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Research Letter

Soil Protein as a Rapid Soil Health Indicator of Potentially Available Organic Nitrogen

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Core Ideas

- The extraction protocol for "glomalin" extracts protein from a wide variety of sources.
- The term *glomalin* or *glomalin-related soil protein* is inaccurate and limits the utility of the method.
- The extracted protein pool should be viewed more broadly as a soil health indicator of potentially available organic N.

Abstract: Increased interest in practical, routine evaluation of soil health has created a need for rapid and inexpensive indicators that reflect soil nitrogen (N) status. Here we propose a soil protein measurement as an indicator of a functionally relevant and sensitive pool of organic N that can be rapidly quantified in soil testing laboratories. The procedure is based on a method that was historically used to measure "glomalin," a pool putatively of arbuscular mycorrhizal fungal origin. Laboratory validation experiments demonstrate that the procedure extracts proteins from a wide range of sources, not just glomalin, and that continued use of the term *glomalin* is inaccurate and limits the application of the method. Therefore, we propose that the pool of proteins extracted by this method can be viewed more broadly as a soil health indicator that reflects the primary pool of organically bound N in soil and thus as potentially available organic N. We provide a laboratory protocol that details autoclaving soil in a neutral sodium citrate buffer solution followed by clarification and protein quantification steps.

Rapid and inexpensive tests are needed for routine use in soil health assessment. Despite the significance of nitrogen (N) from both agronomic and environmental perspectives, most routine soil nutrient tests performed by commercial soil testing laboratories do not include measurements of total soil N or a labile fraction of organic N. Although most commercial laboratories will offer soil inorganic N (nitrate and ammonium), these measurements are not typically included in their routine soil nutrient test, which most often includes pH, organic matter and extractable P, base cations, and micronutrients. The lack of this type of test creates large uncertainty for farmers and can lead to a management framework of mitigating production risks by applying N fertilizer in excess of crop needs (Scharf, 2015). Why then, after decades of work, has the scientific community not converged on an accepted method (or set of methods) to measure soil N routinely on production fields? The reasons are numerous and complex, but the lack of rapid and cost-effective methods identified for soil N testing, along with a meaningful interpretation that can provide growers with useful management decisions, is a primary constraint. An effective N measurement tool must have the following characteristics: (i) be sensitive to management, (ii) be rapid and inexpensive, (iii) be amenable to the high-throughput framework of commercial soil testing labs, and (iv) reflect a functionally important N pool (Doran and Zeiss, 2000).

Numerous soil test methods that reflect N status in soil exist. Table 1 shows tests that measure total soil N or some fraction or pool of soil N. These methods have been developed over the years for different goals. Incubation-based N mineralization methods provide results that more accurately reflect N supply potential over a growing season (Keeney and Bremner, 1966; Stanford and Smith, 1972); however, laboratory incubations of soil are not attractive

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Abbreviations: ACE, autoclaved-citrate extractable; AMF, arbuscular mycorrhizal fungi; EEG, easily extractable glomalin; GRSP, glomalin-related soil protein; PBS, phosphate buffered saline.

where timeliness is an important consideration. Inorganic N (nitrate and ammonium) is a good indicator of immediate plant-available N, but this pool is too ephemeral and thus can be an inadequate indicator of plant available N over the growing season, as weather-related environmental losses and plant uptake can lead to significant changes in a matter of days (Culman et al., 2013; Sela et al., 2017). In contrast, total soil N is too stable to reliably reflect seasonal N availability, as management-induced changes can occur very slowly over time.

Chemically extractable labile organic N fractions are another means to assess the capacity of soils to supply N (Ros et al., 2011). The labile, organic N pool is a good candidate for a soil health assessment as it would be responsive to management changes and seasonal N availability (Wander, 2004). However, current commercially available N tests that measure amino sugars, such as the Illinois soil N test (Khan et al., 2001; Mulvaney et al., 2001) have had limited success at estimating mineralizable N (e.g., Barker et al., 2006; Marriott and Wander, 2006; Osterhaus et al., 2008; Spargo et al., 2009). From a review and meta-analysis of published data, Ros et al. (2011) found that extractable organic N fractions measured using various chemical methods were positively related to mineralizable N but explained only a small proportion (47% on average) of the variation in mineralizable N. Thus, continued work is essential to identify and develop a simple, reliable indicator (or set of indicators) of soil N availability.

Of the compounds that constitute soil organic matter, proteins represent by far the largest pool of organic N in soil (Weintraub and Schimel, 2005; Jan et al., 2009; Nannipieri and Paul, 2009). Depolymerization (rate of amino acid supply), rather than the breakdown of amino acids to ammonium N, is considered to be the rate-limiting step in soil N cycling (Schimel and Bennett, 2004; Weintraub and Schimel, 2005; Jan et al., 2009; Mooshammer et al., 2012). Therefore, soil proteins measure the size of the pool being depolymerized (i.e., the source) and serve as a reservoir of N that is subsequently released through mineralization processes (Roberts and Jones, 2008). Soil protein has been identified as an effective soil health indicator of bioavailable N by recently established initiatives, such as the Soil Health Institute (https://soilhealthinstitute.org/). The soil protein procedure, as presented here (see Supplemental Material), is readily amenable to routine quantification and is already an indicator included in commercially available soil health assessments, such as the Cornell Assessment of Soil Health Framework (Moebius-Clune et al., 2016; Schindelbeck et al., 2016).

The soil protein extraction protocol is based on a neutral sodium citrate buffer solution, the most frequently used extraction technique for "glomalin"– a protein reportedly produced in large quantities by arbuscular mycorrhizal fungi (AMF; Wright et al., 1996). Until now, glomalin-related soil protein (GRSP) has been only operationally defined by its extraction method and quantification assays used to detect it. A key assumption is that the extraction procedure destroys most non-heat stable soil protein except glomalin (Wright and Upadhyaya, 1996, 1998; Rillig et al., 2003). However, a study by Rosier et al. (2006) and others since (Purin and Rillig, 2007; Schindler et al., 2007; Gillespie et al., 2011) suggested that the methodology for glomalin extraction extracts more than just glomalin. Despite these reports, many authors still assume the protein pool extracted using sodium citrate buffer is of AMF origin. Web of Science (accessed in January 2018 using "glomalin*" as a primary search term) reported 398 publications since 1996, when Wright and Upadhyaya (1996) first demonstrated that glomalin can be extracted from soil. Of these, >300 were published after the paper by Rosier et al. (2006) that clearly demonstrated how the glomalin extraction protocol is capable of extracting a range of proteins, not just glomalin. Therefore, the basic question remains the same: Is the protocol used to extract glomalin exclusive to mycorrhizal fungi?

Table 1. Summary of soil test methods that reflect nitrogen status in soils.

	Total soil N	Organic matter	Inorganic N (nitrate and ammonium)	Mineralizable N	Organic labile N
Method	Dry combustion†	Loss on ignition‡	2 M KCl extraction§	Aerobic 28+-d incubations, anaerobic 7-d incubation	Soil protein, hydrolyzable amino sugars
Responsiveness to management changes¶	Slowly, several years	Slowly, several years	Very rapidly, within days	Intermediate, within 1–3 yr	Intermediate, within 1–3 yr
High-throughput	No	Yes	Yes	No	Yes
Rapid and inexpensive	No	Yes	Yes	No	Yes
Soil function the test reflects	Total pool of soil N	Total pool of C (and N)	Immediately plant available N pool	Mineralizable pool of soil N	Mineralizable pool of soil N
Overall potential as a soil health indicator	Expensive as routine soil test; management-induced changes occur very slowly over time	Management-induced changes occur very slowly over time; low analytical precision; not ideal indicator of nutrient availability	Ephemeral; large changes possible over days; thus, not robust indicator	Expensive and time consuming; thus, not suitable for high-throughput	Relatively new methodologies, promising for practical and routine soil health testing

† Nelson and Sommers (1996).

‡ Ball (1964); Combs and Nathan (1998).

§ Keeney and Nelson (1982).

¶ "Responsiveness to management changes" here refers to the length of time it takes to detect management-induced changes using the various soil test methods.

The overall goals of this paper are (i) to provide preliminary evidence for using the soil protein test as a soil health indicator of potentially available organic N, and (ii) to test the hypothesis that the glomalin extraction method extracts proteins from a wide range of sources that are not of AMF origin.

Materials and Methods

Data Sources

For this study, we used sand amended with a known organic matter type, along with soil (<2 mm) collected from a long-term tillage/residue trial in New York to test the hypothesis whether proteins can be extracted in significant quantities from sources other than AMF. Leaves from corn (*Zea mays* L.), bean (*Phaseolus vulgaris* L.), and common weeds (*Thlaspi arvense* L., *Amaranthus retroflexus* L., and *Malva* sp.) were used to represent plant samples; beef and chicken meat to represent animal tissues; and white button mushroom (*Agaricus bitorquis*) and oyster mushroom (*Pleurotus ostreatus*) to represent fungal samples. Plant, animal tissue, and fungal samples were washed with deionized water, dried (60°C) to constant weight, and ground to a coarse powder (<0.5 mm). For each organic matter type, 5 mg of dried substrate and 95 mg acid-washed fine white quartz (Sigma) were added into a 20-mL centrifuge tube. A 1.0-g sand-only blank was included as a control. Each experimental unit, including the control, was replicated in six tubes ($n = 6$).

Extraction and Measurement of Protein

All materials were extracted using the "easily extractable glomalin" protocol of Wright and Upadhyaya (1996, 1998). Briefly, 8 mL of 0.02 mol L^{-1} sodium citrate (pH 7.0) was added to each tube containing 1.0 g of either sand amended with a single substrate (plant, animal, or fungal sample), soil sample, or sand only (control) and mixed well. The tubes were autoclaved (121°C, 15 psi) for 30 min. Following autoclaving, the tubes were cooled and centrifuged (3100 × *g*) for 15 min. The supernatants were decanted and stored at 4°C until analysis. Protein measurements in extracts were made using the Bradford assay (Bio Rad Laboratories). Five microliters of each sample extract was pipetted into individual wells of a 96-well microtiter well plate containing 195 μL phosphate buffered saline (PBS). Then, 50 μL of undiluted Bradford reagent (Bio-Rad G-250 dye) was added to each well. Plates were read 5 min later at 590 nm using a Packard SpectraCount colorimetric microplate reader (Packard Instrument Co.). Protein concentration in each sample was calculated by comparing absorbance values to a standard curve of 0 to 500 μg mL^{-1} of bovine serum albumin in 0.02 mol L^{-1} sodium citrate (pH 7.0) and diluted in PBS. Bonferroni's pairwise comparisons of means were used to separate significant differences in protein concentrations between samples at $\alpha = 0.05$. For further details of the laboratory experiment, see Clune (2007).

Results and Discussion

Measurable quantities of protein were extracted from numerous sources unrelated to mycorrhizal fungi. Protein concentrations in plant tissue, animal tissue, and non-AMF fungal materials were significantly higher than those from soil samples and the sand-only control (Fig. 1). These results are consistent with the findings of Rosier et al. (2006), who measured significantly more protein from soils spiked with a nonmicrobial protein and from leaves of various plants compared with a control soil. These findings collectively demonstrate that the method used to extract the "easily extractable glomalin" (EEG) in fact extracts protein from a wide variety of sources. However, it is possible that heat-sensitive, mineral-associated, and membrane-bound proteins

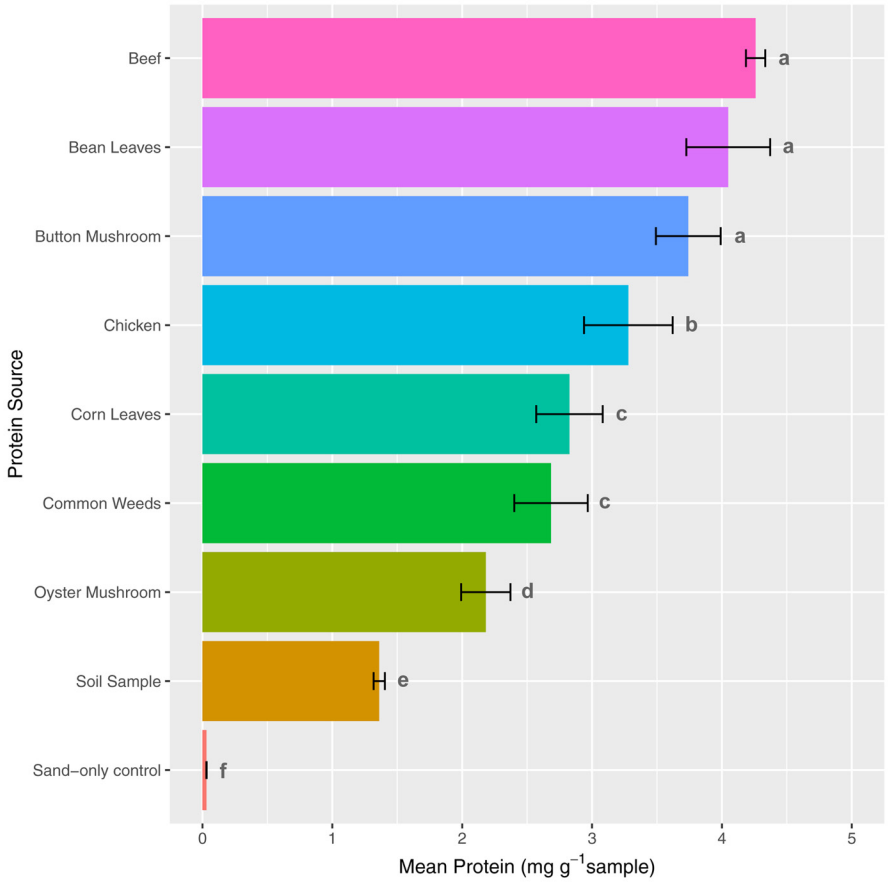

Fig. 1. Mean protein concentrations in control (pure sand), sand amended with different substrates (plant, animal, or fungal samples), and soil subjected to the standard extraction protocol for "easily extractable glomalin" and quantified using the Bradford assay with bovine serum albumin as a standard protein. Mean values with different lowercase letters were significantly different at the $p < 0.05$ level. Error bars represent standard errors of the mean ($n = 6$).

may not be recovered by the citrate buffer extraction protocol. Specific protein types recovered or not recovered by this protocol should be a topic for further research. Thus, we suggest that the extracted protein pool in autoclaved-sodium citrate extracts is more accurately referred to as *autoclaved-citrate extractable soil protein* (ACE protein) or *soil protein*, not *glomalin* or *GRSP*. The continued use of the terms *glomalin*, *EEG*, or *GRSP* perpetuates the misconception that the extracted proteins are primarily associated with the AMF of the phylum Glomeromycota. This is not only misleading and scientifically imprecise, but it also limits the utility, interpretation, and application of the method. Hereafter, we will use the terms *soil protein* and *ACE protein* interchangeably.

Broader Implications for Soil Protein as a Soil Health Metric

A large body of literature has demonstrated the utility of soil protein as an important soil N pool that is sensitive to management practices, a key criterion of a useful soil health indicator. Numerous studies have shown, for example, that soil protein is responsive to tillage and crop rotational diversity (Rillig et al., 2003; Borie et al., 2006; Liebig et al., 2006; Moebius et al., 2007; Wright et al., 2007; Moebius-Clune et al., 2008; Emran et al., 2012; Nichols and Millar, 2013). In an analysis of data from a recent study by Roper et al. (2017), soil protein was strongly affected by tillage intensity and strongly related to corn grain yields (r = 0.94), more than 24-h respiration (r = 0.87) and total organic matter (r = 0.61). Moreover, soil protein is often positively correlated with aggregate stability (r = 0.53–0.84; Wright and Upadhyaya, 1998; Wright et al., 1999; Wright and Anderson, 2000; Rillig et al., 2001; Wu et al., 2014), another key indicator of soil physical structure and overall soil health. Recently, Fine et al. (2017) reported soil protein values for 2451 samples from a wide range of soils in the US Mid-Atlantic, Midwest, and Northeast regions. Median values were 9.0 (range: 1.3–31.1), 11.7 (range: 0–242.2), and 14.5 (range: 2.2–60) mg g^{-1} soil for coarse-, medium-, and fine-textured soils, respectively. In addition, preliminary results of ongoing research from corn fields on Ohio farms (n = 146) showed that soil protein is significantly related ($p < 0.01$) to both total soil N (r = 0.52) and 7-d anaerobic mineralizable N (r = 0.13). The latter relationship may be lower than anticipated due to the high variability of the 7-d anaerobic incubation method. Understanding the relationship between soil protein and other measures of soil N availability, such as mineralizable N by long-term aerobic incubation is essential for soil protein to be an index of the N supplying capacity of soil, which is currently being addressed in several studies (Hurisso et al., unpublished data).

Conclusion

The soil protein procedure (see Supplemental Material) is a rapid method that requires only a small amount of soil for analysis, uses inexpensive reagents, requires relatively simple instrumentation, and is amenable to a high-throughput framework necessary for adoption into commercial soil testing laboratories. However, future work is needed to better understand the functional role soil protein plays and how this method relates to more established methods of soil N availability. For example, the extent that soil protein reflects N mineralization versus N stabilization processes is not yet clear.

Data presented here demonstrate that the procedure extracts proteins from a wide range of sources, not just glomalin. We suggest discontinuing the use of the terms *glomalin*, *EEG*, and *GRSP* in favor of the broader and more accurate terms *soil protein* or *ACE protein*, as the former language is imprecise and misleading. Rather, the pool of proteins extracted by this procedure should be considered to reflect a much broader soil protein pool, which has potential to serve as an indicator of the primary pool of organically bound N in the soil and thus as potentially available organic N and overall soil health.

Supplemental Material

Supplemental material is available online, providing the full soil protein procedure that details autoclaving soil in a neutral sodium citrate buffer solution followed by extract clarification and protein quantification steps.

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