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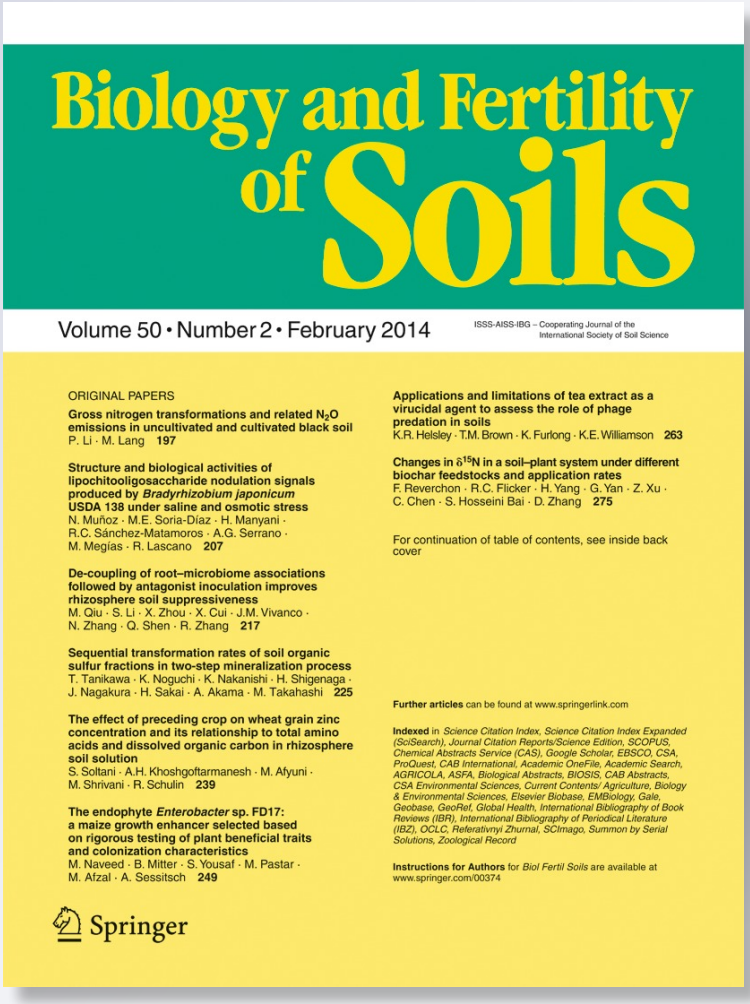

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SHORT COMMUNICATION

Revision of two colorimetric methods to quantify glomalin-related compounds in soils subjected to different managements

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Abstract The aim of this study was to analyze two colorimetric methods used to determine easily extracted glomalin-related soil proteins (EE-GRSP). The historically and most commonly used method for measurement of EE-GRSP as total protein has been the Bradford assay. After some troubles/inconsistencies with this method, we carefully analyzed the Bradford assay, measuring a dilution series of the EE-GRSP fraction and analyzing the time stability of the product. In addition, we did similar analysis of another colorimetric method that quantifies total protein, the bicinchoninic acid (BCA) assay. Unexpectedly, we found that the EE-GRSP concentration values determined by Bradford assay were dependent and variable with the dilution level of the soil extract; moreover, the Bradford assay shows a great instability with the time when soil samples were analyzed but not when protein solution as bovine serum albumin (BSA) was used as control. On the contrary, the BCA assay was independent of the dilution levels of the soil extract and showed stability in the time either for soil samples or BSA protein quantification. These results were consistent and independent on the different type of soils corresponding to different locations and with different textures.

Keywords GRSP · Bradford assay · Bicinchoninic acid (BCA) assay · Soil proteins

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Introduction

Glomalin is a glycoprotein produced in hyphae cell walls of arbuscular mycorrhizal fungi (AMF) (Wright et al. 1996), which belong to the phylum Glomeromycota (Schubler et al. 2001) and form mutualistic associations with roots of the majority of higher plants and occur in all ecosystems (Barea et al. 1997). Glomalin, which is a putative homolog of plant heat shock protein 60 (Gadkar and Rillig 2006), is a hydrophobic protein resistant to proteolysis and at temperature, pH, and detergents denaturation (Wright and Upadhyaya 1996). It contains high Fe concentrations (from 0.8 to 8.8 %) which were hypothesized to represent a structural component important on soil Fe accumulation (Wright and Upadhyaya 1998). Glomalin can be extracted from soil with a protocol involving a harsh extraction of soil by autoclaving in a sodium citrate buffer (Wright and Upadhyaya 1996); it was present in high concentrations (2 to 15 mg/g of soil and up to >60 mg/g of soil) in a wide range of soils (acidic, calcareous, grassland, and cropland) (Wright and Upadhyaya 1998; Wright et al. 1999) and with a turnover time of at least 7 to 42 years in undisturbed soils (Halvorson and Gonzalez 2006; Rillig et al. 2001). Probably, the accumulation of glomalin in soil depends on its physicochemical properties, and glomalin may represent long-term C and N storage in the soil organic matter (Lovelock et al. 2004a; Nichols and Wright 2005, 2006). Other important features have been proposed: it also acts as a glue of soil particles, and its concentration in soil is positively correlated with soil aggregate water stability (Rillig 2004; Wright and Upadhyaya 1996, 1998; Wright et al. 2007); glomalin forming complex with metals might influence both soil fertility (Nichols and Wright 2005; Wright and Upadhyaya 1998) and remediation of contaminated soils (Cornejo et al. 2008; Gonzalez-Chavez et al. 2004); glomalin can be used as an index of arbuscular mycorrhizal fungal biomasa (Krivtsov et al. 2004; Lovelock et al. 2004b) because of the labor and limitations of assays for measuring abundance of AMF (Negrete-Yankelevich et al. 2013; Ren et al. 2013).

According to Rillig (2004), the term "glomalin" must only refer to the protein synthesized by the putative gene of AMF, while the fractions extracted from soil should be named as glomalin-related soil proteins (GRSP). Two methods have been used to quantify the amount of glomalin in soil extracts: the Bradford assay and an enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody, MAb 32B11, that was raised against crushed spores of *Glomus intraradices* FL208 (Wright et al. 1996). The Bradford assay is simple, quick, and reproducible for protein determination (Bradford 1976; Halvorson and Gonzalez 2006); the detection of glomalin by Bradford method is based on the assumption that the extraction procedure fully denatures all proteins other than glomalin which was inferred from similarity of SDS-PAGE profiles of arbuscular mycorrhizal fungus hypha and soil extracts (Wright and Upadhyaya 1998). The monoclonal antibody MAb 32B11 quantifies inmunoreactive glomalin protein and it being considered much more specific than Bradford assay, although it is operationally more complex (Rillig and Mummey 2006).

However, both the Bradford assay and the ELISA assay are affected by other compounds present in the GRSP extract (Rosier et al. 2006; Schindler et al. 2007; Whiffen et al. 2007). This is important due to that, in the glomalin extraction procedure, humic and fulvic acids (Nichols and Wright 2006, 2005), non-mycorrhizal-related heat-stable proteins, and lipids are co-extracted (Gillespie et al. 2011, Rosier et al. 2006). The accurate assessment of the absolute amount of glomalin in soil is problematic and requires improvement of extraction protocols and of the quantification methods. Since MAb32B11 is not a commercial product, GRSP is generally measured by the Bradford total protein assay.

In this study, we aim to assess the precision and the degree of reproducibility and repeatability of GRSP quantification by two different colorimetric methods used to determine proteins in soil extracts: Bradford assay and bicinchoninic acid (BCA) assay. The precision of the method was critical for our purpose because we were aiming to compare quite similar agriculture soils with different history of use. GRSP is one of the parameters studied as part of a multidisciplinary project, BIOSPAS (www.biospas.org/en), aimed to find biological indicators of soil quality and sustainability in agricultural soils under no-till management in Argentina (Wall 2011).

Material and methods

Soil samples

The soil samples were obtained from BIOSPAS Project which study three different soil treatments replicated four times (blocks) in agricultural fields located across a west–east transect along the most productive region in the Argentinean Pampas, specifically at Bengolea at Córdoba Province (33°01′31″S; 63°37′53″W), Monte Buey at Córdoba Province (32°58′14″S; 62°27′06″W), Pergamino at Buenos Aires Province (33°56′36″S; 60°33′57″W), and Viale at Entre Ríos Province (31°52′59,6″S; 59°40′07″W) (see Figuerola et al. 2012). Within 3 days after collection, samples were sieved to remove roots and plant detritus through a 2-mm mesh and stored at 4 °C until processing.

Glomalin extraction

Glomalin was extracted from soil as reported by Wright and Upadhyaya (1996), considering suggestions by Janos et al. (2008) and that is (1) using equal volumes of extracting solution for all samples, (2) employing the same autoclave time and removing samples from the autoclave promptly at the end of the cycle, and (3) immediately centrifuging after autoclaving to separate supernatant from soil. Soil (1 g) was mixed with 8 ml 20 mM sodium citrate at pH 7.00 (citric acid, tri-sodium salt dehydrate), in 50 ml glass centrifuge tubes. Tubes were autoclaved at 121 °C for 30 min. (table autoclave, Model EA-21, Stoord, Buenos Aires, Argentina) and were immediately centrifuged at 5,000×*g* for 15 min (Multi (RF) Series Multipurpose Centrifuge, Thermo ELECTRON CORPORATION, Milford, USA). The supernatant represents the easily extracted glomalin-related soil proteins (EE-GRSP) (Wright and Upadhyaya 1996).

Glomalin quantification

We have used two colorimetric methods to determine glomalin as total protein. The Bradford assay has been the most commonly used method for glomalin quantification and is based on measuring absorbance at 595 nm by using Bio-Rad protein dye reagent (Bio-Rad 500-0006, Mellville, USA) in 96-well, flat-bottomed microplates and bovine serum albumin (BSA) (1.28–12.8 μg of protein per well) as standard; BSA has been usually used as standard for glomalin assays (Gadkar and Rillig 2006; Rosier et al. 2006). Wells contained 50 μl of dye reagent and 160 μl of standard solutions or different dilutions of the soil extract. The second method is based on the use of BCA assay with determination of absorbance at 562 nm (Stoscheck 1990) and BSA as standard (0.625–25 μg of protein per well). Wells contained 200 μl of standard working reagent and 10 μl of standard solution or different dilutions of the soil extract. The plates were incubated at 37 °C for 2 h before measurement. Different dilutions of the soil extract were assayed to accurate estimation of protein content in samples according to calibration standard curve: undiluted; 1/2, 1/4, 1/8, 1/16, and 1/32. Dilutions were performed with the extraction buffer, 20 mM sodium citrate at pH 7.00. All standards and each diluted extract were replicated three times by using different wells in the same plate. All values reported are normalized to 1 g of dry soil. Absorbance of samples was read with a multifunctional microplate reader (FLUOstar Omega, BMG LABTECH, Offenburg, Germany) at 27 °C and

without agitation. To compare both protein determination methods, Bradford and BCA, the same soil sample was used.

Statistical analysis

The data were analyzed by generalized linear models (GLIM) using the software InfoStat (Di Rienzo et al. 2012). Some data showed heteroscedasticity of variances which was modeled by applying the variance function of identity (varIdent in InfoStat) using as criterion lower AIC values (Akaike criterion) and Bayesian information criterion. The normal distributions were controlled by normal Q–Q plot of standardized residuals and by standardized residuals vs. predicted values plot. Analysis by GLIM was followed, when appropriate, with Fisher least significant difference (LSD) tests and contrasts tests to evaluate significant differences between values. Differences were considered significant at $P<0.05$.

Results and Discussion

The quantification of GRSP in fractions extracted from soil as total protein has been generally performed by the Bradford assay protocol as reported by Wright and Upadhyaya (1996) in which

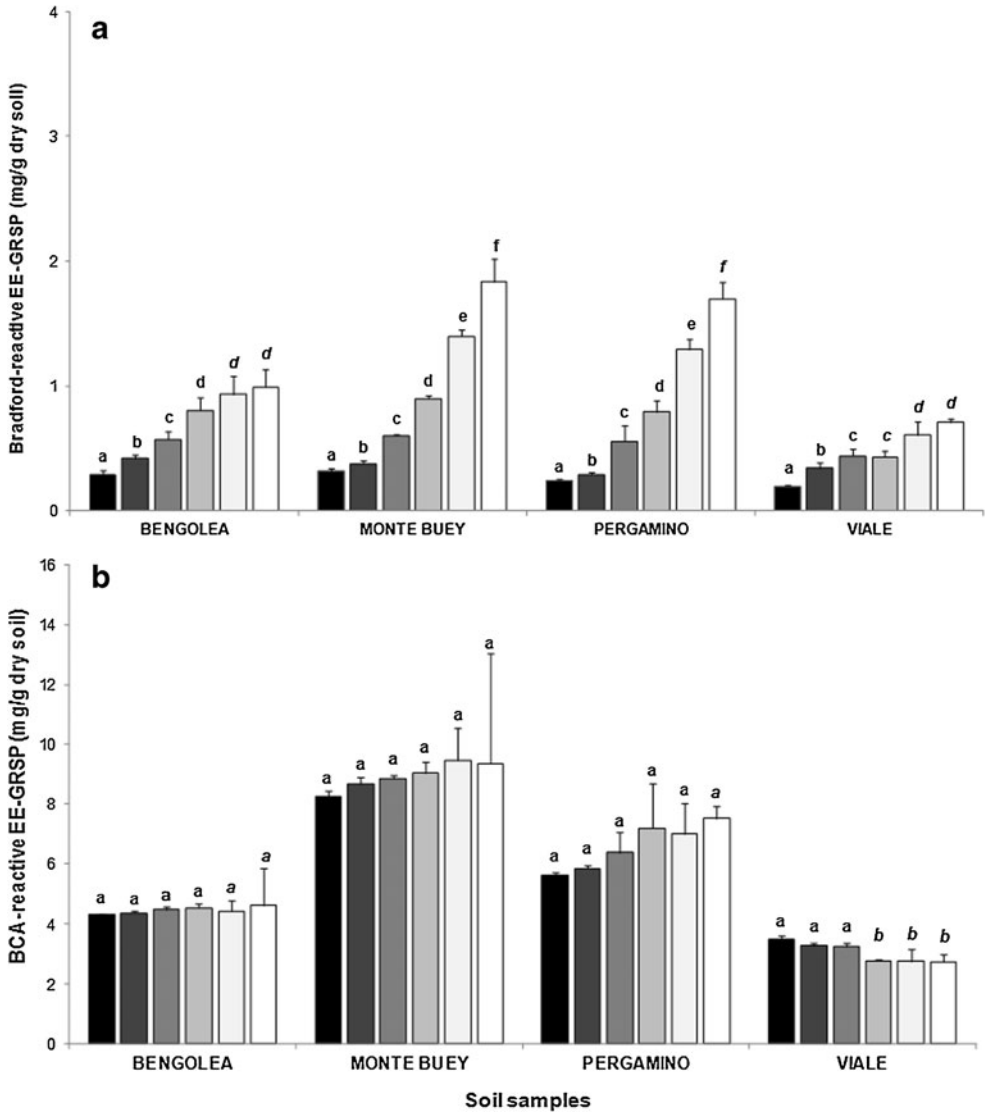

Fig. 1 Concentration of easily extractable glomalin-related soil protein (EE-GRSP), expressed as milligram protein per gram of dry soil, after different dilutions of the soil extract with 20 mM citrate at pH 7.00. Dilutions were ■, undiluted; ■, $d=1/2$; ■, $d=1/4$; □, $d=1/8$; □, $d=1/16$; and □, $d=1/32$. The amount of protein was determined by **a** Bradford assay and **b** BCA assay, using BSA as standard. Data are mean values of three replicates and *bars* represent standard deviations. *Letters* above columns represent significant differences ($P<0.05$) in EE-GRSP between different dilutions of soil extracts, as determined by LSD Fisher's pairwise comparisons and contrasts tests. The values outside the linear range of each assay for BSA are indicated in italics

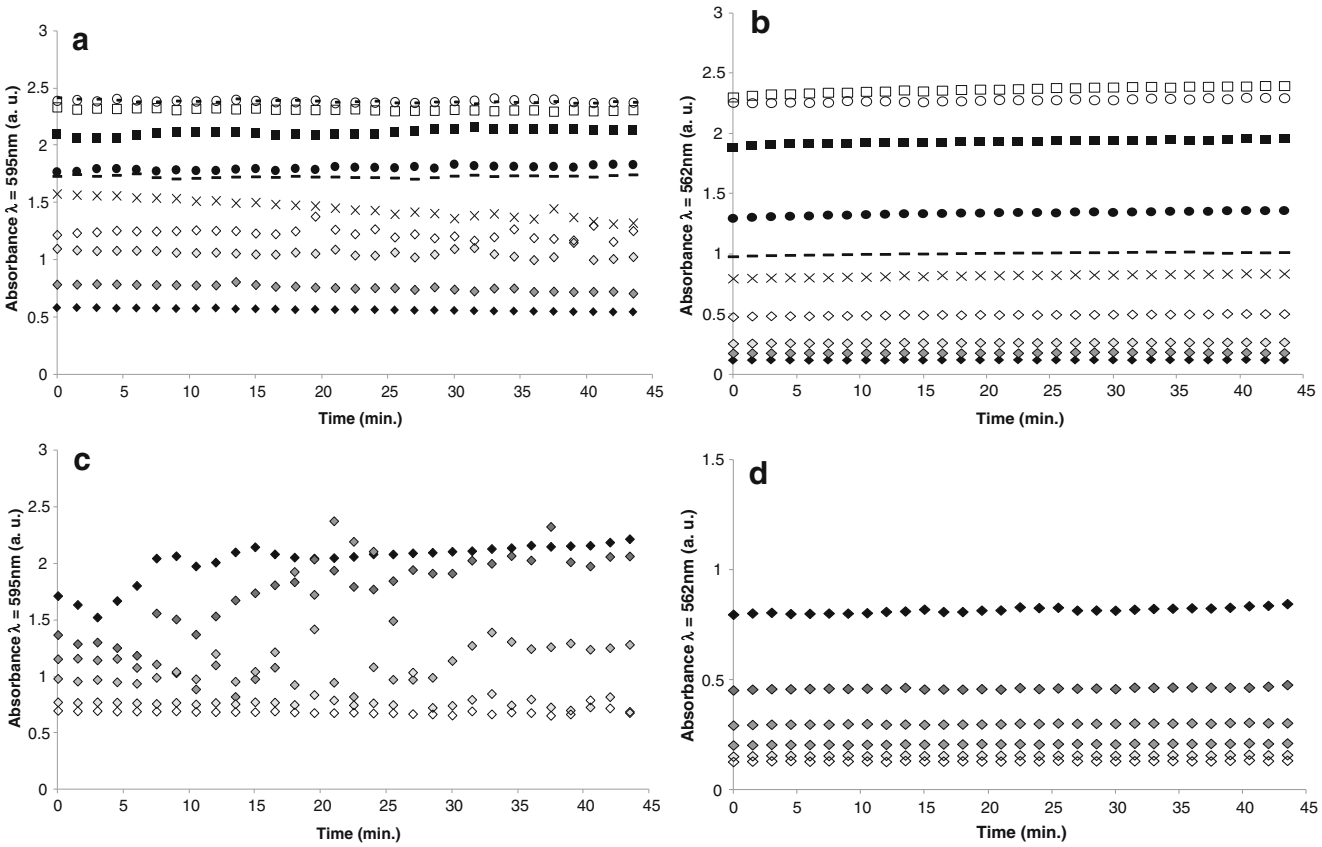

Fig. 2 Time dependence of the absorption peak intensity at **a** 595 nm for Bradford assay and **b** 562 nm for bicinchoninic acid assay for different concentrations of BSA used as standard in each assay. Standard concentrations of BSA used were (***a***) ◆, 0 μg/ml; ◆, 8 μg/ml; ◇, 16 μg/ml; ◇, 24 μg/ml; ×, 32 μg/ml; —, 40 μg/ml; ●, 48 μg/ml; ■, 56 μg/ml; □, 64 μg/ml; ○, 72 μg/ml; and –, 80 μg/ml for Bradford assay and (*b*) ◆, 0 μg/ml; ◆, 50 μg/ml; ◇, 100 μg/ml; ◇, 250 μg/ml; ×, 500 μg/ml; —, 750 μg/ml; ● 1,000 μg/ml; ■, 1,500 μg/ml; □, 2,000 μg/ml; and ○, 2,500 μg/ml for bicinchoninic acid assay. Time dependence of the absorption peak intensity at **c** 595 nm for Bradford assay and **d** 562 nm for bicinchoninic acid assay for different dilutions of soil (NE-9/2010-Bengolea (Córdoba)) extract in 20 mM citrate at pH 7.00. Dilutions of extract were ◆, undiluted; ◆, *d*=1/2; ◆, *d*=1/4; ◇, *d*=1/8; ◇, *d*=1/16; and ◇, *d*=1/32

the fraction extracted is diluted 1/100 in phosphate buffer saline (PBS) at pH 7.40, and the absorbance of the sample is read at 590 nm using BSA as standard. This protocol has been extensively used to determine glomalin fraction in different soils (Halvorson and Gonzalez 2006; Harner et al. 2004; Janos et al. 2008; Nichols and Wright 2005, 2006; Rillig et al. 2003; Rosier et al. 2006; Wright et al. 2006; Steinberg and Rillig 2003; Wright and Upadhyaya 1998). We compared this classical Bradford assay-based protocol to a similar one but using the BCA assay to quantify GRSPs by testing different dilutions of EE-GRSP fraction obtained from four soil samples from different geographical sites sampled at natural environments.

In Bradford assay, the values for glomalin concentration calculated from the different dilutions differed significantly ($P<0.05$) (Fig. 1a). Concentration of the Bradford-reactive EE-GRSP increased between 10–50 % between successive dilutions (undiluted; 1/2, 1/4, 1/8, 1/16, and 1/32). For instance, values for the soil sample of Monte Buey were (a) 0.32±0.02, (b) 0.38±0.02, (c) 0.60±0.01, (d) 0.9±0.3, (e) 1.4±0.05, and (f) 1.84±0.18 mg/g (letters refer to each column in Fig. 1a). The same trend was observed in the different type of soils corresponding to different locations (sandy, loamy, clayed). In a second experiment, ten different dilutions of the GRSP fraction were used (undiluted; 1/2, 1/4, 1/8, 1/16, 1/32, 1/50, 1/100, 1/200, and 1/500) and an increase in concentration data were estimated by the Bradford assay, as a function of the dilution of the extract, similar to the trend observed in Fig. 1a (data not shown). The increment varied between 10–40 % in successive dilutions of samples with absorbance values within the linear range for the standard BSA (from undiluted to 1/32) and between 70–270 % when the absorbance value was outside the linear range for the standard BSA (from 1/50 to 1/500). The same trend was observed regardless of the solvent used to make the dilutions, 20 mM sodium citrate at pH 7.00 or phosphate-buffered saline at pH 7.40. Due to this variability, we hypothesized that the apparent increase in reactivity to Bradford by dilution could depend on a disaggregation of glomalin protein complexes by increasing dilution of the

fraction. To test this hypothesis, we used detergents to break the potential aggregation of glomalin in solution, as sodium dodecyl sulfate (SDS) or Triton X in concentrations of 0.2/100 ml and 0.01 g/100 ml, respectively. EDTA (0.1, 0.05, and 0.01 M) and Dipyridyl (0.025 M) were also used as chelating agents considering that iron is a structural component of glomalin and may be involved in the potential aggregation fact. The addition of detergents or chelating agents did not modify the results.

Alternatively, when the EE-GRSP fraction was determined by the BCA method, the values estimated from the different dilutions did not vary significantly ($P>0.05$) (Fig. 1b). This was particularly true for values within the limits of linear detection of the method. In an independent experiment to confirm the methodological observations, two extra soil samples were used, one from Balcarce at Buenos Aires Province (47°45′S, 58°18′W) and the other from Pereyra Iraola Park at Buenos Aires Province (34°50′22″S, 58°9′47″W) declared Biosphere Reserve by UNESCO in 2008. Again, concentration determinated by the Bradford assay did increase significantly ($P<0.05$) between the successive dilutions while that concentration determinate by BCA assay did not vary significantly ($P>0.05$) with dilution (data not shown). To explore the stability of the colored product of both colorimetric methods, Bradford assay and BCA assay, the intensities of the absorption peaks at 595 and 562 nm, respectively, were read between 0 to 45 min each 90 s from the moment of mixing colorimetric reagents. Figure 2 shows the intensity of the absorption peaks as a function of time for different dilutions of GRSP extract and for different dilutions of BSA used as standard in the two assays. The Bradford assay was very time unstable for soil sample (Fig. 2c) while the BCA assay had a time stability e (Fig. 2d). Interestingly, for the standard BSA protein, time stability was equal for both Bradford assay and BCA assay (Fig. 2a and b).

In conclusion, a remarkable difference was found between EE-GRSP concentrations determined by using the Bradford assay in different dilutions of the same fraction extracted from soil (Fig. 1a). This inconsistency was observed in different soils with different physicochemical characteristics, but this problem was not found when standard BSA solutions were assayed. The metallo-glycoproteic nature of the glomalin, which is related with the possible aggregation of the protein in the soil, could explain the results observed in the Bradford assay. However, it should also be considered that we are not measuring the content of pure proteins but a mixture of different compounds extracted from soil and that the colorimetric methods used in the determination of proteins in soil samples can give artifacts (Gillespie et al. 2011; Nannipieri and Eldor 2009; Nichols and Wright 2005, 2006; Rosier et al. 2006). Alternatively, we found that the BCA assay was a precise and reproducible method to quantify EE-GRSP extracted from the same samples of soil since (1) we did not observed significant differences between values determinate in the different dilutions tested with BCA method (Fig. 1b) and (2) the stability in the time of BCA assay in comparison with the instability of Bradford assay (Fig. 2). These results suggest that the BCA assay possesses a good level of resolution, being appropriate to detect small differences in the quantity of the substance, and therefore could be more appropriate that Bradford assay to quantify EE-GRSP when comparing similar agriculture soils.

It is worth noting that the differences between the absolute values of the GRSP concentrations determined with the two methods, Bradford or BCA (compare Fig. 1 vs. Fig. 2). This difference could be due to differences between the basic chemical reactions of both methods. The Bradford assay involved a reaction under acid conditions in which the anionic form of the dye is stabilized by hydrophobic and ionic interactions, principally with arginine residues and to a lesser extent with histidine, lysine, tyrosine, and phenylalanine residues. The BCA assay involved a reaction under alkaline conditions in which the Cu^{++} forms a complex with the peptide bonds of proteins and becomes Cu^{+}. The Cu^{+} as well as R groups of tyrosine, tryptophan, and cysteine residues then react with the BCA reagent (Stoscheck 1990). More information on glomalin structure is needed to understand these interactions.

Conclusion

Glomalin is a glycoprotein highly stable in soil, and its content has been suggested to be a soil quality indicator (Gillespie et al. 2011). Several studies show that the accuracy measurement of the absolute amount of glomalin from soil is critical; this fact is more relevant when comparing similar agriculture soils with slightly different histories of use, as in this study. We found that the use of BCA assay may be more appropriate than the Bradford assay to quantify EE-GRSP because it showed higher precision and reproducibility for determining the concentration values, and it had greater stability in time.

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