cation causes these complexes to dissociate) (Clapp and Hayes, 1999). Glomalin, which is similar to humic material and may form organo-mineral complexes in the soil and may be

solubilized by the same reactions as humic materials. Pyrophosphate is also a metal chelator, like citrate, and may solubilize glomalin by binding to iron in glomalin. Since iron concentrations in glomalin from hyphae are lower than glomalin from soil (0.2% compared 4.1%), glomalin was not expected to be bound in complexes and pyrophosphate was not expected to extract more glomalin than citrate (as was seen for three of the four species).

Two reasons why pyrophosphate may have extracted more glomalin from hyphae than citrate are: (1) sodium in solution binds to glomalin at the normal binding sites for iron and the concentration of the sodium ion in pyrophosphate is higher than in citrate (35% compared to 23%) or (2) pyrophosphate (i.e. phosphate) may be interacting with glomalin in some unknown manner that increases solubility. Since one of the functions of AM fungi is improved phosphorus nutrition (Bolan, 1991), the interaction with pyrophosphate may indicate a mechanism for the involvement of glomalin in P-acquisition. The sodium ion concentration alone does not result in improved extraction of glomalin because borate, which also had 23% sodium, extracted about the same amount of glomalin as pyrophosphate for only two of the fungal species (*A. morrowiae* and *Gi. rosea*).

The data does not show any definitive pattern except that pyrophosphate extracted more glomalin as measured by the total protein assay. When the immunoreactive protein concentrations are examined, it becomes even more difficult to establish a pattern. It is highly probably that there are variations in the structure of glomalin produced by different species or genera that influence the ability to extract glomalin and/or to retain immunoreactivity.

Another problem with the immunoreactivity data was that the concentration of immunoreactive protein in glomalin extracted from hyphae of *G. intraradices* by both Tris and citrate was much higher than the total protein concentration. Frequently, it has been noted that immunoreactive protein concentrations are less than 100 percent of the total protein concentrations. (See Chapters 1 and 2 for a discussion on how conformational changes may interfere with immunoreactivity.) Values of over 100 percent may have occurred because: (1) the sensitivity of the standard curve for the total protein assay (0 to 5 μg) is more than 100 times less than that for the immunoreactive protein assay (0 to 0.04 μg), (2) the concentrations, especially for immunoreactive protein, being measured are so small that a difference of 0.01 $\mu\text{g } \mu\text{l}^{-1}$ in the assay would be equivalent to 5 μg in a 500 μl sample, and (3) there may be more than one epitope (i.e. binding site) for the antibody, but it is unlikely that multiple molecular configurations react with a monoclonal antibody.

Despite these questions, this experiment showed that inorganic buffers extract glomalin. A further study was conducted in our laboratory to extract glomalin from soil using these same buffer solutions. Carbon values for glomalin extracted from soil were not significantly higher in glomalin extracted with citrate. This indicated that only a small amount of citrate binds to glomalin. Therefore, citrate was not a major contributor to the gravimetric or carbon weights of glomalin.

References

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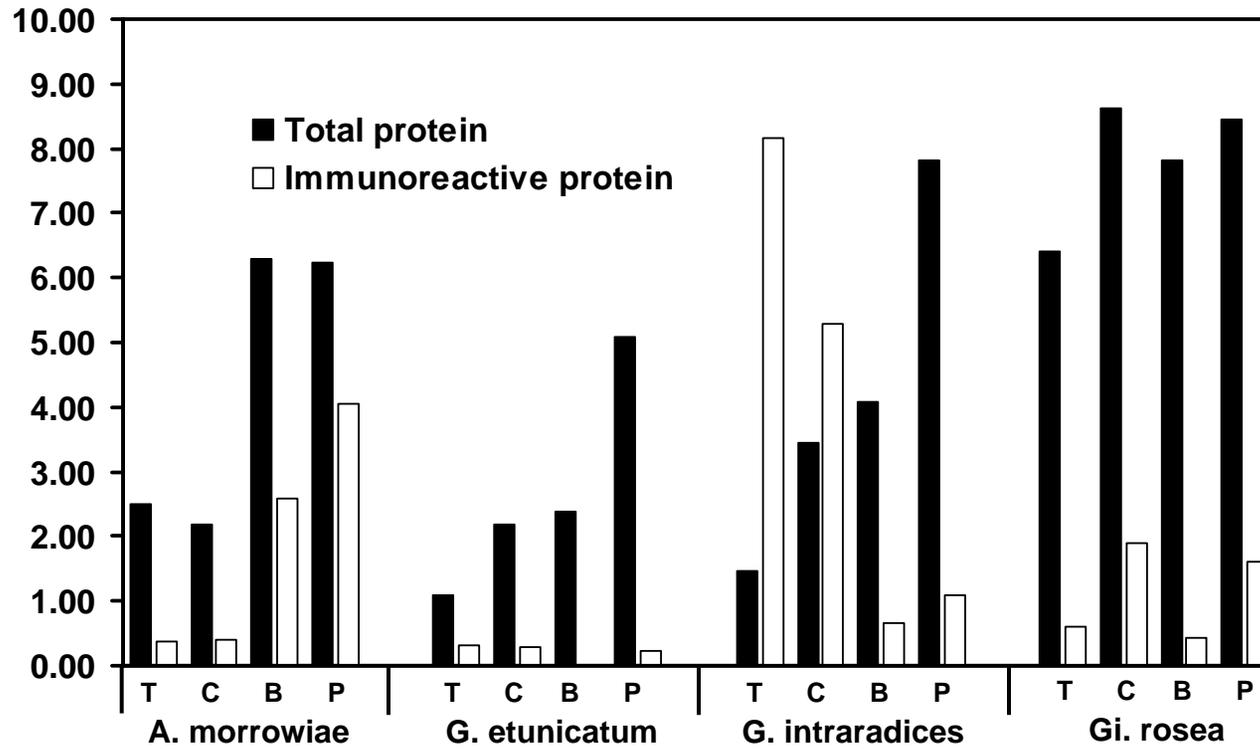
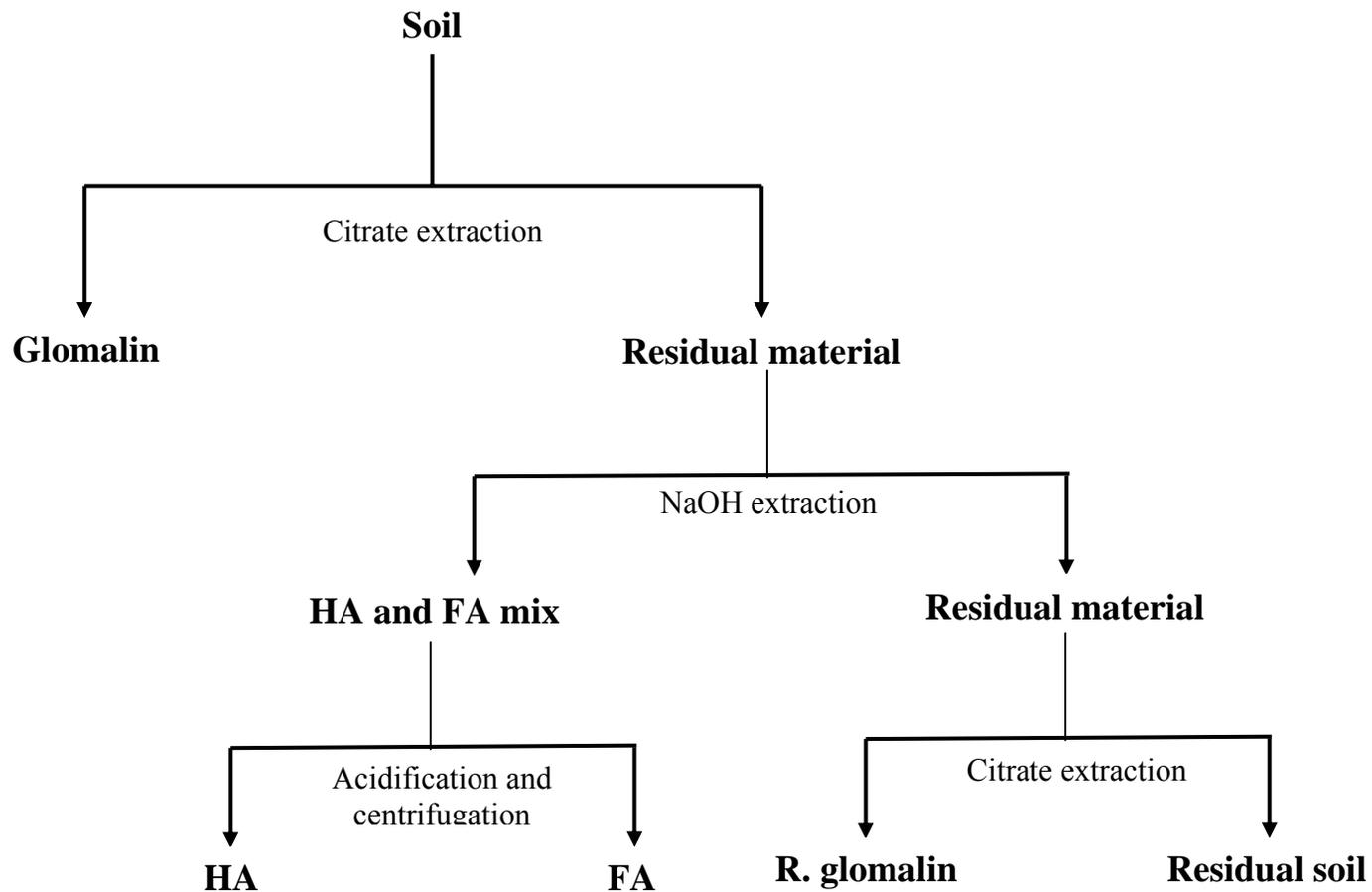


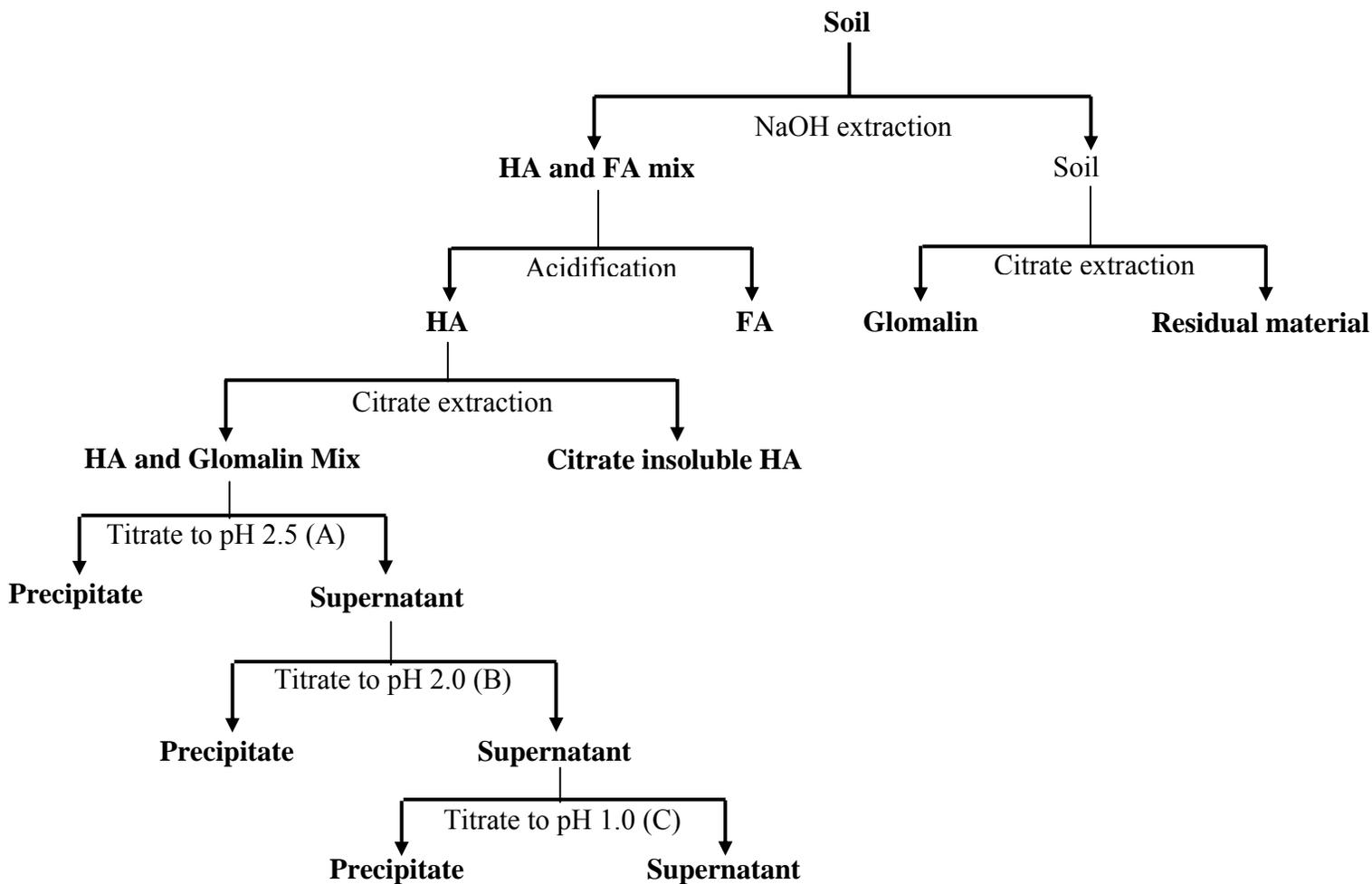
Figure G1. Total and immunoreactive protein measured on glomalin extracted from hyphae of four arbuscular mycorrhizal species (*Acaulospora morrowiae*, *Glomus etunicatum*, *G. intraradices*, and *Gigaspora rosea*) using one of four buffer solutions [Tris (T), citrate (C), borate (B), pyrophosphate (P)].

Appendix H. Diagrams of sequential extraction

Appendix H1. Diagram of Extraction Sequence 1: citrate extraction of glomalin before NaOH extraction of humic acid (HA) and fulvic acid (FA) followed by citrate extraction of recalcitrant glomalin (R. glomalin).



Appendix H2. Diagram of Extraction Sequence 2: citrate extraction of glomalin after NaOH extraction of humic acid (HA) and fulvic acid (FA) followed by citrate extraction and pH level separation of HA.



Appendix I. Characteristics of eight U.S. soils extracted for glomalin and humic acid. †

	Baltimore (site <i>a</i>)	Baltimore (site <i>b</i>)	Wymore	Pawnee	Sampson	Haxtun	Pacolet	Cecil
Mean annual temperature (°C)	12	12	13	12	9	9	17	17
Mean annual precipitation (mm)	1067	1067	838	762	381	432	1250	1250
% sand	26	27	15	21	48	78	NA	75
% silt	49	47	57	59	36	12	NA	14
% clay	25	26	28	20	16	10	NA	11
% OM	6.3	5.0	4.9	4.9	4.2	1.9	7.7	4.0
Mg (ppm)	52.0	32.4	150.2	150.2	150.2	150.2	150.2	18.0
K (ppm)	196.6	64.4	210.9	104.2	225.2	225.2	137.6	43.7
Ca (ppm)	134.5	13.8	1505.1	1505.1	1505.1	944.8	850.0	13.8
Zn (ppm)	6.9	3.6	1.6	1.1	1.2	0.6	16.5	1.0
Mn (ppm)	136.0	92.0	42.5	46.0	25.5	18.0	48.5	18.0
Cu (ppm)	1.3	1.2	0.5	0.4	0.2	0.2	0.7	0.4
SO ₄ (ppm)	15.4	20.6	1.6	1.1	1.8	1.0	NA	2.6
NO ₃ (ppm)	19.2	4.6	1.0	0.8	2.2	1.3	NA	0.7

Appendix J. Gravimetric weights (g kg⁻¹ soil) for the seven organic matter fractions – Residual particulate organic matter (POM), glomalin extracted from POM (P. glomalin), glomalin, glomalin extracted after humic and fulvic acid extraction (R. glomalin), humic acid (HA), and fulvic acid (FA) – extracted from three aggregate size classes [1-2 (A), 0.5-1 (B), and 0.25-0.5 (C) mm] collected from five undisturbed U.S. soils.

Soil	Size	Residual POM	P. glomalin	Glomalin	R. glomalin	HA	FA
Baltimore	A	2.02	0.12	9.19	1.91	0.83	0.88
Sampson	A	24.72	1.86	6.17	2.41	0.31	2.61
Haxtun	A	19.46	2.35	5.45	1.62	0.33	0.03
Pacolet	A	9.12	1.26	5.81	2.82	1.51	0.86
Cecil	A	9.84	0.84	2.99	0.82	0.11	0.34
Baltimore	B	2.35	0.72	9.03	2.09	1.40	0.84
Sampson	B	19.70	2.64	6.31	1.72	0.07	0.41
Haxtun	B	26.76	3.19	6.05	1.71	0.20	0.22
Pacolet	B	9.56	1.18	5.21	3.13	1.19	1.26
Cecil	B	7.85	1.37	2.65	1.30	0.04	0.01
Baltimore	C	NA [†]	0.37	13.17	3.22	1.60	0.82
Sampson	C	14.50	1.10	8.17	3.02	0.54	2.08
Haxtun	C	3.37	0.48	4.08	0.78	0.04	0.07
Pacolet	C	7.61	1.00	7.76	5.55	2.48	1.38
Cecil	C	6.89	1.14	4.55	1.38	0.18	0.03

[†] NA = quantity not sufficient for assay.

Appendix K. Total protein weights (g protein kg⁻¹ soil) in glomalin extracted from the particulate organic matter fraction (P. glomalin), glomalin, glomalin extracted after humic and fulvic acid extraction (R. glomalin), and humic acid (HA) extracted from three aggregate size classes [1-2 (A), 0.5-1 (B), and 0.25-0.5 (C) mm] collected from five undisturbed U.S. soils.†

Soil Series	Size	P. glomalin	Glomalin	R. glomalin	HA
Baltimore	A	0.13 (4)	1.87 (26)	0.48 (17)	0.81 (1)
Sampson	A	2.33 (22)	1.84 (39)	0.69 (23)	0.16 (47)
Haxtun	A	1.58 (21)	1.04 (23)	0.59 (8)	0.15 (43)
Pacolet	A	0.17 (25)	2.39 (17)	0.82 (19)	0.04 (71)
Cecil	A	0.65 (9)	1.62 (8)	0.23 (8)	0.08 (23)
Mean‡	A	0.97 ± 0.43 (16 ± 4)	1.75 ± 0.22 (23 ± 5)	0.56 ± 0.10 (15 ± 3)	0.25 ± 0.14 (37 ± 12)
Baltimore	B	0.10 (6)	1.62 (29)	0.53 (17)	0.77 (NA)
Sampson	B	1.87 (25)	1.35 (31)	0.71 (21)	0.27 (45)
Haxtun	B	1.83 (21)	1.11 (23)	0.62 (6)	0.20 (34)
Pacolet	B	0.21 (26)	2.72 (12)	0.91 (16)	0.08 (NA)
Cecil	B	0.65 (6)	0.77 (13)	0.32 (6)	0.08 (27)
Mean‡	B	0.93 ± 0.39 (17 ± 4)	1.51 ± 0.33 (21)	0.62 ± 0.10 (13)	0.28 ± 0.13 (35 ± 5)
Baltimore	C	0.15 (6)	1.38 (19)	0.75 (22)	0.68 (1)
Sampson	C	1.38 (26)	1.83 (25)	1.09 (19)	0.39 (46)
Haxtun	C	0.44 (16)	0.80 (10)	0.24 (7)	0.10 (40)
Pacolet	C	0.21 (29)	3.36 (16)	1.93 (13)	0.15 (26)
Cecil	C	0.66 (11)	1.57 (8)	0.45 (5)	0.22 (17)
Mean‡	C	0.57 ± 0.22 (18 ± 4)	1.79 ± 0.43 (16 ± 3)	0.89 ± 0.30 (13 ± 3)	0.31 ± 0.10 (26 ± 8)

† Values in parentheses are percentage of immunoreactive (IR) protein: % IR = (IR ÷ TP) * 100

‡ Means ± SEs

Appendix L. Plot Design for the Farming Systems Project, Beltsville, MD

Background: The entire 16 ha site was planted to no-till corn for three years. A detailed soil survey was conducted at 47 sampling points in a non-uniform grid in 1993 (Fig. L1). In 1993, 1994 and 1995, numerous crop, soil and weed characteristics were measured at up to 298 sampling points. Areas of minimum variability were identified by this data. The lowest block variability was with blocking on soil drainage class. Four plots for each treatment system and rotation year were established in strips (110m long and 9.1 m wide) in the field (Figs. L2 and L3). All crops in a rotation are present at the same time giving 2 to 4 subsystems for each system. Maps and plot design were provided courtesy of Michel Cavigelli at USDA-ARS, Beltsville, MD.



Symbol	Map Unit Name
CeA	Christina Silt Loam, 0-3% slope
CeB	Christina Silt Loam, 3-8% slope
DoA	Downer Sandy Loam, 0-3% slope
EkA	Elkton Silt Loam, 0-3% slope
KeA	Keyport Silt Loam, 0-3% slope
KxA	Keyport Silt Loam variant, 0-3% slope
KxB	Keyport Silt Loam variant, 3-8% slope
MkA	Matapeake Silt Loam, 0-3% slope
MkB	Matapeake Silt Loam, 3-8% slope
MxA	Mattapex Silt Loam, 0-3% slope
Ota	Othello Silt Loam, 0-3% slope

Figure L1. The soil survey map showed that the Farming Systems Project site contained several different soil types distributed throughout the field. If you overlay the plot design in Figs. L2 and L3, you can see that each of the soil types is represented in each system.

Svs.	Plot	Crop	Svs.	Plot	Crop
2.1	101	W/SB	7.1	107	C
2.2	102	C	7.2	108	SB
1.1	103	W/SB	7.3	109	W/H
1.2	104	C	7.4	110	H
5.2	105	C	3.1	111	W/SB
5.1	106	SB	3.2	112	C
			6.1	113	W/F
4.2	201	C	6.2	114	C
4.1	202	W/SB	6.3	115	SB
7.2	203	SB	4.1	116	W/SB
7.1	204	C	4.2	117	C
7.3	205	W/H			
7.4	206	H			
5.2	207	C			
5.1	208	SB			
6.3	209	SB			
6.2	210	C			
6.1	211	W/F			
2.2	212	C			
2.1	213	W/SB			
3.2	214	C			
3.1	215	W/SB			
1.1	216	W/SB			
1.2	217	C			

Crop Designations (Crop in system at time of sampling)

- C – Corn
- H – Hay
- SB – Soybean, full season
- W/SB – Wheat-Soybean, double crop
- W/F –Wheat, fallow
- W/H – Wheat – Stubble
- Hay

System Designations
See Table 4A.

Sys.	Plot	Crop	Sys.	Plot	crop
3.1	401	W/SB	6.3	301	SB
3.2	402	C	6.1	302	W/F
4.2	403	C	6.2	303	C
4.1	404	W/SB	1.2	304	C
2.2	405	C	1.1	305	W/SB
2.1	406	W/SB	4.2	306	C
1.1	407	W/SB	4.1	307	W/SB
1.2	408	C	7.4	308	H
6.2	409	C	7.2	309	SB
6.3	410	SB	7.3	310	W/H
6.1	411	W/F	7.1	311	C
7.3	412	W/H	3.1	312	W/SB
7.1	413	C	3.2	313	C
7.2	414	SB	2.1	314	W/SB
7.4	415	H	2.2	315	C
5.2	416	C	5.1	316	SB
5.1	417	SB	5.2	317	C

Figure L2. Farming Systems Project - 2000 Plot Plan

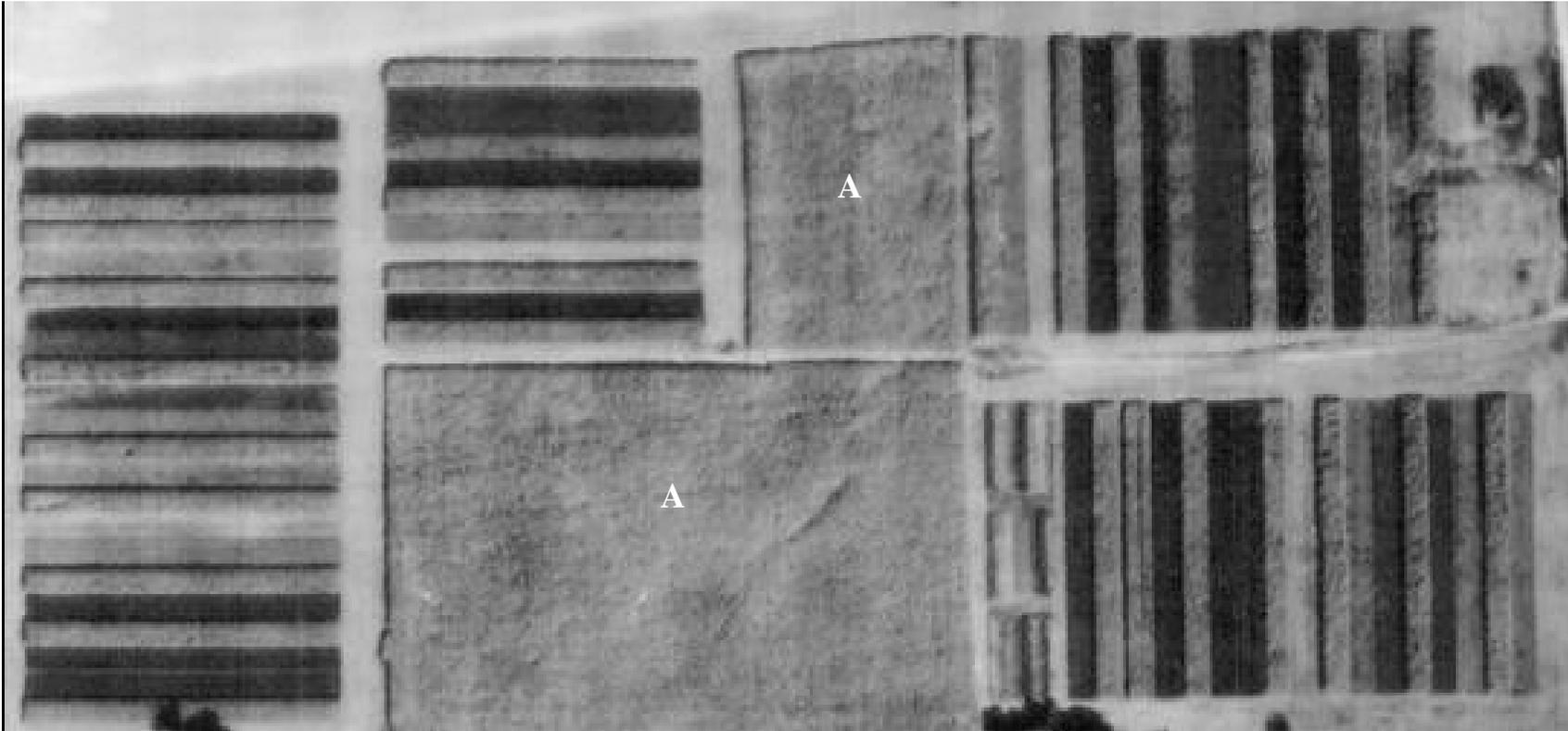


Figure L3. Aerial picture of the Farming Systems Project site, the open areas (A) were locations of high variability based on the pre-assessment and were not included in the experimental design.

Appendix M. Equations used to calculate the Normalized Stability Index (NSI)

Formula for calculation of disruption level in each size class is:

$$DLS_i = \frac{\left[\frac{((P_{io} - S_{io}) - (P_i - S_i))_+}{|(P_{io} - S_{io}) - (P_i - S_i)|} \right]}{2} \times \frac{[1]}{[P_{io} - S_{io}]} \quad (1)$$

where DLS_i = disruption level for each size class I; P_{io} = proportion of total sample weight in size class I before disruption (i.e. capillary rewetted); P_i = proportion of total sample weight in size class I after disruption (i.e. slaked); S_{io} = proportion of sand with size I in aggregates of size I (= aggregate-sized sand) before disruption; S_i = proportion of sand with size I in aggregates of size I after disruption. All proportions are expressed on a soil weight basis (g fraction g^{-1} soil). The size classes in this study were I = 1 = 53-250 μm , I = 2 = 250-2000 μm , and I = 3 = > 2000 μm .

Formula for the whole soil disruption level (DL) is:

$$DL = 1/n \sum_i^n [(n+1) - I] \times DLS_i \quad (2)$$

where n = number of aggregate size classes.

Formula for the calculation of the maximum disruption in each size class [DLS_i (max)] is:

$$DLS_i(\text{max}) = \frac{[(P_{io} - P_p) + |(P_{io} - P_p)|]}{2} \times \frac{[1]}{[P_{io} - S_{io}]} \quad (3)$$

where P_p = primary sand particle content with the same size as the aggregate size class after complete disruption of the whole soil.

Formula for the whole soil maximum disruption [$DL(\max)$] is:

$$DL(\max) = 1/n \sum_i^n [(n+1) - I] \times DLS_i(\max) \quad (4)$$

where n = number of aggregate size classes.

Normalized stability indexed is calculated as:

$$NSI = 1 - \left[\frac{DL}{DL(\max)} \right] \quad (5)$$

Reference

Six, J., E.T. Elliott, K. Paustian. 2000. Soil structure and soil organic matter: II. A normalized stability index and the effect of mineralogy. *Soil Sci. Soc. Am. J.* 64:1042-1049.

Appendix N. Total and immunoreactive protein weights (g protein kg⁻¹ soil) of glomalin extracted from the particulate organic matter fraction (P. glomalin), glomalin and humic acid (HA) in three farming systems (System 1.1 – Synthetic NT C-W-SB, System 2.1 – Synthetic CT C-W-SB, and System 6.1 – Organic MT C-SB-W) at the Farming Systems Project site in Beltsville, MD in July, 2000.†

System	P. glomalin	Glomalin	HA	P
Total Protein Weight				
1.1	0.05 ± 0.01c	1.01 ± 0.06a	0.37 ± 0.02b	<0.0001
2.1	0.05 ± 0.01c	1.08 ± 0.04a	0.38 ± 0.06b	<0.0001
6.1	0.08 ± 0.02c	1.20 ± 0.06a	0.43 ± 0.02b	<0.0001
Immunoreactive Protein Weight				
1.1	0.01 ± 0.00a	0.84 ± 0.18a	0.37 ± 0.03a	0.1352
2.1	0.01 ± 0.00c	1.23 ± 0.04a	0.41 ± 0.08b	<0.0001
6.1	0.02 ± 0.00a	1.13 ± 0.11a	0.28 ± 0.02b	0.0386

† Mean ± SE.

‡ Different letters in a row indicate significant differences according to REML.

Appendix O. Total (TP) and immunoreactive (IRP) protein weights (g protein kg⁻¹ soil) in glomalin extracted from nine systems at the Farming Systems Project site in Beltsville, MD in April, 2001.† ‡

Experiment	System/Plots Sampled	TP	IRP
Controls§	1.1 – Synthetic NT C-W-SB	1.60 ± 0.07Aa	0.72 ± 0.06Aa
	2.1 – Synthetic CT C-W-SB	1.66 ± 0.06Aa	0.79 ± 0.09Aa
Fertilizer Treatment	3.1 – Synthetic MT C-W-SB, 2X raw manure	1.64 ± 0.10A	0.76 ± 0.04A
	3.2 – Synthetic MT C-W-SB, 1X raw manure	1.61 ± 0.06A	0.77 ± 0.06A
	4.1 – Synthetic MT C-W-SB, 2X composted manure	1.57 ± 0.09A	0.71 ± 0.02A
	4.2 – Synthetic MT C-W-SB, 2X composted manure	1.45 ± 0.04A	0.70 ± 0.04A
Prob>F		0.3868	0.8092
Rotation Length	5.1 – Organic MT C-SB	1.61 ± 0.11a	0.65 ± 0.07a
	6.3 – Organic MT C-SB-W	1.74 ± 0.15a	0.80 ± 0.10a
	7.2 – Organic MT C-SB-W-H	1.53 ± 0.04a	0.74 ± 0.03a
Prob>F		0.6042	0.6147

† Mean ± SE.

‡ Different letters in a column indicate significant differences according to REML.

Uppercase letters are used for the Fertilizer Treatment experiment plus controls and lowercase letters for the Rotation Length experiment plus controls.

§ These were the positive [no-till (NT)] and negative [conventional tillage (CT)] treatments that were used as controls the statistics for both April, 2001 experiments.

Appendix P. Immunofluorescence assay

Introduction

An indirect immunofluorescence procedure is used to determine the location of the protein on fungal structures, roots, plastic mesh, soil particles, etc. The monoclonal antibody (MAb 32B11, and IgM class of antibody) is incubated with the sample. A fluorescent tagged secondary is a commercially available reagent (FITC-tagged goat anti-mouse IgM). A microscope with an ultraviolet-emitting light source is used to view the location of green color as an assessment of the location of the protein.

Materials and methods

1. Place root fragments or sand aggregates in small sieve (made from 10 mm ID polyvinyl chloride or other plastic tubing with a 40 μ m nylon mesh glued to the bottom) or place the mesh pieces themselves in wells of a 12-well plate.
2. Add 2% (w/v) skim milk (Carnation®) in phosphate buffered saline (PBS), pH 7.4, to wells – enough to cover roots, sand or mesh
3. Incubate while shaking (50-75 rpm) for 30 min
4. Remove samples – place sieves or mesh on paper towels – and invert and blot plate to remove milk (via hard taps)
5. Add monoclonal antibody 32B11 diluted in PBS – enough to cover – and incubate on shaker for 1 h
6. Remove samples – place sieves or mesh on paper towels – and invert and blot plate to remove antibody solution
7. Add PBS with Tween 20 (PBST) to cover and incubate on shaker for 5 min.
Repeat PBST incubation twice.

8. Add FITC tagged goat anti-mouse IgM diluted in PBS plus 1% (w/v) bovine serum albumin (BSA) and incubate on shaker for 1 hour.
9. Remove samples – place sieves or mesh on paper towels – and invert and blot plate to remove antibody solution
10. Add PBST and incubate on shaker for 5 min. Repeat PBST incubation twice, followed by a 5-min incubation with PBS.
11. Mount on slides with VectaShield® (Vector Laboratories, Burlingame, CA) mounting medium
12. Examine using an epi-fluorescence microscope with band pass combination BP450-BP490 exciter filter, a dichroic chromatic beam splitter FT-150 filter, and a longwave pass LP-520 barrier filter
13. Use a digital camera with a frame grabber to capture and save images

Appendix Q. Plant height vs. light intensity

Background

By the end of the third culturing period for *Gi. rosea*, an obvious difference was seen in plant height for those plants directly under the sodium vapor lights in the greenhouse and those not under supplemental lighting (Fig. 5B). This culturing period was from December 12 to March 15 and had the lowest ambient irradiance throughout all but the last two weeks of the culturing period (Fig. 5C). Because this represented an ideal situation to test the effects of supplemental lighting, plant height and irradiance at the pot level were measured at harvest. Irradiance and plant height were linearly related (Fig. Q1).

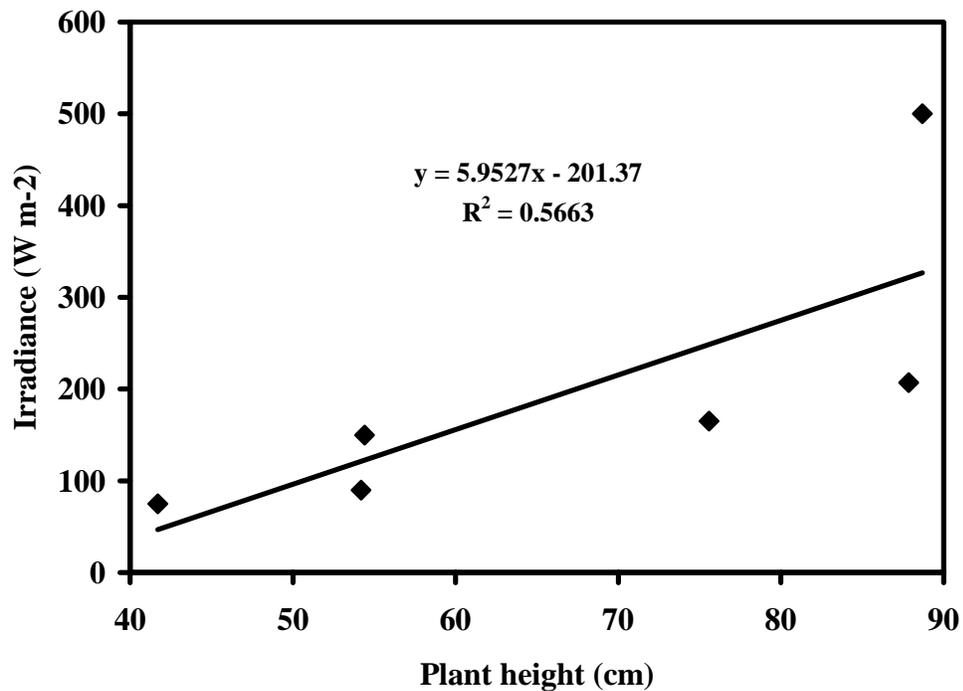


Figure Q1. Relationship between the average height (cm) of corn plants per pot and irradiance (W m^{-2}) at the pot level.

Appendix R. Glomalin weight per weight of hyphae

Introduction

An accurate assessment of extraradical arbuscular mycorrhizal (AM) hyphae is important in evaluating fungal contributions to plant growth and soil structure (Bethlenfalvay et al., 1987; Miller et al., 1995; Stahl et al., 1995). Hyphal length in soil is frequently measured by direct microscopic counts. Shaking manually or grinding in a buffer solution is used to separate hyphal from soil and homogenize the extract (Miller et al., 1995; Rillig et al., 2002; Stahl et al., 1995). Glomalin is a substance produced by AM fungi that may assist in the formation and stabilization of aggregates. Measuring glomalin production in relation to hyphal growth will help determine the role of glomalin in phenomena such as soil aggregation and source-sink relationships with the plant host.

Measuring hyphal length is a tedious process and has inherent sources of error such as: (i) separating hyphae from soil, (ii) getting a homogeneous sample, and (iii) human error (i.e. having more than one person making counts, counting at different times or observer subjectivity) (Stahl et al., 1995). Hyphal weight is often difficult to measure for amounts that are obtainable from pot cultures and may not be within the weight error of a balance that measures to 10^{-6} g.

Unusually large amounts of fungal hyphae, enough to get an accurate weight, were produced in a soilless pot culture experiment after two 14-week culturing periods (Chapter 6). Several methods were developed to isolate hyphae from this material and to obtain a more accurate measure of glomalin per unit of hyphae. The hyphae fraction collected by floatation and wet-sieving frequently contained a large number of spores,

fine sand:coal particles, and other debris. The objective of this work was to get as accurate a measure of hyphal mass as possible and to relate glomalin concentration to hyphal mass.

Materials and Methods

Three fractions (hyphae fraction, spore fraction and hyphae sand fraction) were collected with forced-water washing and wet-sieving using a series of small screens. The hyphae sand fraction was material left behind following forced-water rinsing and decanting over a 53 μm screen. Hyphae collected on this screen were rinsed through a series of stacked screens depending on spore size (*G. etunicatum* 60 μm and 53 μm screens and *Gi. rosea* 125 μm and 53 μm screen). The spore fraction consisted of material on the larger, top screen, while the hyphae fraction was on the smaller, bottom screen. Sand particles and small debris were removed from the hyphae fraction by repeated washing over screens and transferring to new beakers for density separation (i.e. hyphae floats and sand sinks to the bottom of the beaker).

Samples were examined under a stereomicroscope, which showed that debris and spores remained attached to hyphae. Hyphae were selectively removed using forceps and transferred to a tissue grinder. Hyphae were partially crushed and separated by grinding the sample in an aqueous solution, which was then passed through a series of screens to collect the purest hyphae fraction on the smallest screen. For *Gi. rosea*, a 125 μm screen was inserted to collect the spores so the subsequent 60 and 53 μm screens contained different sizes of hyphal fractions. Only the 60 and 53 μm screens were used for *G. etunicatum*. Material on the 60 μm screen was placed in the tissue grinder and the grinding and sieving process was repeated until almost all of the hyphae

went through the larger screen. Material on both screens was collected separately, dried, weighed, and extracted for glomalin. In *G. etunicatum* samples, hyphae were ground until almost all of it went through the 60 μm screen, leaving only material on the 53 μm screen for glomalin extraction.

Glomalin concentration was measured on a 1 mL aliquot by total and immunoreactive protein assays. (See Appendix C for assay methods.) The remaining glomalin extract solution was precipitated in acid, dialyzed, freeze-dried and weighed for comparison to hyphae. After extraction, hyphae were collected, dried and weighed.

Results and Discussion

Isolation of fungal hyphae from spores and debris proved to be a tedious process with the problems that are discussed below. The overall results are in Table R1.

Problems:

1. Comparisons using hyphal weight rather than hyphal length may be incorrect, because hyphal weight may be inflated by the weights of sand particles or other debris that are too tightly bound to hyphae to be removed by grinding.
2. Grinding strips away the outer layer of the spore wall, which may be collected with the 'pure' hyphae and inflate the weight. For Glomineae species, this outer layer also may have glomalin, which would bias these values.
3. Grinding also appeared to release glomalin as scum/foam from hyphae as the hyphae break apart. This scum/foam was lost by rinsing the screen.
4. It was difficult and time-consuming, if not impossible, to separate hyphae from spores and other debris. Also, not all of the hyphae were collected as pure hyphae (some remains on the upper screens) using any of the separation

methods, i.e. grinding and sieving, which meant that comparisons cannot be made on a whole pot basis.

5. Autoclaving the hyphal samples resulted in two problems: 1) a small amount of hyphae may lyse in the autoclave and 2) autoclaving appears to fuse hyphae, especially from the *Glomus* species, together creating more problems when trying to measure hyphal length.

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Table R1. Weight of *Gi. rosea* or *G. etunicatum* hyphae isolated on either a 53 or 60 μm screen and extracted for glomalin – measured as gravimetric weight, total protein weight (TP) and immunoreactive protein weight (IRP).

AMF species	<i>Gi. rosea</i>		<i>G. etunicatum</i>
	53 μm screen	60 μm screen	53 μm screen
Hyphae collection			
Weight hyphae extracted (mg)	3.38 \pm 1.26	12.55 \pm 3.64	1.28 \pm 0.21
Weight glomalin ($\mu\text{g mg}^{-1}$ hyphae)	59.14 \pm 9.88	30.01 \pm 10.38	67.17 \pm 13.71
TP ($\mu\text{g mg}^{-1}$ hyphae)	14.86 \pm 2.04	10.15 \pm 2.56	17.91 \pm 2.54
IRP ($\mu\text{g mg}^{-1}$ hyphae)	5.66 \pm 0.82	5.00 \pm 1.38	18.30 \pm 5.53

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CURRICULUM VITAE

KRISTINE NICHOLS
1930 E Capitol Ave, Apt 304
Bismarck, ND 58501
nicholsk@mandan.ars.usda.gov

EDUCATION:

- 08/1999-12/2003 **Ph.D. Soil Science.** University of Maryland, College of Agriculture and Natural Resources, College Park, MD 20742. **Dissertation title:** Characterization of Glomalin: A Unique Soil Organic Matter Constituent.
- 06/1996-12/1999 **M.S. Environmental Microbiology.** West Virginia University, College of Agriculture, Forestry and Consumer Sciences, Morgantown, WV 26056.
Thesis title: Role of Iron in the Accumulation of Glomalin, an Arbuscular Mycorrhizal Fungal Glycoprotein.
- 09/1990-05/1995 **B.S. Plant Biology, B.S. Genetics & Cell Biology.** University of Minnesota, College of Biological Sciences, St. Paul, MN 55108.

RESEARCH WORK EXPERIENCE:

- 06/2003-present **Soil Microbiologist** United States Department of Agriculture (USDA)-Agriculture Research Service (ARS)-Northern Great Plains Research Laboratory, 1701 10th Ave., Mandan, ND 58455.
- 05/2000-05/2003 **Biological Science Laboratory Technician** United States Department of Agriculture (USDA)-Agriculture Research Service (ARS)-Sustainable Agricultural Systems Laboratory (SASL), 10300 Baltimore Ave, Bldg 001, rm. 140, Beltsville, MD 20705.
- 01/1999-05/2000 **Research Associate** University of Maryland, Center for Agricultural Biotechnology, 5115 Plant Sciences Building, College Park, MD 20742.
- 07/1996-12/1998 **Graduate Research Assistant** West Virginia University, Division of Plant and Soil Sciences, 401 Brooks Hall, P.O. Box 6057, Morgantown, WV 26056.
- 05/1995-05/1996 **Senior Laboratory Technician** University of Minnesota, Department of Plant Biology, 1445 Ave, St. Paul, MN 55108.
- 06/1994-05/1995 **Undergraduate Research Assistant** University of Minnesota, Department of Plant Biology, 1445 Gortner Ave, St. Paul, MN 55108.

PROFESSIONAL EXPERIENCE:

- 08/1996 **Instructor.** ICOM I (International Conference on Mycorrhizae), Berkeley, CA. **Classification & Identification of Arbuscular Mycorrhizal Fungi**

TEACHING EXPERIENCE:

1997, 1998 **Laboratory Teaching Assistant.** West Virginia University, Morgantown, WV
Plant Pathology Laboratory

INVITED PRESENTATIONS:

04/2002 **Glomalin and its Significance to Soil Stability and Carbon and Nitrogen Sink**
University of New Hampshire, Department of Natural Resources, Durham, NH

POSTER AND ORAL PRESENTATIONS (Presenter):

- 11/2002 **Glomalin is a Major and previously Underrepresented Pool of Soil Organic Carbon**
K. A. Nichols, S. F. Wright, E. K. Dzantor, W. F. Schmidt, M.A. Cavigelli. Oral.
American Society of Agronomy Annual Meeting, Indianapolis, IN
- 07/2002 **Carbon Contribution and Characteristics of Humic Acid, Fulvic Acid, Particulate Organic Matter, and Glomalin in Diverse Ecosystems**
K.A. Nichols, S.F. Wright, W.F. Schmidt, M.A. Cavigelli, E.K. Dzantor. Poster.
International Humic Substances Society, 20th Anniversary Conference, Boston, MA
- 10/2001 **Using NMR to Distinguish Unique Components of Humic Substances**
K.A. Nichols, S.F. Wright, W.F. Schmidt, A.J. Simpson, E.K. Dzantor. Oral.
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- 03/2001 **Glomalin as a Constituent of Humic Substances**
K.A. Nichols, S.F. Wright, W.F. Schmidt, E.K. Dzantor. Oral.
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- 11/2000 **Glomalin: An Important, Distinct Component of Soil Organic Matter**
K.A. Nichols, S.F. Wright, W.F. Schmidt. Oral.
American Society of Agronomy Annual Meeting. Minneapolis, MN
- 10/1998 **Effects of Iron Availability on Spore Germination of Arbuscular Mycorrhizal Fungi**
K.A. Nichols, J.B. Morton, S.F. Wright, D.K. Bhumbla. Poster.
American Society of Agronomy Annual Meeting, Baltimore, MD

POSTER AND ORAL PRESENTATIONS (Contributing Author):

- 10/2001 **Glomalin Production by Arbuscular Mycorrhizal Fungi.** S.F. Wright, K.A. Nichols, L. Jawson. Oral. American Society of Agronomy Annual Meetings, Charlotte, NC
- 10/2001 **Soil Quality in No-till, Till, and Organic Cropping Systems.** M.A. Cavigelli, K.A. Nichols. Poster. American Society of Agronomy Annual Meetings, Charlotte, NC
- 10/2001 **Soil Nitrogen and Carbon Pools after Five Years of Till, No-till and Organic Cropping Systems Management: The USDA-ARS Farming Systems Project.** M.A. Cavigelli, K.A. Nichols. Poster. Second International Nitrogen Conference, Potomac, MD
- 11/2000 **Glomalin: An Abundant, Unusual and Important Organic Matter Constituent.** S.F. Wright, L. Jawson, K.A. Nichols. Poster. American Society of Agronomy Annual Meeting. Minneapolis, MN
- 11/2000 **The USDA-ARS Farming Systems Project: Developing Sustainable Cropping Systems for the Mid-Atlantic Region.** M.A. Cavigelli, J.R. Teasdale, T.H. Dao, J. Radhakrishnan, J.E. Buyer, K. Nichols, S. Liang, C. Shuey. Poster. American Society of Agronomy Annual Meeting. Minneapolis, MN
- 08/2000 **Glomalin: A Soil Protein Important in Carbon Sequestration.** S.F. Wright, M.C. Rillig, K.A. Nichols. Poster. American Chemical Society National Meeting, Washington, DC

PUBLISHED MANUSCRIPTS:

- Gonzalez-Chavez M.C., R. Carillo-Gonzalez, S.F. Wright, and K.A. Nichols. 2003. **Glomalin: A Mechanism for Heavy-Metal Sequestration by Arbuscular Mycorrhizal Fungi.** Environmental Pollution. In review.
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PUBLISHED RESEARCH REPORTS:

- Charvat, I.D., T. Pawlowska, M. Smith, D.L. Stenlund, K. Nichols. 1995. Re-introduction of soil mycorrhizae into roadside prairie planting. Minnesota Department of Transportation, Office of Research Administration.

HONORS AND AWARDS:

- 07/2002 Best Poster Award, International Humic Substances Society 20th Anniversary Conference
03/2002 Certificate of Merit, USDA-ARS
03/2001 Certificate of Merit, USDA-ARS
10/1995 President's Leadership and Service Award, University of Minnesota
08/1994 Eloise Pittman Scholarship for Excellence in Plant Biology, University of Minnesota

SOCIETAL AFFILIATIONS:

Agronomy Society of America
International Humic Substances Society
Mycological Society of America
Soil Science Society of America
Soil Ecology Society
Soil Water Conservation Society
Future Harvest-Chesapeake Alliance for Sustainable Agriculture