

# Tannic acid reduces recovery of water-soluble carbon and nitrogen from soil and affects the composition of Bradford-reactive soil protein

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## Abstract

Tannins are plant-derived polyphenolic compounds that precipitate proteins, bind to metals and complex with other compounds. Solutions of tannic acid, or other phenolic compounds, were added to soil samples to determine if they would affect recovery of soluble soil carbon (WSC) or –nitrogen (WSN) or influence the extraction and composition of Bradford-reactive soil protein (BRSP), associated with glomalin. Tannic acid-C added with water was not completely recovered from samples and the amount of total net WSC and WSN recovered was reduced, suggesting formation of insoluble complexes. By comparison, non-tannin phenolics like gallic acid, or methyl gallate, had little effect on extraction of WSC or WSN while a simple gallotannin derived from tannic acid, 1,2,3,4,6-penta-*O*-galloyl-*D*-glucose (PGG), inhibited extraction most. The C and N concentrations in BRSP increased when soil samples were treated with tannic acid or PGG before extraction, a procedure that includes autoclaving. Increases were greatest in the 10–20 cm compared to 0–5 cm depth. Accompanying these were declines in the ratio of absorbance at 465 and 665 nm (*E*<sub>4</sub>/*E*<sub>6</sub> ratio) of BRSP extracts suggesting formation of larger or heavier molecules. In contrast, C and N composition in lyophilized BRSP was unaffected or even slightly reduced when tannic acid or PGG were added to the BRSP extract solution after the extraction process. We conclude that some tannins can reduce the solubility of labile soil C and N, at least temporarily and given unpredictability of response associated with phenolic substances, the Bradford assay should not be relied on to quantify pools or composition of soil proteins like glomalin.

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**Keywords:** Tannins; Water-soluble-C; Water-soluble-N; Bradford assay; Glomalin;  $\beta$ -1,2,3,4,6-penta-*O*-galloyl-*D*-glucose; PGG

## 1. Introduction

Tannins are a diverse class of polyphenolic plant secondary compounds, reported to account for up to 20–60% of the composition of leaves and bark of some species (Cates and Rhoades, 1977; Swain, 1979; Kuiters, 1990; Matthews et al., 1997). Tannins are chemically diverse and somewhat difficult to precisely define but are often split into two broad classes, hydrolyzable and condensed or proanthocyanidins (Haslam, 1981; Khanbabaee and van Ree, 2001). One useful definition of tannins is any phenolic compound of sufficiently high molecular weight containing sufficient hydroxyls and other suitable groups, such as carboxyls, to form effectively strong complexes with protein and other macromolecules under

the particular environmental conditions being studied (Horvath, 1981).

Because of their reactivity, specific tannins are economically and medically important (e.g. Balandrin et al., 1985; Chung et al., 1998; Khanbabaee and van Ree, 2001; Okuda, 2005) and influence important ecosystem processes at scales ranging from the individual organism to ecosystem (Appel, 1993; Hättenschwiler and Vitousek, 2000; Kraus et al., 2003a). Tannins can also influence biogeochemical processes in soils depending in part upon specific structural characteristics (Lewis and Starkey, 1968; Roux et al., 1980; Horner et al., 1987; Fierer et al., 2001; Kraus et al., 2003b; Nierop et al., 2006) because they can form complexes with many classes of biologically important molecules including carbohydrates, proteins, and bacterial cell membranes which affect decomposition rates and nutrient cycling (Horner et al., 1988; Hättenschwiler and Vitousek, 2000; Souto et al., 2000; Hagerman, 2002;

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Kraus et al., 2003a). Due to their widespread occurrence, tannins and related phenolic substances comprise a substantial pool of carbon (C) in the soil that can be used by heterotrophic soil microorganisms leading to increased microbial activity and subsequent short-term immobilization of nitrogen (N) in microbial biomass (Schimel et al., 1998; Castells et al., 2003; Kraus et al., 2004) but can also inhibit microbial processes themselves (Scalbert, 1991; Joshua et al., 1998; Cowan, 1999). Inhibition/facilitation of soil microorganisms by tannins appears complex and may relate to characteristics of the tannins (Schimel et al., 1998) or plant genetics (Schweitzer et al., 2005), and may vary among different soil microbial groups (Souto et al., 2000).

Understanding of fate of tannins after they enter the soil, especially the formation of complexes with organic and inorganic components is incomplete (Schofield et al., 1998; Kogel-Knabner, 2002; Hättenschwiler et al., 2003) but seems important to a basic understanding the formation and transformations of soil organic matter. Of particular interest are their effects on water-soluble pools of soil organic matter and interactions with more recalcitrant forms such as glomalin. Glomalin is an important soil glycoprotein produced by arbuscular mycorrhizal fungi thought to promote soil aggregate formation and represent a significant pool of stable soil organic matter (Wright and Upadhyaya, 1996, 1998; Rillig et al., 2001; Nichols, 2003; Wander, 2004). Bradford-reactive soil protein (BRSP) has been adopted as an operational definition of glomalin and routinely used to estimate standing stocks and dynamics of glomalin-C and -N (e.g. Rillig et al., 2001, 2003; Nichols, 2003; Harner et al., 2004; Lovelock et al., 2004). Estimates of the composition of BRSP, used to calculate the amount of glomalin-C and -N in soil organic matter, range broadly and vary as a function of the amount of BRSP or depth (Lovelock et al., 2004; Halvorson and Gonzalez, 2006). Although earlier work seemed to show no relationship between BRSP (glomalin) and tannins (tannic acid) per se (Rillig et al., 2001), recent studies suggest tannic acid may affect molecular characteristics of BRSP and can bias estimates of glomalin with the Bradford assay (Halvorson and Gonzalez, 2006; Rosier et al., 2006; Whiffen et al., 2007).

Increased knowledge of the interactions between plant secondary compounds such as tannins and soil organic matter formation and nutrient cycling is important especially for land uses, such as silvopasture, that feature intentional combinations of trees, forage plants and livestock managed together to improve spatial and temporal availability of forage production while maintaining ecosystem integrity (Neel and Belesky, 2003). Specific objectives of this study were to determine if tannic acid or other hydrolyzable tannins affect the recovery of soluble soil-C and -N or influence the composition of identifiable pools of soil organic matter such as BRSP. To meet these objectives we conducted four experiments to answer these questions (1) Does tannic acid affect the recovery of water-

soluble-C and -N? (2) Are the patterns of extraction of water-soluble-C and -N due to tannins or other non-tannin phenolic compounds? (3) Does tannic acid affect C and N composition of BRSP? (4) Is C and N composition in BRSP affected by the timing of tannin additions?

## 2. Materials and methods

### 2.1. Soil sample collection and preparation

Experiments 1 and 3 were conducted using samples collected from 5 farm units in Southern West Virginia, each with areas of forest (mixed deciduous or pine), pasture, and cultivated land (cultivated crops including long-term family gardens). Soils were generally classified as fine or sandy loam on slight to moderate slopes and are described in further detail by Halvorson and Gonzalez (2006). For each sample (5 farm units  $\times$  3 areas = 15), 10 soil cores (6.35 cm in diameter) were collected along one or two transects at 50 ft intervals. Cores were subdivided in the field into 0–5, and 10–20-cm depth increments and bulked. In the lab, samples were sieved (2 mm), dried at 55 °C, and stored until further analysis. Experiment 4 was conducted using composite samples, for the 0–5 and 10–20 cm depths, prepared by combining equal portions of the forest ( $n = 5$ ) and pasture ( $n = 5$ ) samples and mixing overnight on a roller mixer.

Experiment 2 was conducted on samples of soil collected from a fescue pasture located on a research farm in southern West Virginia, USA (37°47'55"N, 80°58'19"W) that had been managed as a hayfield and cattle pasture for more than 30 years. Initial plant cover consisted almost exclusively of tall fescue (*Festuca arundinacea*) an important cool-season forage grass typically infected with an endophytic fungus, *Neotyphodium coenophialum*. The soil is classed as a fine loamy, mixed, mesic Typic Hapludult. On May 26, 2003, an area of about 0.28 ha was killed with a nonselective herbicide (Roundup, 7 L/ha) with follow-up applications on August 21, 2003 and June 9, 2004 to control re-growth of weeds. Soil samples were collected on May 18, 2006 from live and killed fescue areas. In each area, 12 soil cores (6.35 cm in diameter) were collected near referenced locations, subdivided into 0–10 and 10–20 cm depth increments and bulked. Composite samples were sieved (2 mm) and stored at 4 °C until analysis. Selected soil properties of the composite samples are listed in Table 1.

Table 1  
Properties of soil used in Experiment 2

Fescue sward	Depth	pH	%C	%N
Live	0–10 cm	6.2	2.81	0.306
	10–20 cm	6.5	1.58	0.169
Killed	0–10 cm	5.7	2.71	0.278
	10–20 cm	6.2	1.47	0.124

Samples were collected from live and killed tall fescue swards.

## 2.2. Experiment 1

Water-soluble organic carbon (WSC) and water-soluble nitrogen (WSN) were determined for forest, pasture and cultivated samples from the 5 farms ( $n = 15$ ) collected at 0–5 and 10–20 cm depth using a sequential cool (23 °C) and hot (80 °C) water procedure (Ghani et al., 2003; Curtin et al., 2006). Three grams of soil were first extracted with either 30 mL of cool deionized water (23 °C) alone (control) or with a mixture of water + 10 mg tannic acid  $\text{g}^{-1}$  soil (Acros Organics, Geel, Belgium). After reciprocal shaking at 200 rpm for 30 min, samples were centrifuged (3 min at 17,000g), decanted and analyzed with a Shimadzu TOC-VCPN analyzer equipped with a TNM-1 module (Shimadzu Scientific Instruments, Columbia, MD). More deionized water (30 mL) was added to soil samples which were then vortexed, incubated in a hot water bath (80 °C for 16 h) and assayed as before. For this study, WSC was determined as the difference between total soluble and inorganic C, but we did not distinguish between inorganic and organic forms of WSN. Net values of WSC and WSN were determined by subtracting the amount of C and N added to samples in water or the tannin-mixture.

## 2.3. Experiment 2

Both WSC and WSN were extracted as above (in duplicate) from the composite samples collected in “live” and “killed” tall fescue fields, treated with tannins or other non-tannin phenolic compounds. Two grams of soil were first extracted with 20 mL cool deionized water (23 °C) alone (control) or with a mixture of water + 10 mg test compound  $\text{g}^{-1}$  soil. Test compounds included gallic acid (GA) (Fisher Scientific, Pittsburgh, PA), methyl gallate (MG) (INDOFINE Chemical Company, Inc., Hillsborough, NJ), tannic acid (Fisher Scientific, Pittsburgh, PA), and  $\beta$ -1,2,3,4,6-penta-*O*-galloyl-D-glucose (PGG) purified from the tannic acid (Hagerman et al., 1997). These compounds were selected because of their postulated role on plant nutrient cycling and because they vary in complexity. GA is a relatively simple phenol and one of the building blocks of hydrolyzable tannins, MG is a phenol that may behave like a tannin, tannic acid (TA) is a commercially available, but imprecisely defined, mixture of hydrolyzable tannins that varies with source (Hagerman, 2002) and PGG is a simple hydrolyzable tannin with well-defined characteristics (Fig. 1, Table 2).

## 2.4. Experiment 3

C and N content of lyophilized BRSP extracts were determined for forest and pasture samples from the 5 farms ( $n = 10$ ) collected at 0–5 and 10–20 cm depth. Samples were treated with tannic acid–water solutions before the first of two extractions. BRSP was extracted from samples using methods similar to Wright and Upadhyaya (1996, 1998). Typically, 1 part soil is autoclaved (127 °C) for 1

hour in 8 parts 50 mM sodium citrate buffer (adjusted to pH = 8.0), centrifuged, and the supernatant collected. The extraction process is repeated per the goal of the study and extracts are centrifuged and assayed colorimetrically (Bradford, 1976) with a commercially available Bradford assay using bovine serum albumin as standards (Biorad). For this experiment, 2 g samples of soil were shaken (180 rpm for 30 min) with 8 mL of a tannic acid (Fisher Scientific, Pittsburgh, PA) solution (0, 5, 10, or 20 mg tannic acid  $\text{g}^{-1}$  soil) with a subsequent addition of another 8 mL of 100 mM (double strength) buffer before the first extraction. A second extraction cycle used only sodium citrate buffer.

The BRSP was recovered from sodium citrate extracts following the method described by Nichols (2003). In brief, sodium citrate extracts were precipitated with 12 M HCl, redissolved in 1.0 M NaOH, dialyzed against deionized water (Spectra/Por 6 Membrane MWCO: 10,000 flat width 45 mm), lyophilized, and analyzed for total C and N with a FlashEA 1112 NC Analyzer (CE Elantech, Lakewood, NJ). Results are reported as a percent of gravimetric weight of the lyophilized material. The C and N composition data were used together with estimates of BRSP to calculate of BRSP C and N pools in soil.

We also measured *E4/E6* ratios, the ratio of absorbance at 465 and 665 nm of BRSP extracts with a Shimadzu UV 1700 spectrophotometer because changes in this ratio have been related to changes in molecular size or weight (Chen et al., 1977).

## 2.5. Experiment 4

The composition of BRSP, extracted from soil samples with 50 mM sodium citrate alone (control), was compared to BRSP from samples with tannins added to soil before autoclaving (pre) or to the soil extract following autoclaving (post). Tannin treatments included two types of tannic acid (Acros, Fisher) and PGG. BRSP was extracted, in duplicate, from composite soil samples described above. Three g samples were first shaken (180 rpm for 30 min) together with 12 mL of deionized water (for control and post-treatments) or with 12 mL of a water mixture supplying 10 mg tannin  $\text{g}^{-1}$  soil (pre-treatment). After shaking, 12 mL of 100 mM sodium citrate, adjusted to pH 8.0 was added to each sample which was then vortexed, and autoclaved for 1 h at 127 °C. Cooled samples were centrifuged (3 min at 17,000g) and supernatant decanted. A second extraction process was repeated after more (24 mL) buffered 50 mM sodium citrate was added to each sample. Supernatants from both extracts were combined yielding about 48 mL of extract. Following the second extraction, 12 mL of deionized water were added to each of the control and pre-extracts. Post-treatment extracts samples received 12 mL of a water + 10 mg tannin mixture  $\text{g}^{-1}$  soil, were shaken for 30 min and allowed to stand so that time of exposure to the tannin mixture was similar for pre- and post-treatments. The BRSP was precipitated from extracts,

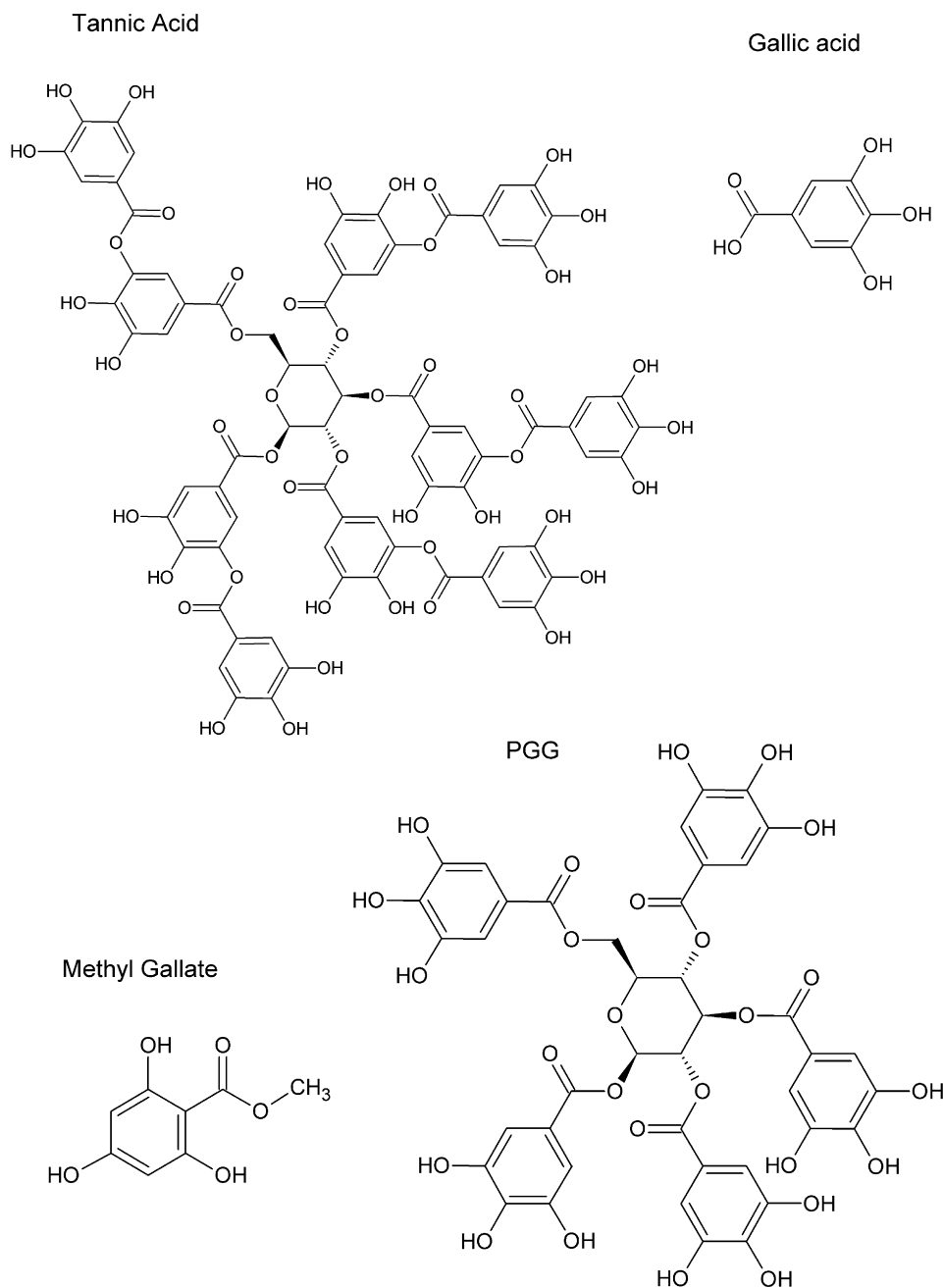


Fig. 1. Chemical structures for tannic acid (TA), gallic acid (GA) methyl gallate (MG) and penta-galloyl-glucose (PGG). The structure shown for tannic acid is a representative molecule for tannic acid, an imprecisely defined mixture of hydrolyzable tannins (Hagerman, 2002).

dialyzed, lyophilized and analyzed for total C and N content as above.

## 2.6. Data analysis

Data were analyzed by analysis of variance (ANOVA) using SAS 9.1 and PROC MIXED using a model that contained both fixed (soil type and depth) and random (sample location) effects (Littell et al., 1996; SAS, 1999). The Kenward–Roger (KR) option was used to calculate degrees of freedom and the compound symmetry (CS)

covariance structure was selected. When appropriate, multiple pairwise comparisons of means were performed using Tukey's HSD test. A value of 5% was selected as the minimum criterion for significance unless otherwise noted. Assumptions of normality were confirmed by the Shapiro–Wilk test in PROC UNIVARIATE. Where appropriate, data were log<sub>10</sub> or arcsine-square root transformed to reduce heteroscedasticity before analysis. Homogeneity of linear model slopes was tested using PROC REG. Mean values shown in the text and tables are arithmetic values followed by the standard error in parenthesis.

Table 2  
Details about gallic acid, methyl gallate, tannic acid and penta-galloyl-glucose

Amendment	Classification	Source	Catalog no.	Lot no.	%C <sup>a</sup>	%N <sup>a</sup>	MW or FW	Galloyl units per molecule
Gallic acid, certified (GA)	Phenolic organic acid	Fisher Scientific, Pittsburgh, PA	A122-500	53472	47.7	0.106	170	1
Methyl gallate, 98% (MG)	Methyl ester of gallic acid	INDOFINE Chemical Company, Inc. Hillsborough, NJ	26081	606062	51.7	0.084	184	1
Tannic acid, ACS reagent (TA)	Mixture of hydrolyzable gallotannins	Acros Organics Geel, Belgium	1401-55-4	A0205549001	49.6	0.396	1701	10
Tannic acid, certified (TA)	Mixture of hydrolyzable gallotannins	Fisher Scientific, Pittsburgh, PA	A310-500	335511	49.4	0.142	1701	10
$\beta$ -1,2,3,4,6 penta- <i>O</i> -galloyl-D-glucose (PGG)	Hydrolyzable gallotannin	Purified from tannic acid (Fisher)			49.7	0.099	941	5

<sup>a</sup>Total C and N were determined in triplicate with a FlashEA 1112 NC Analyzer (CE Elantech, Lakewood, NJ).

### 3. Results

#### 3.1. Experiment 1: extraction of water-soluble-C and -N

A significant interaction was observed between treatment and depth for net cool WSC ( $P < 0.0001$ ). Because the amount of C initially added to samples was subtracted, the net values for cool WSC from tannic acid-treated samples appear negative at each depth (Fig. 2a, c). However, while more cool WSC was extracted from the 0–5 cm depth in control samples; more was extracted from the 10–20 cm depth in treated samples. Addition of tannic acid had no effect on the extraction of WSN with cool water but a main effect of depth was observed ( $P < 0.0001$ ) with more cool WSN recovered from 0–5 cm than the 10–20 cm samples (Fig. 2b, d). Cool WSC was significantly and positively correlated to WSN in control samples ( $r = 0.72$ ), but negatively correlated to WSN in samples treated with tannic acid ( $r = -0.66$ ) (Fig. 3a).

In contrast to extractions with cool water, the subsequent extraction of WSC with hot water was not affected by tannic acid but there was a main effect of depth ( $P < 0.0001$ ) with the average amount of WSC in the 0–5 cm depth more than three times greater than in 10–20 cm depth (Fig. 2a, c). We found an interaction between treatment and depth for hot WSN ( $P = 0.05$ ). More WSN was extracted from 0–5 than 10–20 cm for both treatments but differences between treatments were observed only in the 0–5 cm depth where the samples treated with tannic acid yielded less WSN than control samples (Fig. 2b, d). Hot WSC was strongly correlated to WSN for control and tannic acid treatments,  $r = 0.91$  and  $0.88$ , respectively (Fig. 3b).

A significant interaction was observed between treatment and depth for total WSC, the sum of both cool and hot extractions ( $P < 0.0005$ ). At both depths, more total WSC was recovered from the control samples than from

the samples treated with tannic acid but meaningful differences between the depths occurred only for the control samples (Fig. 2a, c). Main effects of treatment ( $P < 0.05$ ) and depth ( $P < 0.0001$ ) were observed for total WSN with more total WSN was recovered from the control samples than from the samples treated with tannic acid and more total WSN extracted from 0–5 than 10–20 cm samples (Fig. 2b, d). Tannic acid-treated samples exhibited a weaker correlation between total WSC and WSN ( $r = 0.46$ ) than control samples ( $r = 0.88$ ) (Fig. 3c).

#### 3.2. Experiment 2: tannic acid or phenol effect?

For analysis of variance, duplicate data were averaged and composite samples from the two adjacent fescue fields were assumed to be independent resulting in  $n = 2$  for each test compound–depth combination. Even with such small sample numbers, Main effects of test compound and depth were evident for both total net WSC and WSN (Fig. 4). In comparison to extractions with water alone, recovery of total net WSC was not affected by MG but was increasingly reduced by GA, TA and PGG, respectively (Fig. 4a). Greater amounts of WSC and WSN were recovered from the 0–10 than 10–20 cm depth.

#### 3.3. Experiment 3: effects on C and N composition in BRSP

Adding tannic acid increased the C concentration in the final lyophilized BRSP extract in both depths but to a greater degree in 10–20 cm samples (interaction,  $P < 0.0001$ ) where % C increased in BRSP with tannic acid additions until values for both depths were similar at the 10 and 20 mg amendment  $\text{g}^{-1}$  soil rates (Fig. 5a). Adding tannic acid did not affect the concentration of N in BRSP in the 0–5 cm depth, but N concentrations in BRSP increased with tannic acid concentration in 10–20 cm samples until depths were indistinguishable at the 10 and

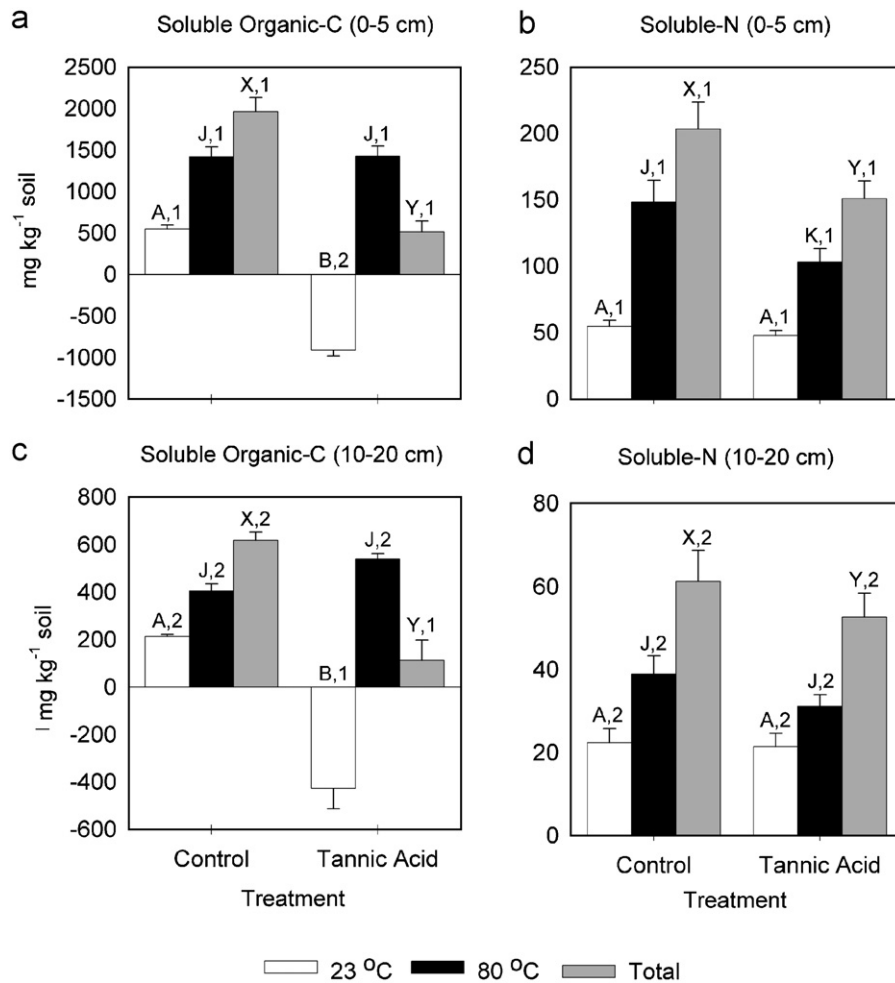


Fig. 2. Average (standard error) net WSC and WSN ( $n = 15$ ). For each depth and bar color, differences between treatments are denoted by letters, differences between depths are denoted by number (Tukey HSD,  $P < 0.05$ ). Net values were calculated by subtracting the C or N initially added by the water or tannic acid from cool WSC or WSN data.

20 mg tannic acid g<sup>-1</sup> soil rates (Fig. 5b). The C to N ratios in BRSP increased with additions of tannic acid ( $P < 0.0001$ ), but were unaffected by depth, from an average of  $10.1 \pm 0.3$  in control ( $n = 20$ ) to  $12.7 \pm 0.4$  at the 20 mg g<sup>-1</sup> amendment rate.

Adding tannic acid also increased the amount of BRSP estimated by the Bradford assay in both depths (Fig. 6a). Consequently, both BRSP-C and -N in soil, calculated with estimates of BRSP and C and N composition data, increased with tannic acid additions (Fig. 6b, c). Conversely, tannic acid decreased the E4/E6 ratios in both depths (Fig. 7).

#### 3.4. Experiment 4: timing of tannin additions on the C and N composition in BRSP

Before analysis, duplicate data were averaged and data for the three tannin treatments were combined so only the effects of the time of tannin addition and depth are considered for further analysis. Even with a narrow inference space, analysis of variance showed main effects

of both time and depth but also an interaction between the two ( $P \leq 0.05$ ). At each depth, % C and N in lyophilized BRSP increased when tannic acid was added before the autoclave-extraction procedure (Fig. 8). In contrast, when tannic acid was added to the BRSP extract solution after autoclaving, only % N in the 10–20 depth was affected; decreasing from control levels.

## 4. Discussion

### 4.1. Extraction of water-soluble-C and -N

C and N extracted from soil with cool water are thought to correlate to recent inputs such as fertilizer, lime, manure, or soluble plant residues while C and N recovered after hot water incubation are positively correlated with soil microbial biomass-C and -N, mineralizable N and total carbohydrates (Ghani et al., 2003). We studied the influence of tannic acid and other compounds on these two pools, reasoning less net soluble C and N extraction with cool water would be evidence of complex formation

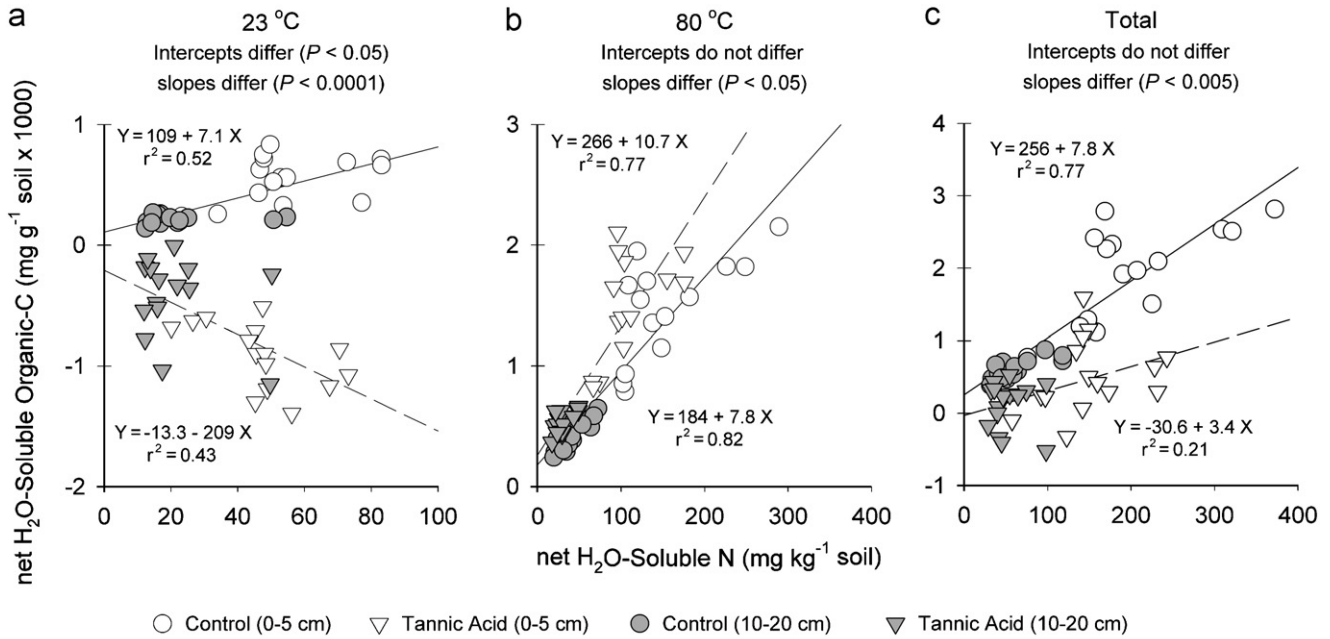


Fig. 3. Relationships between WSC and WSN for (a) cool (23 °C), (b) hot (80 °C), and (c) total extractions made with water alone (control) or with a water + tannic acid solution (tannic acid). Net values were calculated by subtracting the C or N initially added by the water or tannic acid from cool WSC or WSN data.

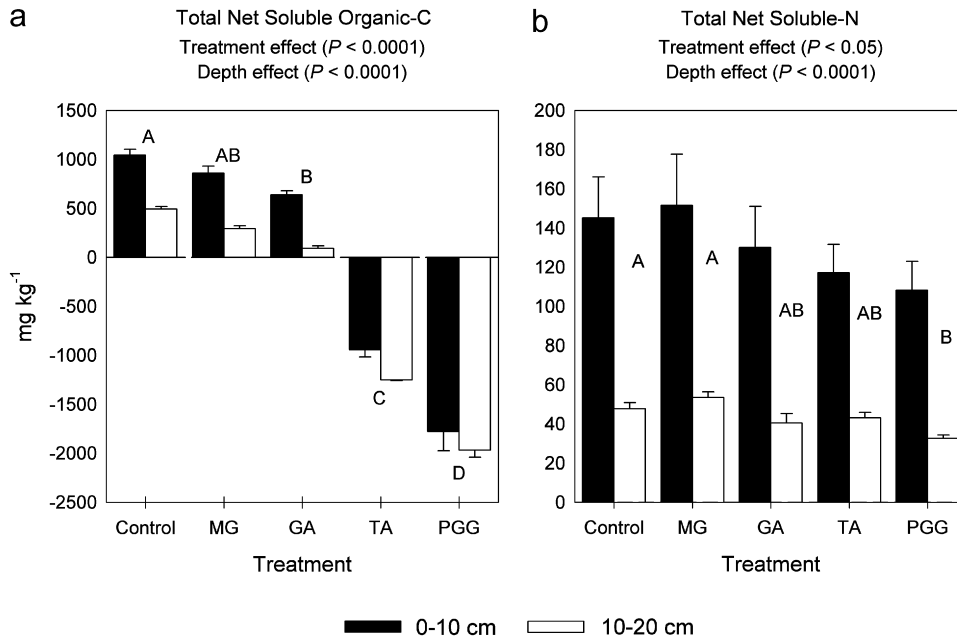


Fig. 4. Average (standard error) net total (a) WSC, and (b) WSN ( $n = 2$ ) extracted by sequential cool (23 °C) and, hot (80 °C) extractions with water (control), or a solution of water + 10 mg phenolic test compound  $g^{-1}$  soil. Phenolic treatments include methyl gallate (MG), gallic acid (GA), tannic acid (TA) or penta-galloyl-glucose (PGG). Calculation of net values as in Fig. 2. In both (a) and (b), main effects of depth are significant, 0–10 greater than 10–20 cm, and differences among treatments are denoted by letters (Tukey HSD,  $P < 0.05$ ).

but that such rapidly formed, and presumably abiotic, complexes might be removed with a second hot water-extraction cycle.

Tannic acid-C, added in an aqueous solution, sorbed rapidly onto the soil matrix but was not extracted by the sequential cool and hot water extractions. Because the

amount of C initially added to samples was subtracted, tannic acid additions resulted in negative values of WSC for extractions with cool water. However, the amount of WSC recovered in the subsequent hot water extraction was similar for both control and treated soil indicating the formation of insoluble complexes (Fig. 2a, c). The chemical

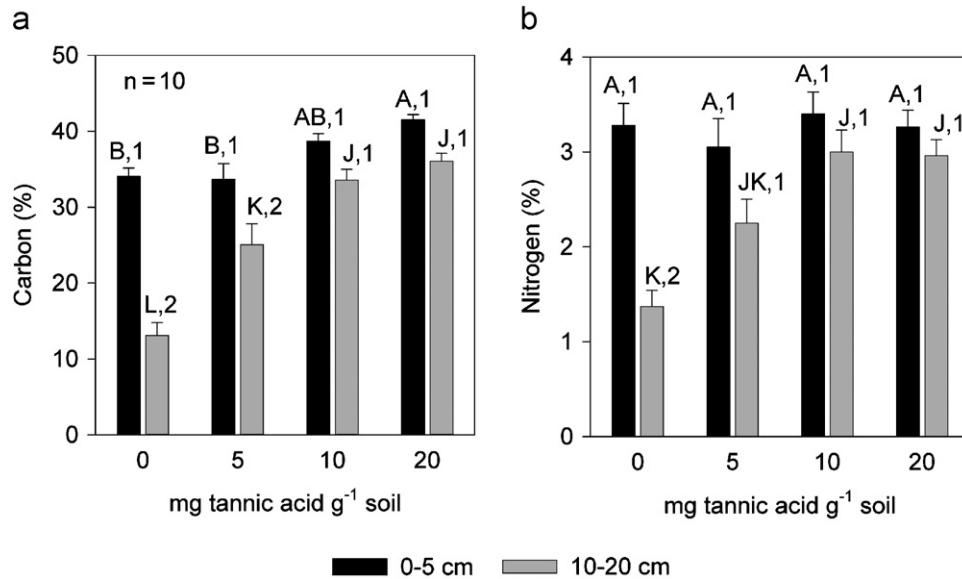


Fig. 5. Average (standard error) %C and N in lyophilized BRSP extracted with 0, 5, 10, or 20 mg tannic acid g<sup>-1</sup> soil added before extraction ( $n = 8-10$ ). Differences between treatments are denoted by letters, differences between depths are denoted by number (Tukey HSD,  $P < 0.05$ ).

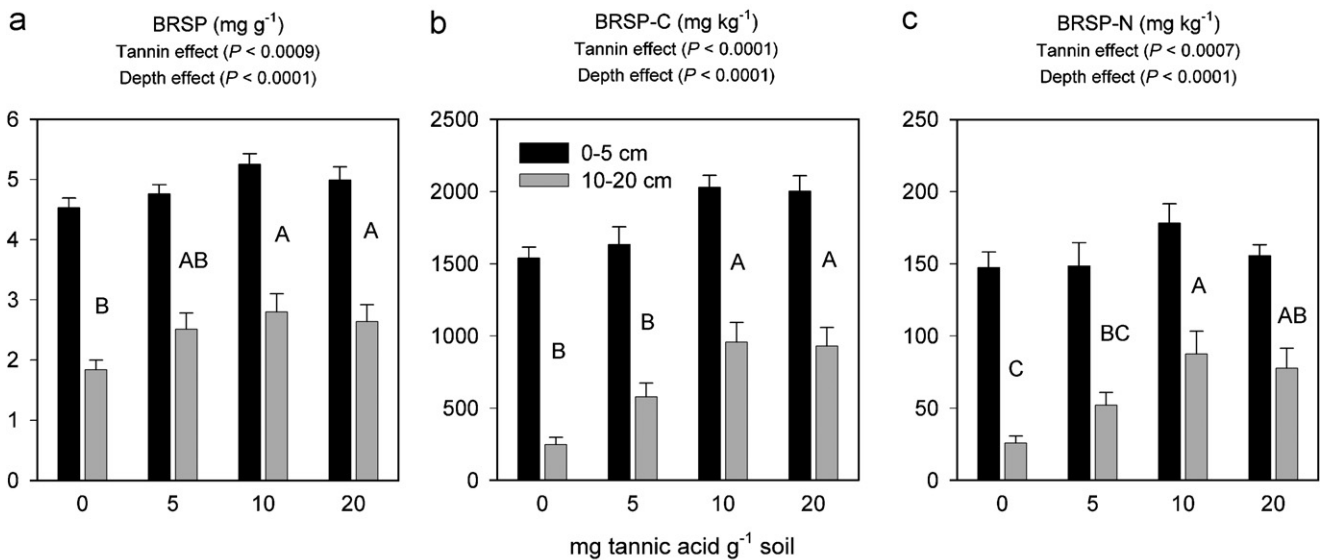


Fig. 6. Average (standard error) (a) estimated BRSP extracted with 50 mM sodium citrate + 0, 5, 10, or 20 mg tannic acid g<sup>-1</sup> soil and resulting (b) BRSP-C and (c) BRSP-N calculated from data in Fig. 5. In each plot, main effects of depth are significant, 0–5 greater than 10–20 cm, and differences among tannic acid levels are denoted by letters (Tukey HSD,  $P < 0.05$ ).

and biological recalcitrance of these rapidly formed tannin–soil complexes remains to be determined as does a more detailed understanding of tannin and soil characteristics that favor their formation.

The amount of N added to soil with tannic acid was negligible and the different patterns of extraction for cool and hot water suggest tannins do not affect all pools of WSN the same way. Tannic acid did not affect the amount of WSN extracted by cool water (Fig. 2b, d) indicating water-soluble forms of inorganic N, such as NO<sub>3</sub>, were not affected. Similar amounts of WSN extracted from control and treated soil also show tannic acid-C did not stimulate

immobilization of soil N by soil microorganisms during the cool water extraction.

Extraction of WSN was reduced by tannic acid in the subsequent hot water extraction (Fig. 2b, d). Because the hot water incubation was at a temperature generally thought too high for microbial activity, this pattern implies interactions between tannic acid and soluble organic forms of N already present at the soil. We did not distinguish between organic and inorganic forms of WSN in this study but both are considered important for plant nutrition (e.g. Jørgen et al., 2003). Curtin et al. (2006) reported hot WSN to be composed mainly (average of 80%) of organic forms

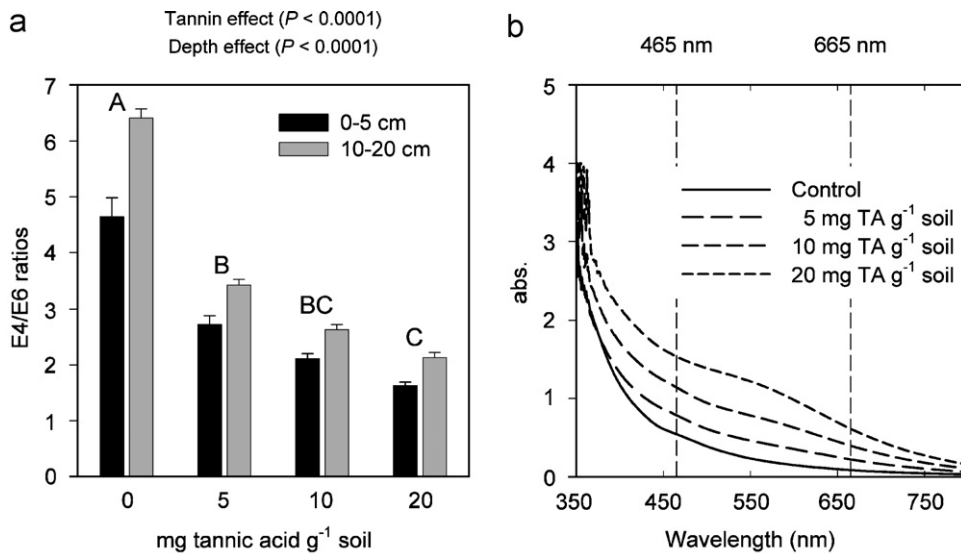


Fig. 7. (a) Average (standard error)  $E4/E6$  ratios of BRSP extracts in relation to tannic acid additions and (b) details of the entire absorbance spectrum for a single soil sample. Data were  $\log_{10}$  transformed before analysis. In (a), main effects of depth are significant, 0–5 less than 10–20 cm, and differences among tannic acid levels are denoted by letters (Tukey HSD,  $P < 0.05$ ).

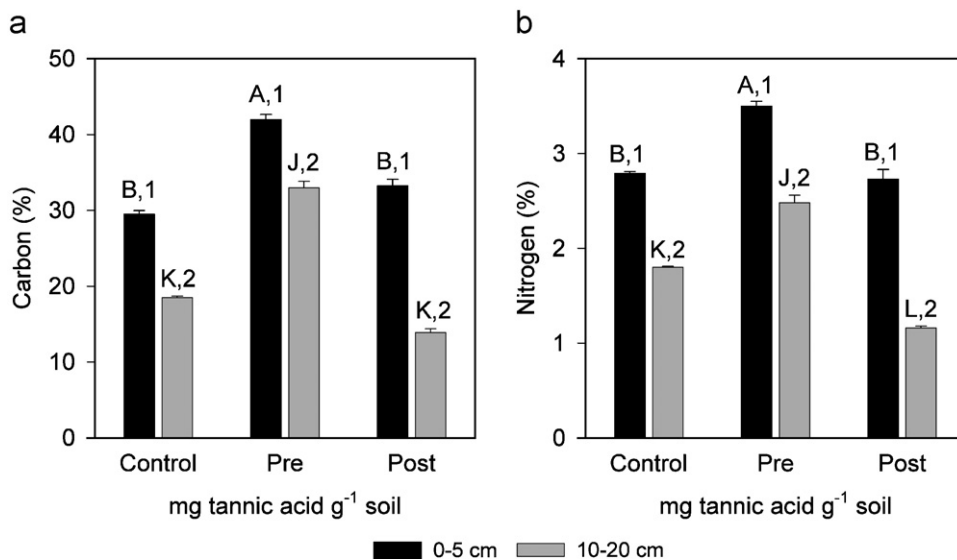


Fig. 8. Average (standard error calculated from all samples) %C and N in lyophilized BRSP extracted with 0 (control), or 10 mg tannic acid  $\text{g}^{-1}$  soil added before (pre) or after (post) extraction. Differences among treatments are denoted by letters, differences between depths are denoted by number (Tukey HSD,  $P < 0.05$ ).

of N with the remainder attributable to hydrolysis of heat-labile organic N compounds.

In view of negative net recoveries of cool WSC, the significant negative correlation observed between cool water WSC and WSN in tannic acid-treated samples (Fig. 3a), suggests sorption of tannic acid-C onto the soil either increased the extraction of WSN or was favored in samples with more labile N, a soil property correlated with soil organic matter in these samples (Halvorson and Gonzalez, 2006). Conversely, more hot WSC was extracted per unit of WSN in samples treated with tannic acid (Fig. 3b) resulting in a higher extract C:N ratio.

#### 4.2. Tannic acid or phenol effect?

Side-by-side comparison of several phenolic compounds reiterated the pattern of lower WSC and WSN recovery following tannic acid additions seen in Experiment 1. Comparisons also illustrate how labile pools of soil C and N respond differently to various related phenolic compounds, or even between sources of tannic acid. For example, positive values for total net recovery of WSC indicate at least some of the C, added with the tannic acid from Acros, was recovered from soil in the first experiment. In contrast, negative net total recovery observed in the second experiment suggests the Fisher tannic acid-C was

less recoverable with our methods. Unlike the other phenolic substances we tested, commercial tannic acids do not have a precisely defined chemical composition but instead are heterogeneous mixtures of galloylglucoses derived from a variety of sources (Kawamoto et al., 1996; Hagerman, 2002).

Lower recoveries of WSC and WSN induced by additions of tannic acid and PGG, but not by GA, seem consistent with the two-stage model for tannin–protein coprecipitation proposed by Kawamoto et al. (1995, 1996). These workers found precipitation of protein by galloylglucoses increased with the number of galloyl groups (see also Haslam, 1974), and was also a function of the tannin/substrate ratio and arrangement of galloyl groups on the phenolic ring. Their further research indicates interactions between tannins and soil organic matter as well as the nature of the resulting complexes will be affected by environmental factors such as soil pH and temperature as well as the concentrations and substance-specific affinities of phenolic substances and soil organic constituents (Kawamoto et al., 1997; Kawamoto and Nakatsubo, 1997a, b).

#### 4.3. Effects of tannic acid on C and N composition in BRSP

Tannins increased not only estimates of BRSP but also the amount of C and N in recovered proteinaceous material when added to samples before they were autoclaved as part of the BRSP extraction process (Experiments 3 and 4). Earlier work by Rillig et al. (2001), established tannic acid is structurally distinct, and does not bind to the glomalin molecule. However, when added to soil, and autoclaved, phenolic compounds including tannic acid may undergo complex and unpredictable reactions including ionization and oxidation that form very reactive substances like phenolate ions or quinones (Appel, 1993; Bittner, 2006; Rimmer, 2006). These substances could polymerize labile pools of C and N in the soil solution and form relatively strong, reversible ionic or irreversible covalent bonds with glomalin, a reportedly heat-resistant glycoprotein, yielding enriched recalcitrant substances.

Further evidence for tannin-induced polymerization of soil organic matter or glomalin is found in decreasing *E4/E6* ratios (Fig. 7a) similar to the data reported by Halvorson and Gonzalez (2006) who found ratios decreased in response to autoclaving alone as well as increasing tannic acid additions. Although lower ratios are associated with the formation of larger or heavier molecules (Chen et al., 1977), aromatic condensation, and relatively long residence times in soil (Stevenson, 1994), this study also shows how additions of tannic acid affect the entire UV–vis absorbance spectrum (Fig. 7b). Increased C concentration in lyophilized BRSP may originate from the tannins themselves bonding to soil proteins but as with its effects on WSN, tannic acid must also interact with soil N in order to affect BRSP-N. Alternatively, additions of

tannic acid may prevent the loss of existing C and N from proteinaceous substances during the Bradford extraction process. Resistance to oxidation might be related to increased recalcitrance in existing soil organic matter (Zibilske and Bradford, 2007).

The C and N content of recovered BRSP was unaffected or even slightly reduced when tannins were added to soil extracts after autoclaving. Neutral phenolic substances are able to form weaker hydrophobic interactions or hydrogen bonds with labile soil C and N (Appel, 1993) that could be disassociated or lost during the separation and dialysis steps employed to purify BRSP. While a simple method for measuring glomalin is desirable, this study and other recent work make clear that plant proteins, tannins and other organic substances can bias estimates of BRSP made with a colorimetric technique like the Bradford assay and can affect the composition of recovered BRSP (Halvorson and Gonzalez, 2006; Rosier et al., 2006; Whiffen et al., 2007). Together with these workers, we question the use of the Bradford assay to estimate glomalin in soil, even in comparative studies.

Though limited in scope, these studies indicate that abiotic reactions between some tannins, and soil organic matter might rapidly decrease the solubility of labile soil C and N and also might affect the composition of organic matter like glomalin. These observations are consistent with each other if tannins mediate the formation of complexes between labile and recalcitrant pools that result in decreased extraction of the former (with water) and enrichment of the latter. These results are also consistent with a view of humic substances as dynamic associations of diverse, relatively low molecular mass components stabilized by hydrophobic interactions and hydrogen bonds (Sutton and Sposito, 2005). However, further research is needed to determine mechanisms of abiotic interactions between tannins and tannin-like phenolics, and soil organic matter, the biotic and abiotic stability of the resulting formation products, and to expand the range of polyphenolics and soils investigated.

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