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Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forest soils

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Abstract

The origins and composition of soil organic matter (SOM) are still largely uncertain. Arbuscular mycorrhizal fungi (AMF) are recognized as indirect contributors through their influence on soil aggregation, plant physiology, and plant community composition. Here we present evidence that AMF can also make large, direct contributions to SOM. Glomalin, a recently discovered glycoprotein produced by AMF hyphae, was detected in tropical soils in concentrations of over 60 mg cm^{-3}. Along a chronosequence of soils spanning ages from 300 to 4.1 Mio years, a pattern of glomalin concentrations is consistent with the hypothesis that this protein accumulates in soil. Carbon dating of glomalin indicated turnover at time scales of several years to decades, much longer than the turnover of AMF hyphae (which is assumed to be on the order of days to weeks). This suggests that contributions of mycorrhizae to soil carbon storage based on hyphal biomass in soil and roots may be an underestimate. The amount of C and N in glomalin represented a sizeable amount (ca. 4–5%) of total soil C and N in the oldest soils. Our results thus indicate that microbial (fungal) carbon that is not derived from above- or below-ground litter can make a significant contribution to soil carbon and nitrogen pools and can far exceed the contributions of soil microbial biomass (ranging from 0.08 to 0.2% of total C for the oldest soils).

Introduction

Arbuscular mycorrhizal fungi (AMF), comprising fungi in the order Glomales (Zygomycota), are ubiquitous symbionts of the majority of higher plants. AMF have been shown to have numerous effects on plant physiology and plant communities (Allen, 1991; Smith and Read, 1997; van der Heijden et al., 1998), which can lead to indirect effects on soil carbon storage. AMF are also very important in the process of soil aggregate stabilization (e.g., Jastrow and Miller, 1997; Tisdall and Oades, 1982). Relatively labile carbon can be protected inside soil aggregates (Cambardella and Elliott, 1992; Jastrow and Miller, 1997; Six et al., 1998), which means AMF have yet another indirect influence on soil carbon storage (Miller and Jastrow, 1992). However, the *direct* contributions of AM fungi to carbon pools, i.e., the carbon content of AMF-derived compounds, rather than the influence of AMF on plants or soil aggregation, are far less studied. Therefore, direct contributions of AMF to C pools are not considered to be of a magnitude immediately relevant to ecosystem level considerations.

Estimates of AMF extraradical hyphal lengths in the field range widely (Rillig and Allen, 1999). One of the highest estimates is 111 m cm^{-3} of soil for a prairie community, for which a hyphal dry weight of less than 0.5 mg g^{-1} was calculated (Miller et al., 1995). Olsson et al. (1999) recently estimated AMF

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hyphal dry weight by phospholipid fatty acid analysis to 0.03–0.35 mg g^{-1}, and concluded that AMF dry weight was the greatest soil microbial biomass component in a linseed field. The turnover rate of AMF hyphae is unknown. In the absence of any strong empirical evidence, the hypothesis has been that lifetime is on the order of weeks. The data leading to this hypothesis was derived from in situ microscopic observations of hyphae growing between glass plates (Friese and Allen, 1991).

Recently, a glycoprotein, named glomalin, was discovered using specialized extraction protocols for soils that revealed amounts up to several mg protein per g of soil (Wright and Upadhyaya, 1996). Glomalin is produced by hyphae of all members of AM genera, but not by other groups of soil fungi so far tested (Wright et al., 1996, unpublished). It is often assumed that proteins do not comprise a major component of soil organic matter (SOM) carbon or nitrogen, because of the ubiquity of proteases in the soil. Plant-derived proteins are rapidly degraded once they come in contact with soil proteases (Paul and Clark, 1989; Swift, 1994). Examining soils from a variety of regions, however, Sowden et al. (1977) found that 40% of soil N was of proteinaceous origin (amino acids). Because hot acid hydrolysis is typically used in these extraction analyses, proteins and peptides are destroyed and only amino acids are found (Schulten and Schnitzer, 1998). ^{15}N NMR has been used widely for the analysis of N compounds in soils (reviewed in Schulten and Schnitzer, 1998). Soil organic matter NMR spectra reveal an abundance (80% of signal intensity) of amide/peptide bonds (Preston, 1996; Schmidt et al., 1997), suggesting that proteins or peptides can become stabilized in soil organic matter. However, when proteins have been extracted from soils, using bicarbonate-extractions (e.g., Lipson et al., 1999) for example, protein amounts are typically in the range of 5–80 $\mu g\ g^{-1}$ soil.

Our knowledge of glomalin in soils is extremely limited, and the protein has not yet been considered in ecological and biogeochemical studies, although concentrations of glomalin in soil seem to be responsive to global change factors such as elevated atmospheric CO_2 (Rillig et al., 1999). Here, in the context of a unique model system of ecosystem development, a tropical soil chronosequence, we address several fundamental questions about this protein. How do glomalin concentrations compare to other soil carbon pools, for example microbial biomass? How does the contribution of glomalin to these pools change over time scales of ecosystem development? Does glomalin concentration across the chronosequence follow a pattern similar to that observed for SOM (Torn et al., 1997), which would be consistent with the hypothesis that glomalin can accumulate in soil? What are turnover times for carbon in glomalin, compared to the presumed turnover of hyphae? What are glomalin concentrations in tropical soils?

The chronosequence is unique in that its six sites are located presently at a constant elevation and climate, with the same dominant arbuscular mycorrhizal plant species, *Metrosideros polymorpha*. We could therefore use the chronosequence as a constrained comparison of sites (being derived from the same parent material), to discern a pattern of glomalin concentrations that might be related to soil mineralogy, substrate age, and ecosystem development.

In order to exclude significant co-extraction of other compounds (e.g., tannins) or other proteins, we also examined the extracted glomalin further with gel electrophoresis and NMR spectroscopy.

Materials and methods

Soils

The six sites along the soil chronosequence used in this study (Table 1) have been described extensively with respect to age, location, climate and plant communities (Crews et al., 1995), plant–soil interactions and mycorrhizae (Treseder and Vitousek, 2001), SOM (Torn et al., 1997), and soil mineralogy and nutrient cycling (Chadwick et al., 1999). The sites are located at similar elevations (range, 1122–1210 m) and climate (mean annual temperature at each site, 16°C; mean annual precipitation at each site, 2500 mm) in areas dominated by *Metrosideros polymorpha* trees. Soil ages range from 300 to 4 100 000 years, and the soils span several soil orders (see Table 1). From each site we examined soil material from five replicate soil pits. Soil material was separated into the litter layer with macro-litter and roots removed (referred to as O horizon), and the top 0.06–0.10 m of soil below the litter layer (organic soil above the obvious B or A2 horizons; referred to here as the A horizon despite the high C content). We thus sampled most of the rooting zone at these sites, where the highest concentrations of AMF are to be expected. Thickness and bulk density of horizons thus defined are given in Table 1.

Table 1. Chronosequence sites and horizon characteristics

Site name	Soil order[a]	Age[a] (10^3 years)	Horizon thickness (cm)		Horizon bulk density (g cm^{-3})	
			O	A	O	A
Thurston	Inceptisol	0.3	10	8	0.32	1.0
Olaa	Inceptisol	2.1	8	5	0.37	0.47
Laupahoehoe	Andisol	20.0	12	8	0.29	0.34
Kohala	Andisol	150	4	7	0.25	0.37
Kolekole	Ultisol	1400	4	3	0.25	0.30
Kokee	Oxisol	4100	7	4	0.25	0.60

[a] Crews et al. (1995).

Glomalin extractions

Glomalin extractions from soils were carried out as described by Wright and Updahyaya (1998): easily-extractable glomalin (EEG) was extracted with 20 mM citrate, pH 7.0 at 121°C for 30 min, and total glomalin (TG) was extracted with 50 mM citrate, pH 8.0 at 121°C in rounds of 60 min each (for up to 23 cycles of 1 h each for the samples containing most glomalin). EEG and TG were extracted from the same soil subsample, with EEG representing the glomalin in the first round of extraction, and TG representing the total extractable pool of glomalin. EEG is considered the most recently deposited fraction of the protein in soil (Wright and Upadhyaya, 1998). Extracts were centrifuged at 10 000$\times g$ to remove soil particles, and protein in the supernatant was determined by the Bradford dye-binding assay with bovine serum albumin as the standard (Wright et al., 1996). An indirect enzyme-linked immuno-sorbent assay (ELISA) with monoclonal antibody MAb 32B11 against glomalin was used to carry out imunoassays, as described in Wright and Upadhyaya (1998).

To (partially) purify extracted glomalin, including removal of carbon-containing extractants, it was precipitated with trichloroacetic acid (TCA) and then dialyzed against 10 mM borate, pH 8.0, as described in Wright et al. (1998). Dialyzed samples were freeze-dried. Thus purified glomalin samples were used for chemical analysis, NMR spectroscopy, and carbon dating. Freeze dried samples were analyzed for C and N on a PE 2400 CHN analyzer (Perkin-Elmer, Norwalk, CT).

For iron analysis, 10 mg of each sample was subjected to microwave digestion in a 3:1 mixture of nitric acid and deionized water at 100 psi for 10 min, 70 psi for 15 min, and 50 psi for 10 min. Samples were diluted to 25 ml with deionized water and then filtered with Whatman # 40 paper. Analysis for iron was by atomic absorption.

Gel electrophoresis

Crude extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A composite of 0.25 g of four to five replicate samples of each of six soils (Thurston O and A, Kohala O and A and Kauai O) was extracted with 50 mM citrate, pH 8.0 at 1-h intervals until the extraction was complete. The dilution buffer was 10 mM Tris with 3% SDS. A 2.5-μg aliquot of extract was diluted to 10 μl in the dilution buffer with the addition of 0.15 M tiron (4,5-dihydroxy-1,3-benzene-disulfonic acid). Extracts were incubated at room temperature overnight. The extracts were heated in a boiling water bath for 8 min followed by the addition of 10 μl of dilution buffer plus 5% dithiothreitol and heated in boiling water for 8 min. Finally, 20 μl of dilution buffer was added to samples. Wells were loaded with 0.25 μg of sample and electrophoresis was performed on a PhastSystem (Amersham–Pharmacia, Piscataway, New Jersey) using a 12.5 T gel. Extracts of hyphae of *Glomus intraradices* EY113/114 and *Gigaspora rosea* FL224 (obtained from pot cultures using Sudan grass) were treated similarly and electrophoresed. Gels were stained with the PhastSystem silver stain. A high pass 3×3 filter was applied to the image using Image Tool 2.0 (The University of Texas Health Science Center in San Antonio).

NMR spectroscopy

Samples (20 mg) of freeze-dried glomalin extracted from the O horizon of Kohala soil, of glomalin extracted from pot cultures containing either *Gigaspora*

rosea or *Glomus intraradices*, and from a Baltimore series soil (for comparison) were reconstituted with D_2O. A sample of commercial tannic acid (10 mg) (Sigma) was also reconstituted with D_2O. All samples were placed in NMR sample tubes. ^{1}H NMR was conducted on a 300-MHz Bruker QE Plus NMR spectrometer with Tecmag Aquerius operating system. Spectra width was 3000 Hz and chemical shifts were referenced relative to the HOD peak at 4.8 ppm (Braun et al. 1998).

Glomalin turnover

The turnover time (inverse of decay rate) of glomalin from the youngest site only (purified glomalin samples from O and A horizons from all five soil pits were composited to make one radiocarbon sample for the site) was explored by measuring the radiocarbon content of the purified glomalin extract and using this value to constrain a stock-flow model of carbon turnover. Radiocarbon is a relatively sensitive tracer of ecosystem carbon cycling on annual to decadal time scales because atmospheric nuclear weapons testing circa 1959–1963 caused a large spike in atmospheric $^{14}CO_2$ concentrations that is declining each year as CO_2 exchanges with the ocean and biosphere. Radiocarbon content was measured by Accelerator Mass Spectrometry at Lawrence Livermore National Laboratory on targets prepared by sealed-tube combustion followed by hydrogen reduction for graphitization (Vogel, 1992). Values are reported as Δ^{14}C, calculated as $(R - 1) \times 1000$, where R=per mil deviation from $^{14}C/^{12}C$ ratio of oxalic acid in 1950 (Stuiver and Polach, 1977). The model of turnover time was based on the same principles as litter decay models, in which the decomposition rate is proportional to the stock and decay constant (i.e., $dC/dt = -kC$, where $T = 1/k$) and was run on annual time steps. We used the following simplifying assumptions about carbon cycling in these soils: annual hyphal production of glomalin is constant (and hence does not need to be estimated), there is a 1-year lag time between plant carbon input to the hyphae and deposition of free (dead) glomalin into the soil; all glomalin is homogenous in terms of decay (in the absence of any data on glomalin decomposition); and material ceases to be measurable as glomalin as soon as it is broken down by microbial action. The model equations can be summarized:

$$\text{Carbon} : C(t) = C_1 - C/T + I \quad (1)$$

$$^{14}C(t) = (C_1R_1 - C_1R_1\lambda - C_1R_1/T + IR_2)/C_2 \quad (2)$$

Where C_i=glomalin carbon stock in year i; R_i=is the radiocarbon content of glomalin in year i; λ=radioactive decay rate of ^{14}C; T=turnover time; and I=annual glomalin production (not estimated; assumed constant).

Microbial biomass

Microbial biomass C was measured by the chloroform fumigation–extraction method (CFEM) on sieved, field moist soil within 1 week of collection (Vance et al., 1987). Approximately 6 g fumigated or non-fumigated (control) soil were extracted with 0.5 m K_2SO_4 and extract carbon content was measured with a Shimadzu TOC-5050A. Microbial biomass C was estimated as the difference between the C in non-fumigated and fumigated soils corrected for an extraction efficiency of 0.25 (Vance et al., 1987). Total carbon and nitrogen content of ground soils was measured using a Carlo Erba elemental analyzer.

Results

Glomalin: evaluation of extracts

Glomalin was found at all sites and horizons across the soil chronosequence with both the ELISA and Bradford assays (Tables 2 and 3). The TG extract, as the measure of total glomalin, was examined further to determine whether there was significant co-extraction of other compounds.

Likely contaminants of proteins isolated from soil include tannins. According to the ^{1}H NMR spectra in Fig. 1, glomalin is the most abundant and best characterized compound citrate extracted from the Kohala O horizon soil. The peaks are all rather broad verifying glomalin is polymeric with some heavy metal (i.e., iron) interference. The two peaks at 0.9 and 1.3 ppm represent methyl (CH_3) and/or methylene (CH_2) groups at about a 1:1 mole ratio. The smaller peak at 2.0 ppm is consistent with aliphatic methine (C**H**) groups. Another peak at 3.6 ppm is present at about two-thirds the area of the peak at 2.0 ppm. All four of these peaks illustrate that glomlain is a polypeptide containing multiple aliphatic amino acids including valine in which the peaks near 3.6 ppm are the peptide backbone methine (N–C**H**–C=O). The broad peak at around 4.6 is indicative of protons associated with

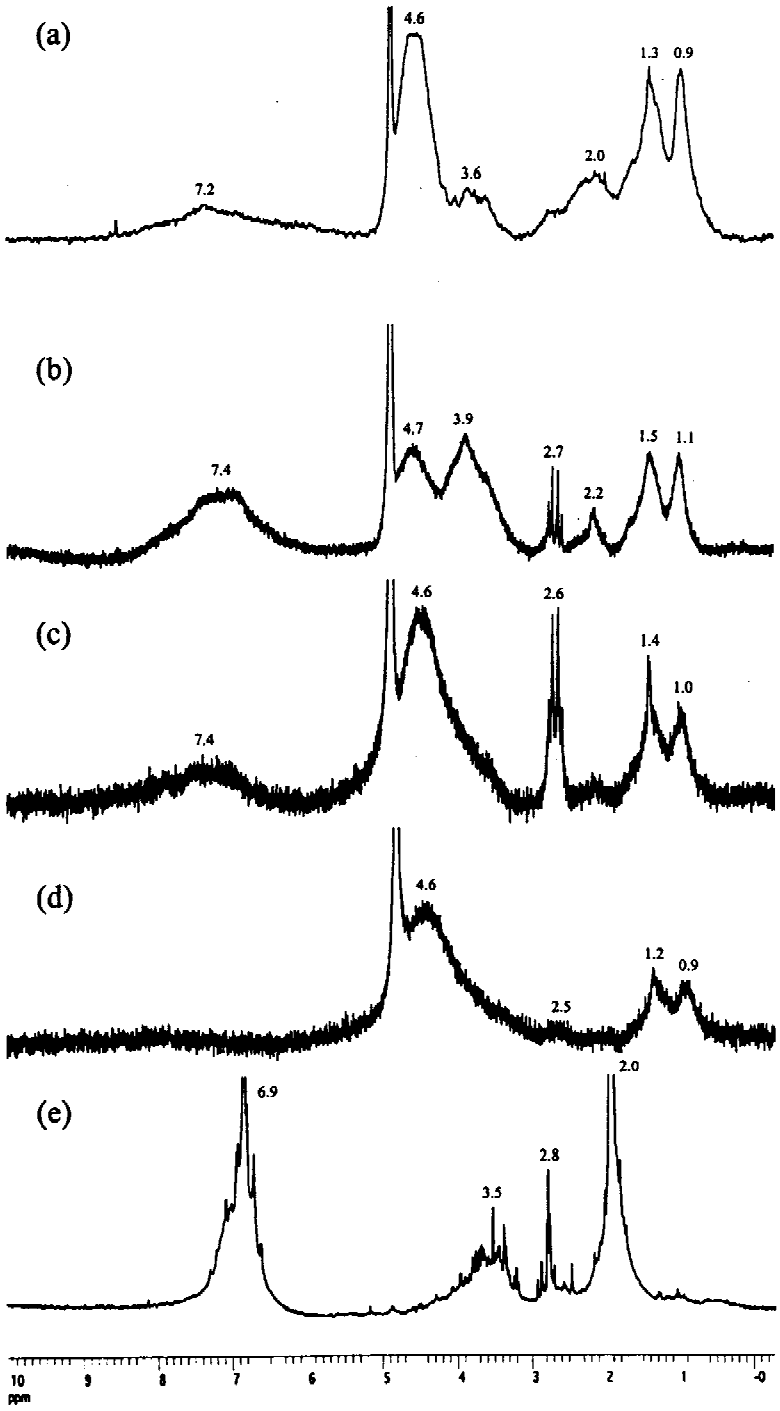

Figure 1. ^{1}H NMR spectra of glomalin extracted from soils and fungal hyphae, and a spectrum for tannic acid: (a) glomalin from O-horizon of Kohala soil, (b) glomalin from hyphae of *G. intraradices*, (c) glomalin from hyphae of *Gi. rosea*, (d) glomalin extracted from Baltimore series soil, and (e) spectrum for tannic acid as comparison.

Table 2. Chemical properties of glomalin (total glomalin pool) extracted from different chronosequence sites and soil horizons (after partial purification by TCA precipitation and borate dialysis). Values are from analysis of one composited sample per horizon and site

Site	Age (10^3 years)	Fe (%)		N (%)		C (%)	
		O	A	O	A	O	A
Thurston	0.3	0.53	2.21	1.1	0.8	17.1	9.9
Olaa	2.1	1.53	2.60	1.2	1.1	15.2	11.5
Laupahoehoe	20.0	0.16	6.46	1.1	1.1	19.4	13.8
Kohala	150	0.62	1.23	1.1	1.5	20.8	22.0
Kolekole	1400	0.04	1.05	0.9	1.4	19.9	18.9
Kokee	4100	0.06	0.34	0.8	1.0	19.9	18.7

Table 3. Microbial, glomalin and soil carbon and nitrogen concentrations (mg cm^{-3}) across the chronosequence. Values are mean and standard error (n=5)

Age (10^3 years)	Microbial C (mg cm^{-3})		Glomalin C and N (mg cm^{-3})				Soil C and N (mg cm^{-3})			
	Carbon		Carbon		Nitrogen		Carbon		Nitrogen	
	O	A	O	A	O	A	O	A	O	A
0.3	0.209	0.191	0.77	1.13	0.062	0.072	61.41	51.8	3.42	3.30
	(0.018)	(0.030)	(0.09)	(0.10)	(0.008)	(0.006)	(3.7)	(8.9)	(0.12)	(0.60)
2.1	0.106	0.162	0.96	0.85	0.092	0.067	54.13	34.4	3.63	2.58
	(0.012)	(0.021)	(0.13)	(0.32)	(0.012)	(0.025)	(5.1)	(3.7)	(0.26)	(0.20)
20.0	0.331	0.279	2.26	2.88	0.181	0.163	121.5	108.2	6.87	6.63
	(0.029)	(0.022)	(0.07)	(0.34)	(0.006)	(0.019)	(4.8)	(7.9)	(0.20)	(0.64)
150	0.327	0.333	3.14	4.53	0.214	0.240	105.2	113.6	5.00	6.91
	(0.028)	(0.014)	(0.31)	(0.64)	(0.021)	(0.033)	(2.8)	(8.2)	(0.32)	(0.33)
1400	0.343	0.226	3.33	2.36	0.247	0.107	114.3	69.1	4.40	4.20
	(0.020)	(0.031)	(0.08)	(0.16)	(0.006)	(0.007)	(2.3)	(7.8)	(0.07)	(0.42)
4100	0.245	0.195	4.92	12.0	0.263	0.484	108.4	233.6	5.00	12.84
	(0.024)	(0.011)	(0.12)	(0.66)	(0.006)	(0.026)	(6.7)	(13.4)	(0.32)	(0.78)

carbohydrates in this glycoprotein. Additional peaks at 7.2 ppm correspond to aromatic ring proton, possibly of aromatic amino acids, in significantly lower mole ratio than the aliphatic protons.

Similar in structure to glomalin from the Kohala soil (Fig. 1a) is glomalin extracted from hyphal samples (Fig. 1b,c) and the Baltimore series soil (Fig. 1d). All four of these spectra have several major structurally related peaks in common including the two peaks at 0.9 and 1.3 ppm at about a 1:1 mole ratio. Also, the hyphal and Hawaiian samples share peaks 7.0–8.0 ppm. These peaks represent the proton in the amide (O=C–N**H**–R) of the peptide backbone and/or aromatic amino acids (Wüthrich, 1986). Glomalin extracted from the hyphal samples (Fig. 1b,c) produces several sharp peaks in the 2.0–3.0-ppm range consistent with peptide methine chains, while glomalin from the Baltimore and, especially, Hawaiian soils have smaller and less sharp representations of these peaks. The hyphal samples can have sharper, more distinct peaks overall than the soil samples because these samples have less heavy metals, especially iron, bound to them. The protein in the soil samples could be in several different conformational states and/or some portions of its sequence could be different. Thus, differences in glomalin origin can produce the differences in peak location (chemical shift) and amplitude observed in various NMR spectra.

In contrast, the tannic acid spectrum (Fig. 1e) is distinctly different from all four of the glomalin spectra. Tannic acid has peaks that are not present on any of the glomalin spectra. These additional peaks are in the

Table 4. Pearson's correlation coefficients (r) of soil age ($\log_{10}$) with glomalin concentrations (mg cm^{-3}) in the O- and A-horizons across the chronosequence. Correlation coefficients in bold indicate significance at $P<0.001$ (df=29)

Glomalin fraction	O-horizon	A-horizon
TG	**0.78**	**0.89**
IRTG	−0.11	**0.85**
EEG	−0.02	**0.63**
IREEG	−0.12	**0.86**

1.5–4.0-ppm region. The tannic acid peak at 6.8 ppm is also present in the glomalin spectra but the amplitude of the tannic acid peak indicates that there is a much higher molar ratio of aromatic protons in tannic acid than in glomalin. Tannic acid does not have the peaks that are seen in all four of the glomalin spectra, namely the two sharp peaks at 0.9–1.5 ppm and the broad peak at 4.0–5.0 ppm. The peaks in the tannic acid spectrum are much sharper than in the glomalin samples because this acid is a smaller molecule with a more uniformly repeating molecular structure. Also, there is a lower concentration of interfering atoms, such as iron. The lack of peaks present in the tannic acid spectrum at their corresponding mole ratio in the glomalin spectra illustrates that not only is tannic acid not present and not bound to the glomalin molecule; glomalin is structurally distinctly dissimilar to tannic acid or tannins.

To determine if other proteins were co-extracted we compared banding patterns of glomalin from known sources (single-fungus isolates) with the crude extract banding patterns. While the typical banding pattern of glomalin was apparent in all extracts, we found no obvious other bands in the gel (Fig. 2).

Glomalin: pattern of concentrations across the chronosequence

Concentrations of all fractions of glomalin were positively correlated with substrate age ($\log_{10}$ transformed) in the A horizon (Fig. 3 and Table 4). In the O horizon, however, only TG was correlated with substrate age (Table 4). No clear pattern emerged with respect to the relative abundance of glomalin in the A and O horizon (Fig. 3). However, the highest glomalin concentrations were observed in the A-horizon of the oldest site, with >60 mg cm^{-3} of TG (this was the equivalent of >100 mg glomalin g^{-1} soil, with soil weight corrected for coarse matter; data not shown).

Concentrations of glomalin in the different fractions were highly positively correlated with each other in the A-horizon, but this was not the case in the O-horizon, where correlations were not very strong and occasionally negative (Table 5). The percentage of TG protein that was immunoreactive was significantly negatively correlated with substrate age ($\log_{10}$) for both the A and O horizon (A horizon: $r=-0.56$; O horizon $r=-0.62$; $P<0.001$). However, no significant correlations were found for the immunreactive percentage of the EEG fraction (A horizon: $r=0.0001$; O horizon: $r=-0.32$; $P>0.05$). The percentage of immunoreactive TG was also highly negatively correlated with the number of extraction cycles required to extract it from soil (A horizon: $r=-0.73$; O horizon: $r=-0.68$; $P<0.001$).

Contribution of glomalin to soil C and N

The amount of C and N in glomalin represented ca. 4–5% of total soil C and N in the oldest soil (Table 3). By contrast, the contributions of soil microbial biomass C to total soil C ranged from only 0.08 to 0.2% for the oldest soil. As a consequence, glomalin-carbon (across the entire chronosequence) was always substantially higher than microbial biomass carbon (Table 3).

Glomalin residence time in soil

The ^{14}C content of total glomalin extracts from the youngest site (O and A horizon combined) was $\Delta^{14}C=173\pm9\%$, respectively. Based on the stock-flow model and its assumptions, this translates to a minimum glomalin residence time in the soil of 6 years, with a possible range of 6–42 years.

Discusssion

We reported here glomalin concentrations that are much higher than the highest values found so far in temperate environments (e.g., Wright and Upadhyaya, 1998). We used comparative gel electrophoresis and NMR spectroscopy to verify that significant co-extraction of other compounds (specifically tannins and other proteins) did not occur.

Controls on the production of glomalin are unknown. Soil factors (e.g., nutrient concentrations, iron concentrations: glomalin contains iron; Wright and Upadhyaya, 1996), climate (growing season length,

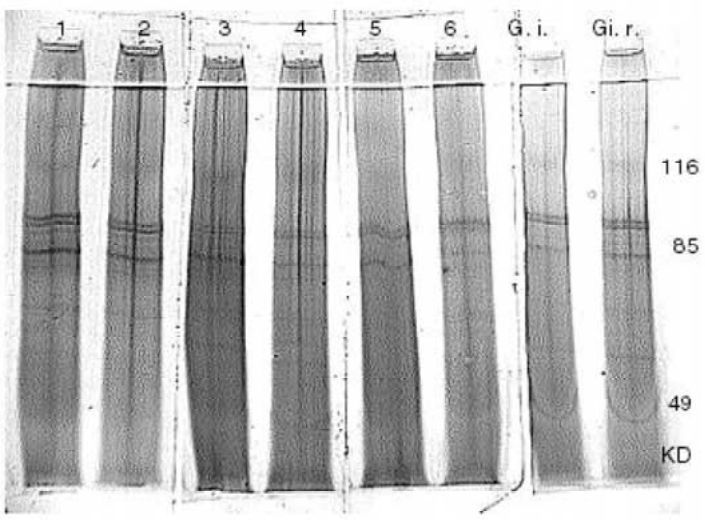

Figure 2. Crude extracts of soils compared to extracts from known sources of glomalin on hyphae (produced on Sudan grass roots) are shown in a photograph enhanced by a high pass 3×3 filter in Image Tool 2.0. Lanes numbered 1–6 are Thurston O, Thurston A, Kohala O, Kohala A, Kauai O and Kauai A, respectively. *Glomus intraradices* EY113/114 (G. i.) and *Gigaspora rosea* FL224 (Gi. r.) are shown for comparison. The positions of molecular weight markers are indicated.

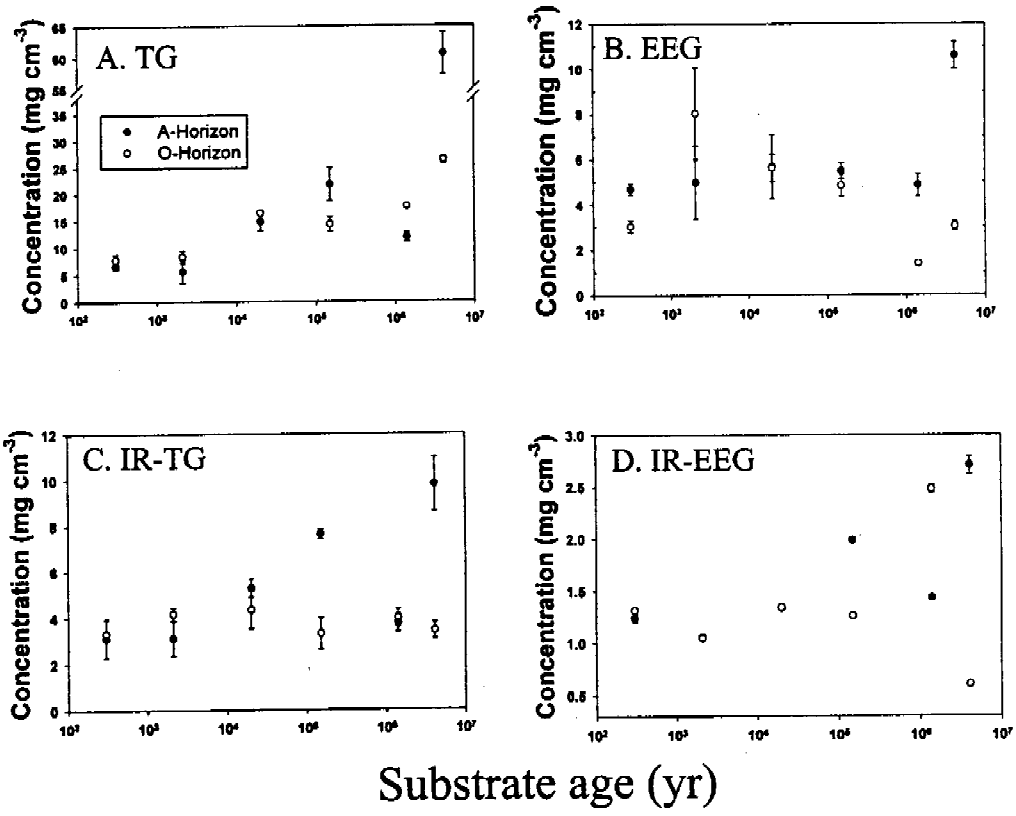

Figure 3. Soil concentrations (g cm^{-3}) of glomalin fractions across the six soil chronosequence sites (A-horizon, filled circles; O-horizon open circles). (A) TG, total glomalin; (B) EEG, easily extractable glomalin; (C) IR-TG, immunoreactive total glomalin fraction; (D) IR-EEG, immunoreactive easily extractable glomalin. Error bars are the standard error of the mean (n=5). When error bars are not shown they are smaller than the symbols. Note log-scale on x-axis.

Table 5. Correlations (Pearson's *r*) of different glomalin fractions in the O- and A-horizons. Bolded ($P<0.001$; df=29) and italicized ($P<0.05$; df=29) correlation coefficients indicate statistical significance

	O-horizon				A-horizon			
	TG	IRTG	EEG	IREEG	TG	IRTG	EEG	IREEG
TG	1.00				1.00			
IRTG	−0.03	1.00			**0.79**	1.00		
EEG	−0.30	0.29	1.00		**0.73**	*0.54*	1.00	
IREEG	*−0.49*	0.27	*0.46*	1.00	**0.90**	**0.81**	*0.68*	1.00

temperature, moisture), the fungi involved (AMF species identity and possibly diversity), and the host plant(s) and their productivity could be important contributors. We suggest that the high concentrations of glomalin in this study can be explained by the particular combination of several factors: the long tropical growing season, the prevalence of arbuscular mycorrhizal plants throughout ecosystem development, the high SOM content, and the high soil iron content.

At least for the A horizon, all glomalin fractions were linearly correlated with the log of substrate age (Table 4); for the O horizon, only TG follows this relationship. It is important to note that this pattern is based on concentrations per soil volume, not total soil stocks. This pattern is consistent with the hypothesis that glomalin accumulated in soil.

This result is also consistent with the alternative hypothesis that glomalin production increased along the chronosequence. Future research goals should clearly include the development of methods to measure production of glomalin in situ. AMF root colonization did not differ between roots obtained from the youngest, oldest, and intermediate (20 000 year) sites (Treseder and Vitousek, 2001), suggesting that at least the dependence of plants on mycorrhizae was not greatly changed across the chronosequence. However, an increase in immunoreactive EEG along the chronosequence suggests that production may have increased. Immunoreactivity of glomalin is gradually lost as the protein is presumably subject to microbial attack, interfering with the site recognized by the antibody (Wright and Upadhyaya, 1996).

The pattern of glomalin concentrations across the sites does not follow the pattern of soil carbon across the sites (Torn et al., 1997), where a maximum was reached at intermediate-age sites, with no apparent trend for increased carbon content in the older sites. Soil mineralogy across these sites was identified as an important contributor to soil carbon storage capacity (Torn et al., 1997). Glomalin sequestration/ stabilization may hence be controlled by mechanisms different from those that control the bulk of soil carbon.

Carbon (C) and nitrogen (N) in glomalin made a substantially larger contribution to the total soil C and N pools than microbial biomass. Soil microbial biomass includes, among many other microbes, the hyphae of AMF. We can therefore safely conclude that the standing hyphal biomass C stock must be much smaller than the glomalin C pool. Glomalin is produced by living hyphae of obligate biotrophic AMF. This means that this carbon arrived in soil via a substantially different pathway than most of the other microbially altered soil carbon: it is not saprobically derived from dead plant organic matter. Our study highlights the importance of AMF and their products in soil carbon storage. Importantly, our results indicate that mycorrhizal fungi may constitute a much larger carbon sink in terrestrial ecosystems than was previously assumed based on the live biomass of these fungi in soils (extraradical hyphae) or in roots (colonizing intraradical mycelium).

Glomalin is present in soils in such an order of magnitude (e.g., >60 mg cm^{-3}, or over 100 mg g^{-1}) that it may have to be considered in studies of plant–soil interactions. For example, glomalin contains iron (see Fig. 1 and its discussion; Table 2). The amount of iron sequestered in glomalin may have interesting consequences for rhizosphere ecology, for example by limiting the amount of iron available for uptake by phytopathogenic fungi. In this context it is interesting to note that iron concentrations varied consistently between O and A horizons (Table 2), perhaps suggesting that glomalin can bind iron in varying amounts based on the soil environment.

It is unknown how available C and N in glomalin are to soil microbial communities (we assume that it cannot be available to AMF due to the fact that they are obligate biotrophs). Decomposition studies have

to be the next step to answer this question, but carbon dating indicated that glomalin carbon (and therefore probably N) may not be turning over very rapidly.

One of the assumptions of the turnover model we used, uniformity of decay, is problematic. It is very likely that there are different pools of glomalin in soil that are stabilized in different ways, resulting in differential turnover. However, in the absence of data on decomposition rates of glomalin, we used this assumption as a first approximation in our model. Our estimate of turnover time could be narrowed to a smaller range if data on glomalin production in soil were available, but currently no methods exist to measure this variable satisfactorily. The estimated turnover time, on the order of several years to decades, is much longer than the turnover time expected for hyphae of AM (on the order or days: Friese and Allen, 1991). This challenges our thinking about AMF contributions to SOM pools, which were typically regarded as short-term, fast turnover (Allen, 1991). We show that AMF products can enter a slower turnover pool of carbon in soil, which would counteract a rapid cycling of carbon in soils, re-entering the atmosphere as CO_2.

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