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Glomalin-related soil protein: Assessment of current detection and quantification tools

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Abstract

Despite the widely acknowledged importance of arbuscular mycorrhizal fungi (AMF) in soil ecology, quantifying their biomass and presence in field soils is hindered by tedious techniques. Hence biochemical markers may be useful, among which glomalin-related soil protein (GRSP) could show a particular promise. Presently GRSP is operationally defined, its identification resting solely on the methods used to extract it from soil (citric acid buffer and autoclaving) and the assays (Bradford/enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody) utilized to detect it. The current assumption is that most non-heat stable soil proteins except glomalin are destroyed during the harsh extraction procedure. However, this critical assumption has not been tested. The purpose of this research was to challenge the GRSP extraction process to determine the accuracy of the Bradford method as a measure of glomalin; and to provide some assessment of the specificity of the ELISA monoclonal antibody. In two studies we spiked soil samples either with known quantities of a glycoprotein (BSA: bovine serum albumin) or with leaf litter from specific sources. After extraction 41–84% of the added BSA was detected with the Bradford method. This suggests that the currently used extraction procedure does not eliminate all non-glomalin proteins. Also, ELISA cross-reactivity against BSA was limited, ranging from 3% to 14%. Additions of leaf litter also significantly influenced GRSP extraction and quantification suggesting that plant-derived proteins, as would occur in the field, had a similar effect as BSA. Litter additions decreased the immunoreactive protein values, suggesting interference with antibody recognition. We conclude that the use of GRSP, especially Bradford-based detection, in the assessment of AMF-derived substances within field soils is problematic, it may be inappropriate in situations of significant organic matter additions.

Keywords: Glomalin; Arbuscular mycorrhizal fungi; ELISA; Bradford method; Monoclonal antibody

1. Introduction

Determining arbuscular mycorrhizal fungi (AMF) biomass in field soils is often difficult and tedious (Jakobsen et al., 1992; Miller et al., 1995; Rillig et al., 1999). As a consequence, biochemical markers such as ergosterol, chitin and glomalin have been considered for the study of AMF. Because several organisms produce ergosterol and chitin, their usage as AMF indicators is somewhat limited (Frey et al., 1994). Furthermore, Olsson et al. (2003) found that AMF may not contain ergosterol. Because glomalin-related soil protein (GRSP) has been linked with AMF, several studies have used this substance to identify AMF presence in greenhouse sand cultures (Wright et al., 1996). AMF hyphal growth has been related to GRSP production in sand cores from tropical forest soils (Lovelock et al., 2004) and horticultural mesh traps from field soils (Wright and Upadhyaya, 1999). Krivtsov et al. (2004) utilized soil GRSP pools to directly estimate AMF biomass changes in forest soils.

Currently GRSP is operationally defined, meaning that the identification of this protein rests on the methods used to extract it (citric acid buffer, autoclaving at pH of either 7.0 or 8.0) and the assays (Bradford method/enzyme-linked immunosorbent assay (ELISA) with MAb32B11) used for quantification (Rillig, 2004). Several studies suggest that the ELISA is quantifying the immunoreactive portion of the total GRSP pool (Wright and Upadhyaya, 1996, 1999; Wright et al., 1996, 1999; Rillig and Steinberg, 2002).

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The current assumption in using the Bradford method is that all or the vast majority of proteins are destroyed during the harsh extraction procedure except glomalin. The ELISA assay relies additionally on a monoclonal antibody (MAb32B11) raised against the crushed spores of the AMF *Glomus intraradices* (Wright et al., 1996). The monoclonal antibody used reacts strongly with all AMF species tested (Wright et al., 1996) and does not significantly cross-react with non-AMF fungal species examined (Wright et al., 1996). Values obtained from the Bradford method and ELISA assay are often well correlated, thus providing circumstantial evidence that the extraction process is mostly measuring glomalin (Wright and Upadhyaya, 1996, 1999; Wright et al., 1996).

The gene for the AMF protein glomalin has recently been sequenced in our laboratory (Gadkar and Rillig, unpublished); yet it remains difficult to assess the relationship between GRSP obtained from soil and glomalin. Hence a new nomenclature has been introduced (Rillig, 2004; Table 1), clearly separating glomalin from soil-derived protein pools (GRSP). In this context, several questions remain: does the extraction method used destroy all other proteins besides glomalin, or do other proteins survive the extraction process and contribute to the GRSP pool? How specific is the monoclonal antibody used in the MAb3211B-based ELISA process?

Here we take an indirect approach to addressing some of these questions: spiking soils with non-glomalin protein or non-glomalin containing substrates (containing a mixture of proteins). We wished to test the response of the different GRSP fractions when challenged with extraneous non-glomalin protein additions, and to provide additional tests of the MAb32B11-ELISA. We conducted two studies: (i) samples from two different soils were spiked with varying quantities of a thermolabile protein (bovine serum albumin (BSA)) of similar size to glomalin and (ii) soil samples were amended with leaf litter from specific sources (*Poa annua*, *Populus trichocarpa* and *Pinus ponderosa*). If the extraction process eliminates all non-heat stable proteins, then the Bradford and ELISA assays will not be strongly influenced by extraneous protein additions of BSA and leaf litter. Furthermore, we hypothesized that the ELISA-based protein values would be influenced by the presence of BSA and leaf litter proteins to a lesser degree.

2. Materials and methods

2.1. Soil description

For experiment 1, we used two different soils (referred to as CB and P22-B) collected from the Nyack Floodplain (western Montana; 48°29′N, 114°00′W on the middle fork of the Flathead River) for which soil OM and GRSP (Table 2) levels differed as a consequence of soil age (Harner et al., 2004). Soils were collected to a depth of 20 cm, air dried, and stored at room temperature prior to use. Experiment 2 utilized only the 1-year old soils.

2.2. Soil extraction

There are currently two detection methods utilized to quantify GRSP: Bradford protein assay, yielding Bradford reactive soil protein (BRSP), and an ELISA, using the monoclonal antibody MAb32B11 developed against crushed spores of *Glomus intraradices* (Wright and Upadhyaya, 1998), yielding the immunoreactive soil protein (IRSP). The first step in the extraction process is to recover the EE-BRSP and EE-IRSP soil fractions (EE = easily extractable). This was done by autoclaving 1.0 g of soil with 20 mM sodium citrate, pH 7.0 at 121 °C for 30 min. Only one autoclave cycle is required to obtain this fraction. Following this extraction process, the BRSP and IRSP fractions were extracted from the same soil sample using 50 mM sodium citrate, pH 8.0 and repeated autoclaving at 121 °C for 60 min. After each extraction/autoclaving cycle, the sample was centrifuged at 5000*g* for 15 min. The supernatant was decanted and stored at 4 °C until analysis. The extraction process continues until the supernatant is clear/light yellow in color. Once the extraction process was complete, each extract was centrifuged at 10,000*g*. The Bradford assay was first utilized to determine the concentration of EE-BRSP and BRSP using BSA as a standard. Immunoreactive protein values were measured using an indirect ELISA with MAb32B11 (Wright and Upadahyaya, 1996).

2.3. Protein addition

We applied BSA (Fisher Scientific, Fair Lawn, New Jersey) to each sample at five and 10 times the background IRSP level detected in each soil. The CB (1-year-old soils) soils received 2.8 or 5.6 mg of BSA, and P22-B (66 year old soils) soils, 11.2 or 25.0 mg of BSA. We selected this level of protein additions in order to effectively challenge the detection methods. BSA was thoroughly mixed into 20 g of field soil at the specified rate (Precision Scientific Shaker) for 2 h. After mixing, 1.0 g of soil was removed from each tube and immediately extracted. This was done in order to decrease the chance of microbial activity influencing the amount of protein in each sample. All treatments were replicated eight times.

2.4. Leaf litter addition

The second portion of our experiment set out to determine the influence of plant-derived proteins on the GRSP pool. Leaf material was collected from *Poa annua*, (annual Bluegrass), *Populus trichocarpa* (Black Cottonwood) and *Pinus ponderosa* (Ponderosa Pine). Samples were air dried at 80 °C for 24 h, and subsequently blended into a fine powder. The blended leaf material (0.15 g) was added to 1.0 g of CB soil and mixed for 2 h (Precision Scientific Shaker). The soil leaf mixture was extracted immediately and analyzed using the methods described above. All treatments were replicated five times.

Table 1
Proposal for new terminology for various fractions of soil proteins and glomalin adapted from Rillig (2004)

Old usage	Identity	Proposed new name/usage	Reason for change
TG (total glomalin)	Bradford-reactive soil protein after extensive extraction	BRSP (Bradford reactive soil protein)	Bradford method measures all protein sources; may be non-specific (see this study)
EEG (easily extractable glomalin)	Bradford-reactive soil protein after mild extraction	EE-BRSP (easily extractable BRSP)	Bradford method measures all protein sources; may be non-specific (see this study)
IRTG (immunoreactive total glomalin)	Immunoreactive (MAb32B11) soil protein identified after extensive extractions	IRSP (immunoreactive MAb32B11 soil protein)	Potential for antibody cross-reactivity or sensitivity issues (see this study)
IREEG (immunoreactive easily extractable glomalin)	Immunoreactive (MAb32B11) soil protein identified after mild extraction	EE- IRSP (easily extractable immunoreactive MAb32B11 soil protein)	Potential for antibody cross-reactivity or sensitivity issues (see this study)
Glomalin	Old term used to identify all protein pools measured by Bradford and ELISA (i.e., TG, EEG, IRTG, and IREEG) and the actual protein	GRSP (Glomalin related soil protein)	To clearly separate soil-derived protein from the putative gene product
Glomalin (*sensu stricto*)	Currently unknown identity, theoretically glomalin should be similar to soil glomalin pools (in particular immunoreactive pools)	Glomalin(s)	The name glomalin should be reserved for the gene product

Table 2
Physical description of Nyack Floodplain soils

Site	Age	pH	Texture	Sand (%)	Clay (%)	Silt (%)	SOM (%)	IRSP ($mg\,g^{-1}$)
CB	1	7.9 (0.03)	Sandy loam	72	14	14	0.80 (0.05)	0.56 (0.01)
P22-B	66	7.7 (0.06)	Sandy loam	66	14	20	2.20 (0.07)	2.50 (0.03)

Values are means (standard error; $n = 3$).

2.5. Data analysis

In order to determine the amount of added BSA remaining after the extraction process (%), as detected by the Bradford method, we utilized the formula (BRSP−background BRSP)/BSA addition × 100. The amount of ELISA cross-reactivity (%) as a result of BSA addition was determined as (IRSP−background IRSP)/BSA addition × 100. ANOVA was used to test for treatment differences. Where *F*-ratios were significant ($P<0.05$), treatment means were compared via Tukey–Kramer test (JMP, SAS Institute). Non-parametric analyses (Kruskal–Wallis test) were utilized if data failed to meet parametric assumptions. Where *H*-values (Kruskal–Wallis test statistic) were significant ($P<0.05$), treatment means were compared via Kruskal–Wallis multiple comparison *Z*-value test (NCSS, 2000). The CB 5×, 10× and P22-B 5×, 10× treatments contained two statistical outliers and the *Poa annua* treatment contained one outlier, all of which were removed prior to analysis. We defined outliers as data points which are two standard deviations above/below the sample mean. Finally, we have restricted comparisons to within soil types.

3. Results

3.1. BSA addition experiment

BRSP concentrations ($mg\,g^{-1}$) across all treatments are shown in Fig. 1A. Significant amounts of BSA persisted after the extraction process and were detected by the Bradford method for both the easily extractable ($P_{CB} = 0.002$ [Kruskal—Wallis]; $P_{P22\text{-}B} = 0.0001$[ANOVA]) and total protein extractions ($P_{CB} = 0.0001$; $P_{P22\text{-}B} = 0.0001$ [ANOVA]). This pattern of increase occurred regardless of soil type.

The proportion of BSA (%) remaining after the extraction process was determined for each treatment and soil type (Table 3). The residual BSA detected by the Bradford method did differ significantly between the CB 5× and CB 10× EE-BRSP extraction. However, we did not detect significant differences in any of the other treatments. The proportion of BSA remaining after extraction was directly influenced by the amendment amount.

The addition of BSA significantly influenced the amount of EE-IRSP ($P_{CB} = 0.001$; $P_{P22\text{-}B} = 0.002$ [Kruskal–Wallis])

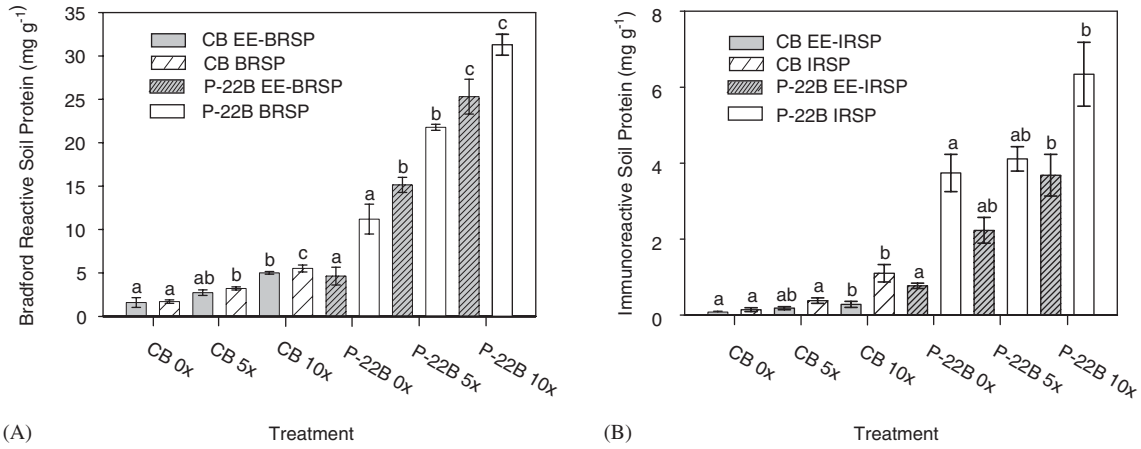

Fig. 1. (A) Bradford reactive soil protein (BRSP) for both sites CB and P-22B BSA amended soil treatments. BRSP concentration was calculated as mg g^{-1} soil. Means (+) SE were compared with Kruskal–Wallis multiple comparison Z-value test (CB EE-BRSP and BRSP) or Tukey–Kramer (P-22B EE-BRSP and BRSP) significance accepted at $P<0.05$. (B) Immunoreactive soil protein (IRSP) for both sites CB and P-22B BSA amended soil treatments. IRSP concentration was calculated as mg g^{-1} soil. Means (+) SE were compared with Kruskal–Wallis multiple comparison Z-value test (CB and P-22B EE-IRSP) or Tukey–Kramer (CB and P-22B IRSP), and significance accepted at $P<0.05$.

Table 3
Percentage of BSA remaining after extraction process

Fraction	Soil	BSA addition				F or H (P)
		5 ×		10 ×		
		Mean	SE	Mean	SE	
EE-BRSP	CB	34.0	6.27	61.1	2.73	18.3 (0.002)
	P22-B	83.0	8.50	74.0	7.50	1.49 (0.25)
BRSP	CB	52.4	6.30	67.4	7.13	2.34 (0.16)
	P22-B	84.7	3.40	83.7	6.62	0.00 (1.00)

Treatment comparisons were restricted to within soil type, e.g. CB (5) EE-BRSP (%) was analyzed against CB (10) EE-BRSP (%).

Table 4
The percentage of ELISA cross-reactivity

Fraction	Soil	BSA addition				F(P)
		5 ×		10 ×		
		Mean	SE	Mean	SE	
EE-IRSP	CB	3.51	1.40	3.55	1.35	0.001 (0.98)
	P22-B	2.71	1.30	10.4	3.34	5.30 (0.05)
IRSP	CB	8.50	2.32	14.2	2.42	2.78 (0.13)
	P22-B	10.6	2.88	11.6	2.21	0.07 (0.80)

Treatment comparisons were restricted to within soil type, e.g. CB (5) EE-IRSP (%) was analyzed against CB (10) EE-IRSP (%).

and IRSP ($P_{CB} = 0.0001$; $P_{P22\text{-}B} = 0.02$ [ANOVA/log transformed]) (Fig. 1B). Significant levels of monoclonal antibody (MAb32B11) cross-reactivity did occur when treatment soils were saturated with BSA (i.e. 10 × treatments).

The percentage of BSA-induced cross-reactivity was calculated for each soil type and treatment (Table 4). Similar to the trend observed for the Bradford method, as BSA increased in concentration so did the percentage of ELISA cross-reactivity. Significant difference in cross-reactivity was observed for the P22-B EE-IRSP treatments. However, no significant differences in percent cross-reactivity occurred between any of the other treatments.

3.2. Leaf litter additions

All of the leaf litter treatments and extractions contained significantly greater proportions of protein than the control ($P_{EE\text{-}BRSP}<0.001$ [Kruskal–Wallis]; and $P_{BRSP}<0.001$ [ANOVA/log transformed]) (Fig. 2A).

The IRSP values differed significantly ($P<0.001$ [ANOVA]) between treatments (Fig. 2B). The control and *Poa annua* treatments had greater concentration of IRSP than the other treatments. The EE-IRSP extraction results did not differ significantly ($P<0.06$ [ANOVA]) between treatments, yet they followed the same pattern as the IRSP results.

4. Discussion

There are several important implications of these results in regards to GRSP detection and quantification. First they challenge the suitability of the Bradford method and the extraction process in measuring glomalin (i.e., the AMF product) pool size. Our results indicate that a significant portion of BSA was not eliminated during the GRSP extraction process and was detected by the Bradford method. BSA is a globular glycoprotein with a molecular weight of ~66 kDa (Relkin, 1996, Kanny et al., 1998), denaturing at 65 °C (Ruegg et al., 1977, Kanny et al., 1998, Aparicio et al., 2005). The extraction of GRSP from soil is considered harsh and would be expected to quickly and effectively denature BSA beyond Bradford detection. However, the Bradford assay is capable of detecting peptides as small as 3000 Da (Sedmak and Grossberg,

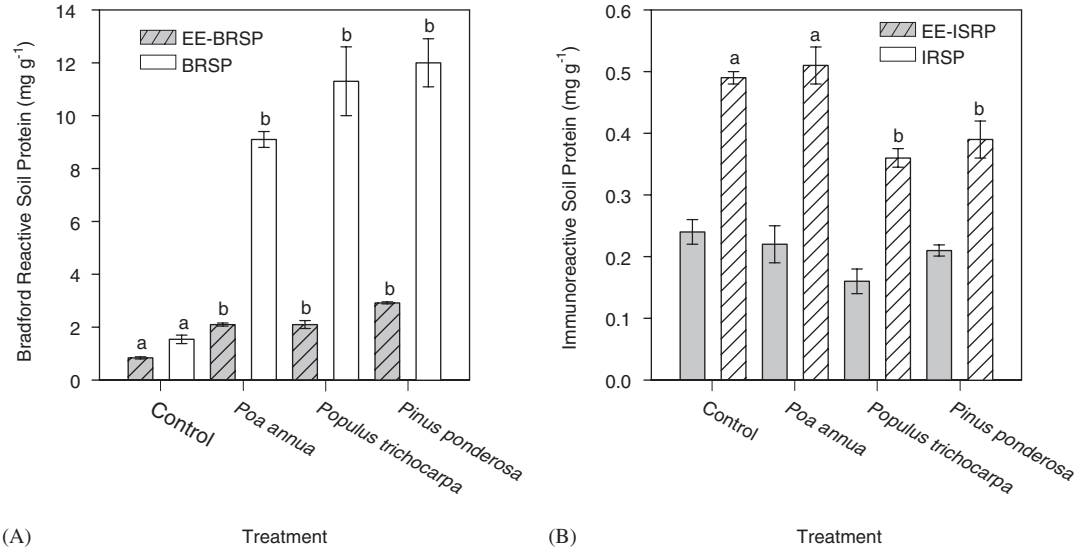

Fig. 2. (A) The amount of leaf litter proteins detected by the Bradford method after GRSP extraction process was caculated as mg g^{-1} soil. Means (+) SE were compared with Kruskal–Wallis multiple comparison Z-value test (EE-BRSP) or Tukey–Kramer (BRSP), and significance accepted at $P<0.05$. (B) The effect of leaf litter proteins on GRSP quantification via ELISA is expressed as mg g^{-1} soil. Means (+) SE were compared with Tukey—Kramer test, and significance was accepted at $P<0.05$.

1977). It is unlikely that the GRSP extraction process would reduce BSA to less than 3000 Da. Also, we found that as BSA concentration increased per treatment, the ability of the GRSP extraction process to denature it beyond Bradford method detection was diminished (Table 3). BSA when heated forms a gelatin matrix through disulfide and non-covalent bonds (Matsudomi et al., 1993).

We have shown that the AMF monoclonal antibody (MAb32b11) is only slightly cross-reactive when extraneous amounts of BSA were added, suggesting that the ELISA is a more accurate reflection of glomalin. Monoclonal antibodies have proven to be highly specific in their ability to differentiate between fungal isolates (Thornton et al., 1993, 1994; Dewey et al., 1996; Thornton and Dewey, 1996; Thornton and Gilligan, 1999). Before the development of a monoclonal antibody, the primary method of detecting AMF involved polyclonal antibodies (Wright et al., 1996). Polyclonal antiserum has mainly been developed against AMF spores (Kough et al., 1983; Hahn et al., 1993; Friese and Allen, 1991) and hyphae (Wilson et al., 1983; Gobel et al., 1995). In all cases, the main limitation with these polyclonal antibodies was their high degree of cross-reactivity.

Our results further suggest that the GRSP extraction process does not denature all plant-derived protein sources. These proteins are of sufficient size and quantity after extraction to be detected by the Bradford method. There are several widespread plant-derived protein classes that may withstand autoclaving, including dehydrins (Robertson et al., 1994; Tabaei-Aghdaei et al., 2000; Volaire, 2002; Pelah et al., 1995; Wisniewski et al., 1996; Caruso et al., 2002; Jarvis et al., 1996; Richard et al., 2000) and heat shock proteins (Wisniewski et al., 1996; Hall, 2002; Burke et al., 1985; Mansfield and Key, 1987; Schöffl et al., 1998). This possibility had not previously been considered in GRSP measurements.

Our results indicate that leaf litter can reduce the sensitivity of the ELISA assay. ELISA sensitivity loss can occur as a result of retention or interference bias (Otten et al., 1997). Retention bias occurs when the antigen becomes attached to organic compounds and is not released during the extraction process (Otten et al., 1997). This is especially critical in soils with high organic matter (Schnitzer, 1982). Interference bias takes place when soluble soil components attach to the wall of the micro titer plate displacing the antigen of interest (Otten et al., 1997). Based on our Bradford results, we know that significant amounts of protein were extracted from each of the leaf litter treatments. It is plausible that some component (i.e. tannins or resins) contained in the *Populus trichocarpa* (Black Cottonwood) and *Pinus ponderosa* (Ponderosa Pine) treatments suppressed detection of GRSP.

In summary, we have shown that the GRSP extraction process does not eliminate all non-glomalin protein sources. Thus the use of the Bradford method can be influenced by soil organic matter and is not an accurate reflection of glomalin (sensu stricto, i.e. the gene product; Rillig (2004)) pool size. Furthermore, the ELISA may be prone to both retention and interference biases depending on the amount of organic matter contained in the soil sample. Based on these findings, the Bradford method and ELISA assay may be useful in measuring glomalin pools, when organic matter concentrations are low such as washed, autoclaved soil (Lovelock et al., 2004; Wright and Upadhyaya, 1999), or in other controlled experimental conditions. However, in many other situations, especially in the field, or under conditions where significant extra-

neous protein additions occur (i.e. manure, sewage, and litter fall), we strongly caution against the use of the Bradford method to assess glomalin pools in soils. Further research should include testing methods for the reduction of retention and interference bias of the ELISA as outlined by Otten et al. (1997).

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