Changes in the structural composition and reactivity of *Acer rubrum* leaf litter tannins exposed to warming and altered precipitation: climatic stress-induced tannins are more reactive

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Received: *2 December 2010*
Accepted: *12 January 2011*

New Phytologist (2011)
doi: 10.1111/j.1469-8137.2011.03667.x

Key words: *Acer rubrum,* climate change, flavonoid, Fourier-transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR), proanthocyanidin, secondary metabolite, tannins.

Summary

- Climate change could increase the frequency with which plants experience abiotic stresses, leading to changes in their metabolic pathways. These stresses may induce the production of compounds that are structurally and biologically different from constitutive compounds.
- We studied how warming and altered precipitation affected the composition, structure, and biological reactivity of leaf litter tannins in *Acer rubrum* at the Boston-Area Climate Experiment, in Massachusetts, USA.
- Warmer and drier climatic conditions led to higher concentrations of protective compounds, including flavonoids and cutin. The abundance and structure of leaf tannins also responded consistently to climatic treatments. Drought and warming in combination doubled the concentration of total tannins, which reached 30% of leaf-litter DW. This treatment also produced condensed tannins with lower polymerization and a greater proportion of procyanidin units, which in turn reduced sequestration of tannins by litter fiber. Furthermore, because of the structural flexibility of these tannins, litter from this treatment exhibited five times more enzyme (β-glucosidase) complexation capacity on a per-weight basis. Warmer and wetter conditions decreased the amount of foliar condensed tannins.
- Our finding that warming and drought result in the production of highly reactive tannins is novel, and highly relevant to climate change research as these tannins, by immobilizing microbial enzymes, could slow litter decomposition and thus carbon and nutrient cycling in a warmer, drier world.

Introduction

Microbial decomposition of plant litter and soil organic matter sustains ecosystem productivity by cycling carbon, nitrogen, and other nutrients. Decomposition, in turn, is primarily controlled by climate and plant litter chemistry. The climate that sustains plant growth and decomposition is rapidly changing; Earth's average surface temperature is projected to increase by 1.4–5.8°C by the end of this century (IPCC, 2007). At the same time, rainfall events are expected to become less frequent and more intense, resulting in longer, more frequent periods of drought. These changes could directly affect ecosystem nutrient cycling by affecting the chemical composition and thus the decomposability of litter produced. As the efflux of CO_2 through microbial decomposition of organic matter is a significant component of the global carbon cycle (Davidson *et al.*, 2000), the climate-induced change in litter chemistry could alter the global carbon budget as well.

Polyphenols, which are synthesized through the phenylpropanoid pathway, represent a diverse and the most abundant class of plant secondary compounds. They are involved in many ecological and physiological functions in plants, including defenses against pathogens and herbivores, lignification, pigmentation, pollination and plant–plant interactions (Dixon *et al.*, 2005). Tannins constitute the second most abundant polyphenolics in vascular plant species after lignin, and are characterized by their capacity to interact with proteins. Structurally, tannins can be divided into two major classes – condensed tannins (CTs) and hydolyzable tannins (HTs; Kraus *et al.*, 2003a). Condensed tannins or proanthocyanidins are produced by both gymnosperms and angiosperms, and are polymeric flavanoids commonly linked by a C–C interflavan bond at C-4–C-8 or C-4–C-6 between the flavan-3-ol monomers (Fig. 1a). Proanthocyanidins can be further subdivided based on their B-ring hydroxylation pattern, with procyanidins having a di-hydroxy and prodelphinidins a tri-hydroxy B-ring. Evolutionarily, hydrolyzable tannins are more advanced than condensed tannins, and are limited to relatively advanced dicotyledonous plant families (Kraus *et al.*, 2003a). Hydrolyzable tannins are complex esters of gallic acid with glucose (Fig. 1b), and based on the presence and absence of intramolecular C–C coupling between the galloyl groups, they are further divided as gallotannins and ellagitannins, respectively (Fig. 1b,c).

In plants, the tannin concentration is highly variable among species, while within species it varies with the age and tissues (Schweitzer *et al.*, 2008). Environmental factors such as nutrient availability, drought, pH, herbivory, ozone and CO_2 concentration also affect tannin concentrations (Herms & Mattson, 1992; Bussotti *et al.*, 1998; Kraus *et al.*, 2003b; Jaakola & Hohtola, 2010; Lindroth, 2010). Tannins may account for up to 25% of foliar DW (Kraus *et al.*, 2003b), and because of their limited resorption during senescence, tannins may undergo further concentration in senesced tissues, thus forming a major fraction of leaf C input to soils. Owing to their protein complexation capacity, tannins can directly influence ecosystem processes such as litter decomposition and nutrient cycling by lowering the catalytic efficacy of enzymes, as well as reducing the substrate suitability for proteolytic enzymes (Kraus *et al.*, 2003b). Tannins act as multidentate ligands binding to proteins through their phenolic groups and/or hydrophobic regions. These interactions with proteins are mostly non-covalent, and HTs, because of their high hydrophobicity, are thought to precipitate proteins through hydrophobic interactions, whereas hydrogen bonding (between phenolic hydroxyl and peptide carbonyl groups) dominates in CT–protein complexes (Hagerman *et al.*, 1998).

This biological activity of tannins depends more strongly on their structure than on their abundance (Kraus *et al.*, 2003a). The reactivity of tannins is affected by the concentration of condensed vs hydrolyzable tannins, the hydroxylation pattern of the B-ring, extent of polymerization, type of cross-linkage between monomeric units, substitution pattern of the A-ring, and *cis* vs *trans* confirmation at C-2–C-3 (Kraus *et al.*, 2003a). Thus, the influence of tannin quantity on nitrogen mineralization is not straightforward. Many studies have found a negative relationship between the amount of tannin and soil N mineralization (Kraus *et al.*, 2004; Nierop *et al.*, 2006), whereas others have reported no effect (McCarty & Bremner, 1986; Schimel *et al.*, 1996) or a positive relationship (Kanerva *et al.*, 2006; Kanerva &

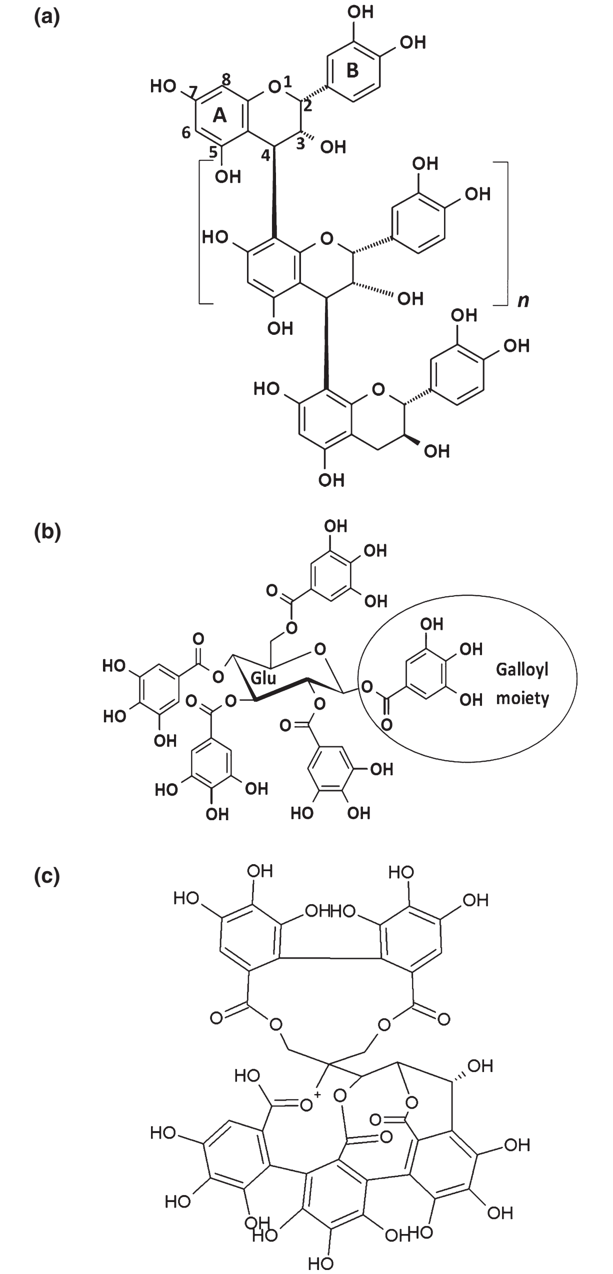

Fig. 1 General structural characteristics of (a) condensed tannins, (b) gallotannins, and (c) ellagitannins.

Smolander, 2008). High-molecular-weight tannins cause enzyme/substrate precipitation (Bradley *et al.*, 2000), whereas low-molecular-weight tannins directly affect microbial metabolism through toxicity (Schimel *et al.*, 1996; Fierer *et al.*, 2001). Some low-molecular-weight tannins are also rapidly utilized by microbes as a C source, which results in microbial immobilization of N (Kraus *et al.*, 2004). Thus, depending on the tannin quality the decrease in N mineralization could be caused by various mechanisms. As the biological function of tannin is strictly structure-dependent, it is difficult to generalize about how a given quantity of tannins will influence ecosystem processes (Kraus *et al.*, 2003a,b, 2004; Hernes & Hedges, 2004; Nierop *et al.*, 2006).

Although tannin quantities are known to respond to environmental stimuli, very little is known about how climate change will affect tannin structure, and consequently biological reactivity. Few studies have looked at the effects of changes in CO_2 concentration on litter chemistry. Liu *et al.* (2005, 2009) found no effect of elevated CO_2 on concentrations of soluble sugars, phenolic and condensed tannins in *Aspen*, and elevated CO_2 had little effect on the decomposability of the litter produced (Norby *et al.*, 2001). Even in the absence of quantitative changes, climatic stress, by altering the phenylpropanoid pathway, could cause structural variations in tannins. Also, the response of plants to CO_2 has been shown to be limited by N availability (Norby *et al.*, 2010), and soil moisture was reported to be the most important factor controlling soil carbon dynamics in a constructed old-field experiment also manipulating temperature and CO_2 (Garten *et al.*, 2009).

The main objectives of this study were to ascertain the influence of predicted warming and altered precipitation on the composition and structural chemistry of tannins, and to quantify the corresponding changes in their biological reactivity. We hypothesized that increased climatic stress resulting from either drought or increased temperatures, by modifying the phenylpropanoid pathway, would induce the production of polyphenols that are structurally different from the constitutive polyphenols. We expected that the differences in structural chemistry would make these stress-induced polyphenols more reactive. Understanding how warming and altered precipitation can affect tannin composition and structure will help us predict how litter decomposition and nutrient dynamics may change in a warmer world.

Materials and Methods

Study site and environmental treatments

We collected *Acer rubrum* litter samples from the Boston-Area Climate Experiment (BACE), located in Waltham, Massachusetts, USA. Within the BACE, old-field plots are subjected to a factorial combination of four levels of warming and three levels of precipitation. The BACE is divided into three replicate blocks, with 12 plots in each block, for a total of 36 plots (Fig. 2). Each block has an ambient, wet and drought zone. Within each zone, groups of four square, 2 m × 2 m plots are arranged in order from ambient temperature to the warmest temperature, with 1 m spacing between plots. Before the experiment, trenches 0.6 m deep were dug around each plot and lined with two layers of polyethylene sheeting to prevent the lateral movement of water and nutrients between individual plots and their surroundings.

The BACE infrastructure used passive removal and active distribution systems to manipulate precipitation. Above the drought zone, a rainout shelter with 15-cm-wide clear polycarbonate slats spaced 15 cm apart removed 50% of incoming precipitation. In the nonfreezing months, this water drained into storage tanks and was immediately transferred to the wet section via a sprinkler system, to achieve a 50% increase in each precipitation event. Ambient and wet treatments lie under a similar structure and receive mild shading to match that of rainout shelters (reducing photosynthetically active radiation by *c.* 6%). During the 2009 growing season (defined here as 1 May to 13 October, the approximate date of leaf fall), we measured volumetric soil moisture in the top 30 cm of the soil profile approximately weekly using time-domain reflectometry (TDR-100; Campbell Scientific, Logan, UT, USA). Growing season soil moisture averaged 22% across the growing season in the ambient, unwarmed treatment. Drought alone reduced soil moisture to 14%, and drought and warming in combination dried soils to 10%. Unwarmed, wet treatment plots averaged 24% soil moisture, and warmed wet plots averaged 20%. Warming alone reduced moisture in ambient precipitation plots to 15%.

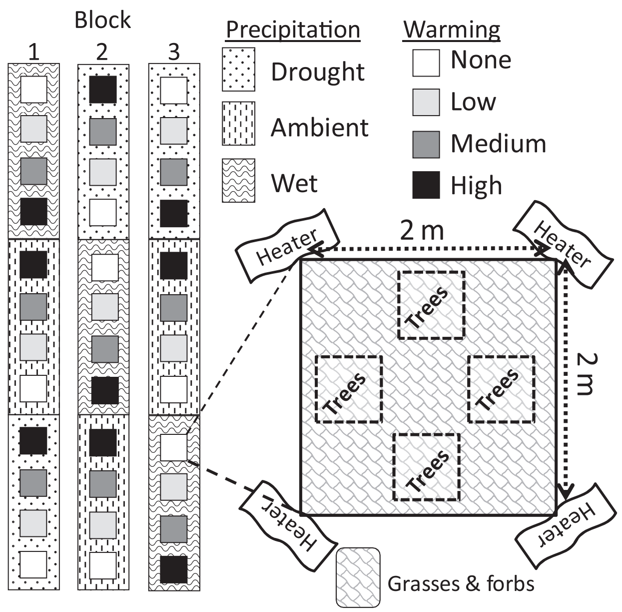

Fig. 2 Plot layout of the Boston-Area Climate Experiment.

Since summer 2008, the temperature of the plant canopy has been manipulated using ceramic infrared heaters; heaters of different wattages are used to achieve the different warming conditions (low warming, 200 W; medium warming, 600 W; high warming, 1000 W). Feedback control is achieved within each group of four plots based on the difference in temperature between the plant canopies of the high warming and ambient plots, as measured every 10 s by infrared radiometers (IRR-PN; Apogee Instruments, Logan, UT, USA) mounted at a 45° downward angle, 1 m above the northern edges of the plots. The system limits warming of the plant canopy to a maximum of 4°C above ambient in the high warming plots. Across the 2009 growing season, the canopy temperature of the high warming treatment averaged *c.* 3°C warmer than in the ambient treatment. There was some variation based on precipitation treatment; while the temperature difference in all treatments averaged between 3.1 and 3.3°C at night, during the day it averaged 2.9°C in the drought plots but only 2.1°C in the wet plots. To date, warming of the top *c.* 10 cm of the soil has been similar to that of the plant canopy.

Litter collection and preparation

In each plot, four seedlings/saplings of each of four tree species (*A. rubrum*, *Betula lenta*, *Quercus rubra* and *Pinus strobes*) grew in four designated subplots within a matrix of grasses and forbs (see dashed squares in Fig. 2). *A. rubrum* was selected for this study based on litter availability. Leaf litter was collected during the fall of 2009, with leaves collected within days of abscission. The *A. rubrum* (hereafter *Acer*) litter used for this study came from the most different temperature treatments (unwarmed and high warming) in the drought, ambient, and wet precipitation treatments. This resulted in six treatments, each with three experimental/treatment replicates (with individual plots as the replicates, and *Acer* litter from up to eight trees pooled within a plot). The three replicates per treatment were maintained in all analyses involving litter. Litter was air-dried to a constant weight, ground in a Wiley mill, and then ground again in a ball mill.

To obtain tannins, litter was extracted with 70% acetone four times. Initial studies showed that 70% acetone extracted significantly higher amounts of condensed tannins compared with 50% or 80% acetone and similar concentrations of methanol. The residual litter after the acetone extraction was washed three times with methanol and dried under nitrogen; this residual fiber was used to determine the HT and CT fractions that were fiber-bound.

Tannin purification

Tannins were purified from litter using the method described in Hagerman & Butler (1980). Because of the limited amount of litter available, three replicates within each treatment were pooled together for tannin purification. One replicate of the droughted and warmed treatment did not have litter for tannin purification; therefore the remaining two replicates were pooled together for this treatment. Briefly, the ground plant tissues were extracted with 70% acetone four times, the extracts were pooled and acetone was evaporated under nitrogen stream. The extract in the aqueous phase was then re-extracted three times each with diethyl ether and ethyl acetate, and the aqueous extract was then loaded on to a Sephadex LH-20 column. Low-molecular-weight compounds were eluted using 70% ethanol, and the bonded tannins were further eluted with 80% acetone. The acetone was evaporated under nitrogen and the aqueous extract was washed with ethyl acetate. The aqueous phase was freeze-dried to obtain purified tannin. Purified tannins were redissolved in 50% MeOH before analysis.

Fourier-transform infrared spectroscopy

Fourier-transform infrared (FTIR) spectroscopy is a robust tool that characterizes the chemical constituents based on the vibrational frequencies of their covalent bonds and functional groups. It has a significant advantage over traditional wet chemistry analyses as it is nondestructive, involves simpler sample preparation and yields information on the relative proportion of various chemical constituents within a matrix (Mascarenhas *et al.*, 2000; Dokken *et al.*, 2005; Artz *et al.*, 2008; Nault *et al.*, 2009).

The infrared spectra of the litter samples as KBr pellets were collected in transmission mode using a Perkin-Elmer Spectrum One FTIR spectrometer. Each sample (treatment replicates) was subdivided into three subsamples before sample preparation. The ball-milled samples and KBr (FT-IR grade) were further powdered separately in an agate mill and then mixed together at a ratio of 2 mg litter to 98 mg KBr (optimized based on preliminary studies), and further homogenized to a very fine powder in an agate mill. The mixture was then placed on a diamond crystal of an attenuated total reflectance (ATR) accessory of the spectrometer and an even contact and distribution of samples was achieved using a flat-tip powder press. Care was taken to apply the same force across all samples. ATR spectra were recorded from 4000 to 650 cm^{-1} at 4 cm^{-1} resolution. In all cases, 50 interferograms per sample were recorded, co-added and averaged and corrected against the spectrum of pure KBr as the background. Thirteen identifiable peaks that correspond to major functional groups were picked for analysis. Since the instrumentation is more sensitive to the qualitative changes in composition, we used relative peak heights for comparison across different samples after baseline correction. Relative peak heights were computed as the ratio of the individual intensity of each peak to the ratio of the total intensities of 13 peaks (Haberhauer & Gerzabek, 1999). Principal component analysis (PCA) was used for easier interpretation of spectra with relation to sample properties, as it provides a reliable way of extracting most of the information in a large data set using only a few principal components (PCs).

Total phenol assay

Total reducing capacity of both litter and tannin samples was measured with the Folin-Ciocalteu Assay (Huang *et al.*, 2005). For litter samples, the treatment replicates were analyzed using gallic acid as a standard, and total phenol was expressed in gallic acid equivalents. For purified tannins, the absorbance data (725 nm) was collected at four concentrations having absorbance values between 0.05 and 0.8 absorbance unit (AU). Data were then plotted as AU × 1000 vs concentration in mg kg^{-1} (Kraus *et al.*, 2003a). Three analytical replicates were processed for each treatment. Linear regressions with intercepts forced through zero were fitted to these data and the slope (1000 × AU/(mg kg^{-1})), standard error of the slope and the R^2 value of the slope were computed.

Condensed tannin assay

The acid-butanol assay was employed as per Porter *et al.* (1986) to quantify the amount of extractable and fiber-bound CT in the acetone extracts of litter and residual fiber, respectively. In this assay the condensed tannins are depolymerized into carbocation and flavan-3-ol units in the presence of diluted mineral acids, and the carbocation units further undergo rapid auto-oxidation to form anthocyanidins with intense red color, which is quantified spectrophotometrically at 550 nm. As the presence of water interferes with color development, standards were prepared in the same solvent matrix as that of samples. Condensed tannins in the treatment replicates of the litter and residual fiber were quantified using purified *Acer* tannin (ambient precipitation × no-warming treatment) as standard. Since the purified *Acer* tannin was found to be a mixture of CT and HT, the CT concentration on a mass basis in the purified tannin standard was determined using acid-depolymerization in the presence of excess phloroglucinol (Koerner *et al.*, 2009). For purified tannins, the slopes of the absorbance data were collected (three analytical samples per treatment) and plotted as described for the Folin assay.

Gallotannins and ellagitannins

For the quantification of gallotannins the samples were acid-hydrolyzed to gallic acid, which was then methylated to yield methyl gallate. Upon acid hydrolysis, the hydroxydiphenoyl residue of ellagitannins in the sample undergoes lactonization to produce ellagic acid. Because of the C–C bonding of the polyphenolic residue with polyol unit, further hydrolysis is prevented. Methanolysis was carried out with 20 mg of litter (residual-fiber for bound HT) in 2.2 ml of 1.8 M methanoic HCl at 85°C as described in Hartzfeld *et al.* (2002). The amounts of methyl gallate and ellagic acid were then quantified using high-pressure liquid chromatography (HPLC). Samples were analyzed with a Shimadzu quaternary pump UFLC system (Shimadzu Corporation, Kyoto, Japan) equipped with an autosampler, inline degasser, and UV–visible diode array detector. Separations were performed on a Gemini C_{18} column (5 μm 110A^0; 250 mm × 4.6 mm ID; Phenomenex, Torrance, CA, USA). The mobile phase consisted of MeOH : MeCN : water (10 : 5 : 85, v/v) buffered at pH 2.2 with 0.5% H_3PO_4. This gave a minimum peak resolution (R_s) of 4. The limit of detection was defined as having a signal-to-noise (S : N) ratio of 10 and all values reported are based on the peak area at 272 nm. For purified tannins three analytical replicates were run per treatment as already described.

Protein complexation capacity

The protein complexation capacity of the litter was assessed by quantifying the amount of tannin–protein complex formed using a radial diffusion assay as described in Hagerman (1987). A tannin concentration for the assay was optimized using preliminary studies. Because of the possible interference of acetone with proteins, the aqueous-acetone extract of the litter was dried under nitrogen and redissolved in 50% aqueous methanol for analysis. The samples were loaded into small wells cut in an agar plate containing protein (0.1%) and incubated at 25°C for 98 h. To make the study more ecologically relevant, along with BSA we tested the complexation capacity of tannin with β-glucosidase, a microbial exo-enzyme that is involved in the degradation of cellulose in soil and also used for monitoring biological soil quality (Stott *et al.*, 2010). In this assay, the opaque ring that develops from the formation of protein–tannin complex is measured length- and depth-wise, and the volume of the opaque region of agar is related to the protein complexation capacity of the tannins.

Solution ^{13}C nuclear magnetic resonance (NMR) spectroscopy

General structural information about purified tannins was obtained using solution ^{13}C NMR spectroscopy of 250 mg purified tannin dissolved in 1.0 ml mixture of 70% deuterated acetone and 30% water. The spectra were obtained at 125.77 MHz on a Bruker Avance 500 spectrometer (NMR facility, Clemson University), using inverse-gated decoupling, 45° pulse, 0.4 s acquisition time, 3.6 s relaxation time and 30 000–40 000 scans. The experimental conditions were optimized based on preliminary studies to obtain an optimal S : N ratio at regions of interest of the spectrum.

These also showed that the *Acer* tannins were mixtures of CT and HT, so that structural properties were assessed using methods developed in Kraus *et al.* (2003a). The ratio of HT to CT was estimated as a proportion of galloyl to CT monomer units, based on peak positions for tannic acid (which is mainly pentagalloyl glucose). This was calculated from the ratio of the sum of the peak intensities (i.e. integrals) at 110, 140 and 165 ppm (contributed by HT C-2′+ C-6′, C-4′ and C′OOH fractions of gallic acid moieties, respectively) to the total intensity from 90 to 172 ppm and after accounting for the C intensities (7 carbon atoms for gallic acid and 12 for CT monomer units). Because the phenolic peak *c.* 144–147 ppm is obscured by signals from HT, the relative proportions of procyanidins to prodelphidins were computed from the peak height ratio 116 : 107. This value was used to determine the percentage of procyanidins directly from a calibration curve developed from 38 ^{13}C NMR analyses of CT from 15 species ($r^2 = 0.95$, Fig. 3), an expansion of the calibration used in Kraus *et al.* (2003a). Chain lengths of tannins were estimated using the area ratio of 65–69 ppm (C3 in a chain terminating position) + 69–75 ppm (C3 in chain extender position) to 69–75 ppm.

Data analysis

To determine the main and interactive effects of warming and altered precipitation on the changes in tannin chemistry, we used a mixed-model maximum likelihood analysis. Significant differences among individual treatments were then determined using Tukey's HSD *post-hoc* test. The relationship between the litter quality parameters and their protein complexation capacity was determined by stepwise multiple regression analysis, and interpretation of the FTIR spectrum was based on PCA. All data analyses were performed using SAS (SAS Institute, Inc., Cary, NC, USA).

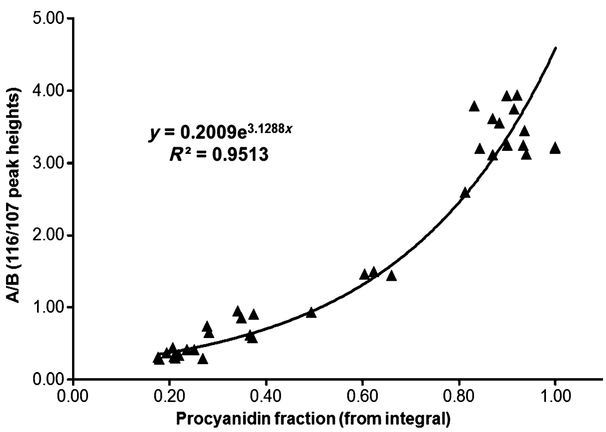

Fig. 3 Calibration curve connecting the ratio of peak height (at 116/107 ppm) of 38 ^{13}C nuclear magnetic resonance (NMR) spectral analyses of tannins from 15 species to their fraction of procyanidin from peak area.

Results

FTIR analysis

A PCA based on the relative peak intensities of FTIR peaks reflected changes in the chemistry of *Acer* litter as affected by different climatic treatments (Fig. 4). PC axes 1 and 2 accounted for 44.0 and 20.2% of the variance in the data set, respectively. The three wave numbers with the highest eigenvector loadings are listed on each PC axis. Infrared spectral characteristics of *Acer* litter exposed to drought + warming differed from those of all other treatments along the PC axis 1 (Table 1). This treatment resulted in a broader peak covering the 1030–1088 cm^{-1} region, which could indicate differences in abundance of polysaccharides (1033 cm^{-1}; C–O stretching; Lammers *et al.*, 2009) and flavanoids (1043–1088 cm^{-1}; =C–O–C and C–C stretching; Wu *et al.*, 2008). Also, this water-stressed treatment resulted in an abundance of saturated hydrocarbon compounds similar to long-chain alkanes, alkyl esters and alkyl alcohols typically found in cuticle and waxes (2850, 2919 cm^{-1}; symmetric and asymmetric stretching of methylene groups, respectively; Lammers *et al.*, 2009). Wave numbers that were common to all other treatments represented an abundance of cellulose (1159 cm$^{-1,}$ antisymmetric bridge C–O–C stretching;

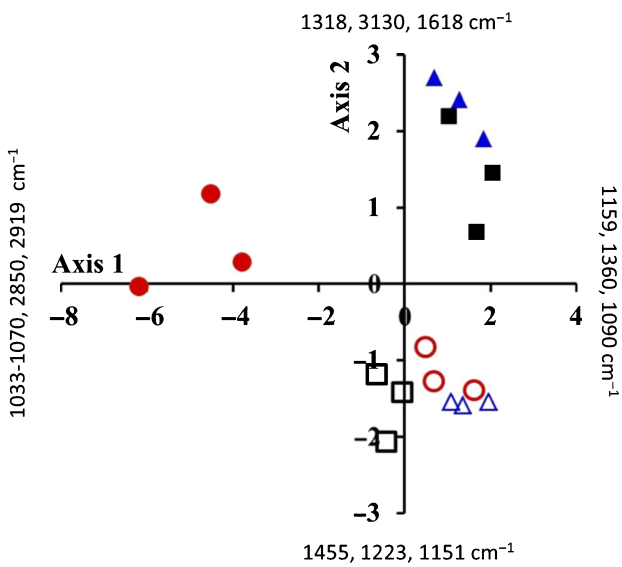

Fig. 4 Principal component analysis plot of the relative intensities of 13 dominant Fourier-transform infrared (FTIR) peaks of leaf litter of *Acer* exposed to different climatic treatments. Axis 1 accounts for 44.0% of variance in the data set and Axis 2 accounts for 20.2%. Compounds in litter that correspond to the first three wave numbers having the highest eigenvector loading are listed on each principal component axis. Closed symbols: circles, drought; squares, ambient; triangles, wet treatments exposed to warming; open symbols represent the respective unwarmed treatments. Points represent replicates (three per treatment).

Table 1 Results of *post-hoc* mean separation test (Tukey's HSD) from ANOVA of principal component axis coordinate scores

Precipitation	Temperature	Scores	Group
PC axis 1			
Ambient	High	1.59	A
Wet	None	1.46	A
Wet	High	1.26	A
Drought	None	0.92	A
Ambient	None	−0.38	A
Drought	High	−4.84	B
PC axis 2			
Wet	High	2.34	A
Ambient	High	1.44	AB
Drought	High	0.48	B
Drought	None	−1.16	C
Wet	None	−1.55	C
Ambient	None	−1.55	C

Treatments that do not share letters differ significantly ($\alpha = 0.05$).

1360 cm^{-1}, CH bending; 1090 cm^{-1}, skeletal vibration involving C–O stretching; Kondo & Sawatari, 1996). The peak at 1360 cm^{-1} could be the result of the CH_3 bending of lignin (Boeriu *et al.*, 2004).

The second PC axis corresponds mainly to the warming treatments. The warmed treatments were distributed towards the positive side of the PC 2 axis and unwarmed treatments towards the negative side (Fig. 4). This grouping was statistically significant at $\alpha = 0.05$ (Tukey's HSD; Table 1). Warming increased abundance of waxes and cutin (1318 cm^{-1}; CH deformation and C–O stretch), various aromatic compounds (3130 cm^{-1} – C–H stretch) and lignin and aromatic carboxylates (1615 cm^{-1}; C=C stretch and C=O stretch, respectively, Haberhauer & Gerzabek, 1999), with the strongest effects in the wetter treatments. The unwarmed treatments were characterized by the relative abundance of pectin (1455 cm^{-1}, C=O stretch; 1151 cm^{-1}, glycosidic bonds of pectin).

Total phenol

The Folin–Ciocalteu assay relies on the capacity of a sample matrix to reduce heteropolyphosphotungstates–molybdates to heteropoly blue complexes in alkaline solution (Mo(VI) + e = Mo(V)). Considering that the Folin–Ciocalteu reagent could be reduced by many compounds in litter, including purines and pyramidine bases, cysteine and ascorbic acid, this assay is nonspecific to the concentration of total phenolics in the sample. Hence, the assay result could be expressed only as the overall reducing capacity of compounds in the litter (Huang *et al.*, 2005). The reduction capacity of the litter varied across the different precipitation treatments ($P < 0.0001$), and was unaffected by temperature ($P = 0.31$; Supporting Information, Fig. S1). Drought had the highest total phenolic content/reducing capacity compared with the ambient and wet precipitation treatments (Tukey's HSD; $P < 0.05$).

Hydrolyzable tannin

Acid hydrolysis converted > 90% of the gallic acid to methyl gallate, and there was no detectable gallic acid in our samples after hydrolysis. The gallotannin content of the senesced foliage ranged from 1 to 10% of DW, whereas the concentration of ellagitannins ranged between 1 and 2.3% (Fig. 5a,b). The amount of fiber-bound hydrolyzable tannins was < 0.5% of leaf DW. There was a significant warming × precipitation effect on the quantity of both gallotannin ($P < 0.0001$) and ellagitannin ($P < 0.02$). Gallotannin content of all precipitation treatments increased when exposed to warming (Tukey's HSD; $P < 0.001$; Fig. 5a), and the foliage from droughted warmed plots had twice the gallotannin content of unwarmed plots (Fig. 5a).

In combination with monomeric ellagitannins that yield ellagic acid upon hydrolysis, oligomeric-ellagitannins are found in some species and are formed by the intermolecular C–O oxidative coupling between galloyl and hexahydroxydiphenoyl moieties. Along with gallic acid, methyl gallate and ellagic acid, our HPLC method was also optimized to detect methyl sanguisorboate that could result from the hydrolysis of these oligomeric units (Vrhovsek *et al.*, 2006). The absence of any methyl sanguisorboate in our hydrolyzed samples indicates that the majority of *Acer* ellagitannins are monomeric.

Condensed tannins

The combination of drought treatment and warming resulted in a higher concentration of CTs (Fig. 5c; Tukey's HSD; $P < 0.001$), whereas warming decreased CTs in the wet treatment (Tukey's HSD; $P = 0.002$). Warming did not affect CTs in the ambient precipitation treatment (Tukey's HSD; $P = 0.99$). Up to 45% of total condensed tannins were fiber-bound and thus nonextractable (Fig. 5d). The percentage of CT sequestration to fibers decreased with warming in wet (Tukey's HSD; $P = 0.001$) and drought treatments (Tukey's HSD; $P = 0.027$).

Total tannins (CTs + HTs)

The amounts of total tannin and extractable total tannin were affected by both precipitation and warming, and there was a significant precipitation × warming interaction ($P < 0.001$; Fig. 6a, b). Drought increased total tannin and extractable total tannin content in warmed and unwarmed plots (Tukey's HSD; $P < 0.01$). Droughted warmed plots had approximately twice the amount of total tannin and extractable total tannin compared with control treatments (ambient × no warming). In wet and ambient precipitation treatments,

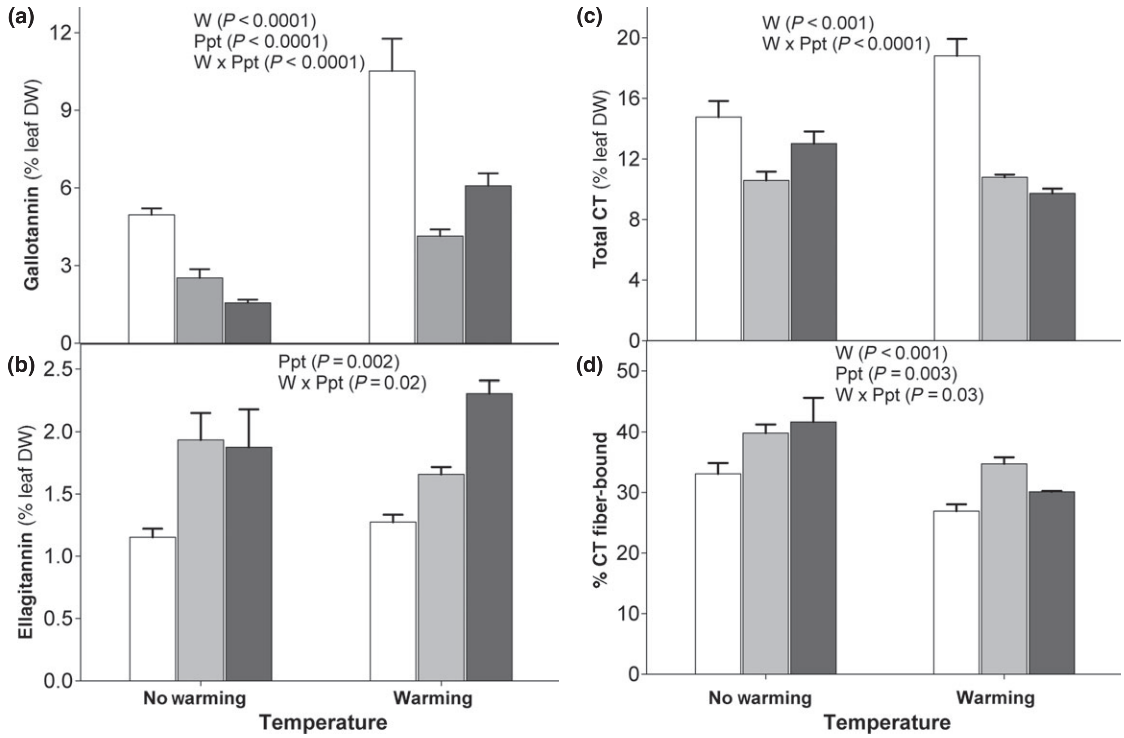

Fig. 5 Effect of warming (W) and precipitation (Ppt) treatments on the foliar concentration of gallotannins (a), ellagitannin (b), total condensed tannin (c) and percentage of nonextractable condensed tannin (d) in *Acer rubrum*. Treatments: open bars, drought; light gray bars, ambient; dark gray bars, wet. Values are means ± SD of treatment replicates (n = 3).

warming had no effect on the foliar total tannin and extractable total tannin contents of *Acer* (Tukey's HSD; $P > 0.05$).

Protein complexation

The protein complexation capacity of the litter was substrate-dependent, and in general the glucosidase complexation capacity of the litter was higher than its BSA complexation capacity (Fig. 6c,d). The drought treatment increased BSA complexation capacity (Tukey's HSD; $P < 0.001$; Fig. 6c), and there was a warming × precipitation interaction on glucosidase complexation ($P < 0.0001$; Fig. 6d). Litter from the drought treatment exhibited a higher glucosidase complexation capacity that increased with warming (Tukey's HSD; $P < 0.001$), whereas glucosidase complexation capacity remained unaffected by warming in both ambient (Tukey's HSD; $P = 1.0$) and wet ($P = 0.26$) treatments (Fig. 6d).

Relationships between litter quality parameters and their protein complexation capacity were determined by stepwise multiple regression analysis. The BSA complexation capacity of litter from various climatic treatments was positively correlated with percentage extractable tannins, whereas glucosidase complexation capacity was positively correlated with percentage bound CTs and total extractable tannins, and negatively correlated with ellagic acid content (Table S1). This negative association of ellagitannins with protein complexation could be explained by its structural rigidity as a result of the presence of intramolecular biphenyl linkages (Fig. 1c; Deaville *et al.*, 2007).

Purified tannin

Spectral interpretation of ^{13}C NMR showed the structural diversity in purified *Acer* tannins caused by the climate treatments (Fig. 7, Table 2). *Acer* plants that experienced ambient and wet precipitation had litter with a higher proportion of condensed tannin monomer units (40–42%) with longer chain lengths (4.8–5.9 units). Drought led to an increase in the proportion of hydrolyzable tannins, accompanied by a decrease in chain length of condensed tannins. Under drought, warming further increased the proportion of hydrolyzable tannins to 80%. The spectra of purified tannins from treatments receiving both drought and warming corresponded reasonably well to the gallic acid moieties of commercially available tannic acid (predominantly HTs), especially in the 110–170 ppm region (Fig. 7). However, as the HT signals in the O- and di-O-alkyl regions could not be identified, the NMR spectra could only be used to determine the relative abundance of monomer units of gallic acid and CT, and not the percentage mass of CT in purified tannins. Using acid-depolymerization with excess phloroglucinal, the purified

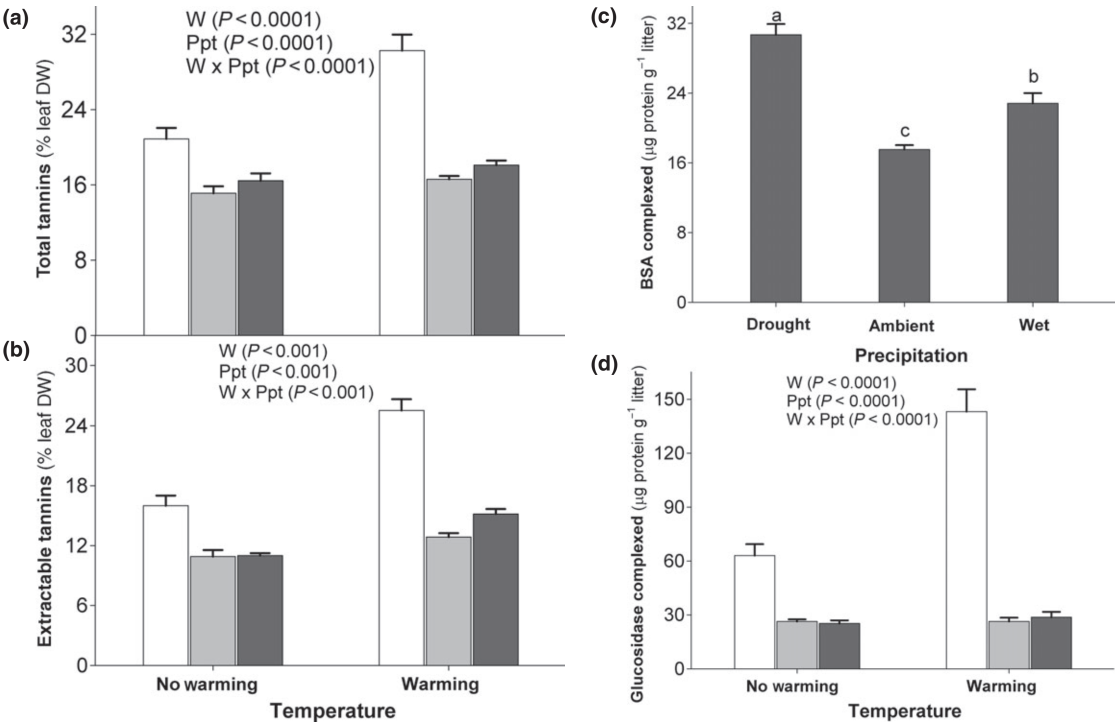

Fig. 6 Effect of warming (W) and precipitation (Ppt) treatments on the leaf litter concentration of total tannins (a) and extractable tannins (b) in *Acer rubrum*, and the BSA complexation capacity (c) and β-glucosidase complexation capacity (d) of the litter. Treatments: open bars, drought; light gray bars, ambient; dark gray bars, wet. Values are means ± SD of treatment replicates (n = 3).

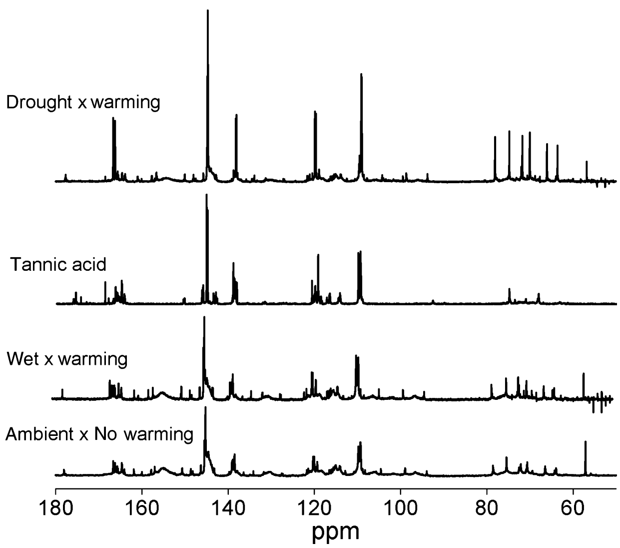

Fig. 7 Solution ^{13}C nuclear magnetic resonance (NMR) spectra of commercial tannin (tannic acid) and purified foliar tannins of *Acer* exposed to different warming and precipitation treatments.

tannin from the ambient precipitation × unwarmed treatments was found to be 54% CT by mass.

Based on the height integral ratio at 116 to 106, as per Kraus *et al.* (2004) and based on the more robust calibration curve (Fig. 3), *Acer* tannins had a higher abundance of procyanidins, and the proportion of procyanidin units was marginally higher in drought + warming and wet, unwarmed treatments. *Acer* CT has been shown to be abundant in procyanidins (Bate-Smith, 1978). Compared with the *cis* region (75–80 ppm), the *trans* region (80–85 ppm) of the spectra did not have any identifiable peaks, reflecting the very high abundance of cis- structures in *Acer* tannins (Fig. 7). The reduction capacity of *Acer* tannin (Folin assay) was similar among purified tannins from different climatic treatments. The differences in reactivity (i.e. slope) for the condensed tannin assay varied between 9.1 and 13.2 among treatments, with drought + warming having the lowest reactivity and wet, unwarmed having the highest (Table 2). The acid methanolysis of the purified tannins reflected a significant increase in the content of gallotannins and ellagitannins in *Acer* subjected to drought + warming treatments. Warming decreased the CT (acid-butanol assay) but increased the gallotannin content within each of the precipitation treatments. Similarly, warming led to a decrease in chain length of CT, regardless of precipitation (Table 2). The β-glucosidase inhibition capacity determined using salicin-hydrolysis inhibition assay (Julkunen-Tiitto & Meier, 1992) demonstrated the purified tannins from warmed, droughted treatments to be two times more reactive compared with ambient treatment (analytical replicate, data not shown).

Table 2 Information about the composition and structure of purified *Acer* leaf litter tannins obtained from wet chemistry analysis and interpretation of ^{13}C nuclear magnetic resonance (NMR) spectra

Treatments		Wet chemistry[a]						NMR spectral interpretation		
		Folin-Ciocalteau		Condensed tannin		Gallotannin	Ellagitannin			
Precipitation	Temperature	Slope[b] (SE)	R^2	Slope (SE)	R^2	% weight	% weight	% HT[c]	Procyanidin fraction	Chain length
Ambient	Unwarmed	3.9 (0.1)	0.98	12.9 (0.1)	0.99	20.2 (2.6)	2.4 (0.1)	59	0.85	5.5
Ambient	Warmed	3.7 (0.1)	0.97	11.2 (0.1)	0.98	24.7 (2.2)	3.3 (0.9)	58	0.84	4.8
Wet	Unwarmed	3.6 (0.1)	0.99	13.2 (0.3)	0.99	19.2 (0.2)	2.7 (0.1)	57	0.86	5.8
Wet	Warmed	3.9 (0.2)	0.97	11.7 (0.2)	0.98	28.9 (1.9)	2.5 (0.2)	59	0.80	5.0
Drought	Unwarmed	3.7 (0.1)	0.99	10.3 (0.1)	0.99	26.1 (1.3)	3.8 (0.3)	67	0.85	3.5
Drought	Warmed	3.7 (0.1)	0.99	9.1 (0.1)	0.99	35.0 (0.7)	4.6 (0.7)	80	0.96	2.5

[a]Values from three analytical replicates.
[b]Slope calculated as 1000 × absorbance unit/concentration (mg kg^{-1}).
[c]Percentage of hydrolyzable tannin (HT) monomer units (not mass basis).

Discussion

Balanced availability of nutrients is required for optimal plant growth; changes in the nutrient acquisition ability of a plant or mineralization rates in the soil can affect the metabolite composition of the plants. In this study, warming and precipitation change caused both qualitative and quantitative changes in the chemistry of *Acer* leaf litter. Tannins in leaf litter from the most water-stressed treatment (drought + warming) underwent structural changes, causing them to be more readily extractable and five times more biologically reactive. Few other studies have examined how the structural chemistry of tannins in leaf litter responds to predicted climatic changes. The dramatic changes we observed could be ecologically significant.

Using FTIR, we found that warming and precipitation altered *Acer* leaf litter chemistry (Fig. 4). Together, drought and warming caused the greatest water stress for plants, and resulted in higher relative concentrations of cellulose and plant defense compounds, such as flavonoids, cutin, and waxes. Similarly, warming increased abundance of waxes, cutin, and polyphenols across all precipitation treatments. Thus, warmer, more water-stressed conditions led to a greater abundance of protective compounds in leaf litter. Polyphenols, including flavonoids, regulate oxidative stress in plants (Hernandez & Van Breusegem, 2010) and act as an herbivore deterrent (Degabriel *et al*., 2009), whereas leaf waxes can reduce water loss (Aharoni *et al*., 2004).

Tannin abundance and quality

Acer leaf litter contained a mixture of HTs and CTs (Fig. 5; Bate-Smith, 1978), with CTs predominating across all climate treatments (Fig. 5). The climate treatments affected leaf tannin profiles of *Acer*, most dramatically in the drought + warming treatment, which caused a doubling of total tannin content, to 30% of leaf DW (Fig. 6a). This change in tannin profile could result from changes in the phenylpropanoid pathway that would help the plant to mitigate water stress. Under nutrient deficiency or drought, plant growth can slow before C assimilation declines (Herms & Mattson, 1992). Under these conditions, flavonoid biosynthesis has been proposed as a pathway for excess energy dissipation and carbon diversion by plants (Hernandez & Van Breusegem, 2010), thus reducing the production of reactive oxygen species. Further, because of their H-atom transfer or one-electron transfer mechanism, flavonoids can quench reactive oxygen species, protecting plant tissues from peroxidation damage (Leopoldini *et al*., 2004). Flavonoids also protect against herbivory as a result of their protein complexation and pro-oxidant capacities, and so help the plant defend previously acquired resources (Herms & Mattson, 1992; Wright *et al*., 2010). We observed a decrease in leaf litter CTs in the treatments providing optimal growth conditions (warmed and wet; Fig. 5c), suggesting lower investment in these defense compounds under the best growth conditions.

Warming and precipitation change affected the quality of CT; the litter of foliage exposed to drought + warming produced CT with a high relative abundance of procyanidins (dihydroxy B-ring) that were less polymerized (Table 2). The hydroxylation pattern of the B-ring is primarily governed by the activity of various plant enzymes, including flavonoid 3′-hydroxylase and flavonoid 3′, 5′-hydroxylase (Menting *et al*., 1994). Owing to the temperature regulation of enzyme induction and activity, cooler temperatures favor flavonoids with higher degrees of hydroxylation (prodelphinidins, Jaakola & Hohtola, 2010), which could in turn result in the observed higher prodelphinidin content of unwarmed treatments. The condensation of monomeric flavan-3-ols, though less understood, is also under strict enzymatic control. Polyphenol oxidases catalyze the conversion of flavan-3-ols to their respective quinones, which are then converted to carbocations. Oligomeric proanthocyanidins are formed following a nucleophilic attack by C-6 or C-8 of catechins on these carbocations (Dixon *et al.*, 2005). In plants, the down-regulation of polyphenol oxidases is associated with an increased drought tolerance (Thipyapong *et al.*, 2004). Given their significant role in proanthocyanidin biosynthesis, this down-regulation of polyphenol oxidases could contribute to the lower degree of polymerization under climatic stress. Changes in condensation patterns can also occur post-enzymatically, when mild acid conditions could result in cleavage of interflavanic bonds, resulting in a reduction in the degree of polymerization (Vidal *et al.*, 2002). The cellular pH of higher plants can become more acidic at higher temperature (Aducci *et al.*, 1982), which could thus reduce the degree of polymerization of CTs in the warmed treatments (Table 2). Further, a reduction of CT polymerization could explain, in part, the low yield of purified tannins from warmed treatments in acid-butanol assay (Table 2). During the oxidative acid-butanol depolymerization of CT, only the flavones at the extender position yield colored anthocyanidins – not the terminal catechin units. Hence, CT that predominantly consists of shorter chains will result in lower color yield, because the terminal units are not detected (Kraus *et al.*, 2003a).

In the drought + warming treatment, the total quantity of CT increased but the amount of CT sequestered to fiber did not increase (data not shown), resulting in a reduced fraction of bound CT (Fig. 5d). In green leaves, tannins are confined to vacuoles (Marles *et al.*, 2003) and sequestered away from the normal cell metabolism. However, during leaf senescence, following vacuole collapse the tannins could interact with cell wall components and could be irreversibly bound. Interaction of tannins with cell walls can occur during the active growth stage as well (Gagne *et al.*, 2006). The smaller fraction of bound CT in the drought + warming treatment (Fig. 5d) could be partially explained by their lower degree of polymerization (Table 2), which would in turn decrease their interaction with cell wall components (Bindon *et al.*, 2010). Phenolic hydroxyl groups of tannins could associate with carboxylic groups of cellulose through hydrogen bonding. The affinity of such associations has been shown to decrease with an increase in temperature because of preferential sorption of tannins to highly energetic sites of cellulose (Espinosajimenez *et al.*, 1987) and this could potentially reduce tannin sequestration in warmed treatments. Also, the CT in droughted + warmed treatments had a higher proportion of less reactive dihydroxy B-rings that have a lesser propensity for forming B-ring quinones (Nierop *et al.*, 2006). This would decrease the association of stressed tannins with cell-wall components. Litter from unwarmed treatments had relatively high contents of pectin (Fig. 4), which is a major component in cell walls that could sequester CT (Bindon *et al.*, 2010). This could explain the high abundance of fiber-bound tannins in unwarmed treatments (Fig. 5d). Unlike CT, leaf mesophyll cell walls are the primary sites of synthesis and deposition of HTs (Grundhofer *et al.*, 2001), and this difference in subcellular localization could result in a greater proportion of HTs being readily extractable.

Biological reactivity of litter tannins

The drought + warming treatment resulted in a doubling of CTs and HTs in litter (Fig. 5), but a fivefold increase in glucosidase complexation capacity of the litter (Fig. 6d), highlighting the importance of tannin quality in determining interactions with proteins. The protein complexation capacity of tannins is governed by the structure of both tannins and proteins. The main attributes of tannins that govern their complexation capacities are their molecular size (higher molecular size, resulting from better cross-linking, results in higher complexation capacity), conformational flexibility (higher flexibility, by providing better accessibility to the binding sites, results in better complexation capacity) and water solubility (higher hydrophobicity, by decreasing the hydration shell around molecules, will result in higher complexation; Spencer *et al.*, 1988).

In our study, despite the lower degree of polymerization of the CT fraction (Table 2), the highest protein complexation capacities were found in litter from the drought treatment, whether unwarmed or warmed (Fig. 6c,d). This could be explained by two mechanisms. First, there is a trade-off between the degree of polymerization and the conformational freedom of a molecule. CTs with a higher degree of polymerization would experience structural rigidity as a result of restricted rotation about the repeating 4–8 or 4–6 interflavan bonds (Fletcher *et al.*, 1976; Spencer *et al.*, 1988). Thus, polymerization beyond a threshold level could negatively affect the conformational freedom of tannins (possible steric hindrance of 3′, 4′-dihydroxyphenyl groups; Haslam, 1974), thereby decreasing their binding capacity to proteins. Second, water-stressed treatments had the highest concentration of HTs during purification (Table 2). This could be attributed to the lower degree of polymerization of CT in these treatments (Table 2), which could result in lower binding of these compounds to the resins and subsequent loss during the methanol-wash step. The preferential concentration of HT during purification indirectly indicates a higher degree of polymerization of HT fractions in the stressed treatments, which could result in their greater influence in protein complexation capacity.

Acer produces a mixture of HTs and CTs (Bate-Smith, 1978), and the concentration of the extractable forms in the drought + warming treatment was approximately double that of the control treatment (Fig. 6b). In our study, the tannic acid precipitated more protein on a per-weight basis than *Acer* tannin from any of the treatments. Based on the

NMR spectra and the acid-butanol reaction, the commercial tannic acid used in this study did not have any detectable CT. A higher protein complexation capacity of tannic acid that far exceeds the complexation capacity of pure CT samples, and samples with a mixture of CTs and HTs have been reported before (Kraus *et al.*, 2003a). Depending on its structural flexibility, HT may have increased the protein complexation ability of our samples as much or more than CT, since structural rigidity could hinder the protein interactions of highly polymerized CT.

The *Acer* litter consistently had higher glucosidase complexation capacity than BSA complexation capacity (Fig. 6c,d). This could be explained by structural differences between the two proteins that could contribute to their reactivity. BSA is a globular protein whose structure, which can be approximated as prolate to oblate spheroids (Jachimska *et al.*, 2008) with limited structural flexibility, causes steric hindrance to protein binding. Glucosidase has an extended random coil confirmation, which increases accessibility to its peptide linkages and thus offers more binding surface to tannins. Similar differences in the reactivity of tannins to protein quality have been reported before. For example, Deaville *et al.* (2007) reported that the binding affinity of conformationally restrained ellagitannins to flexible gelatin was four times greater than to structurally rigid BSA. Thus, the fivefold increase in β-glucosidase precipitation capacity of litter shown in our study could be partially indicative of the structural flexibility of tannins from climatic stress treatments. Although CT with a low degree of polymerization is theoretically considered to be more labile than its long-chain counterpart, recent studies have shown that short- and long-chained CTs have similar recalcitrance (Kraus *et al.*, 2004). Our study demonstrates that warming and altered precipitation can alter the composition and structure of *Acer* tannins, with implications for their reactivity.

Implications

Although climatic changes eventually restructure plant communities, responses of individual species can strongly influence ecosystem functioning in the intermediate term. Where plant communities change slowly, changes in litter chemistry of individual forest species may affect biogeochemical cycling more strongly than changes in species composition. To our knowledge, this is the first study to examine the changes in leaf litter chemistry of plants exposed to simulated warming and precipitation changes. Tannin quantities have previously been shown to respond to environmental stimuli. However, our results, by profiling the subclasses of tannins, depict this variation at a finer level and identify consequences of these changes for biological reactivity. This study clearly documents variations in allocation to foliar compounds and strong changes in the quality and reactivity of tannins induced by climatic stresses. Our results in *Acer* suggest that tannins produced under climatic stress can have higher enzyme complexation capacities, and hence the quantity alone cannot be taken as a benchmark for predicting the ecosystem properties of tannins. Higher reactivity could inhibit litter decomposition by immobilizing the microbial enzymes that catalyze the catabolic reactions during decomposition, and by protecting protein substrates from proteolytic enzymes. Similarly, the higher mobility and reactivity of stress-induced tannins could affect the decomposability of resident soil organic matter. This would mean decreased nitrogen availability to plants, potentially weakening net terrestrial carbon uptake. If these mechanisms operate similarly across many species, this mechanism could have widespread consequences for nutrient cycling and soil carbon sequestration, potentially providing an important medium-term feedback to climate change.

Acknowledgements

We thank Drs Ann Hagerman and James Kennedy for their valuable input on methodology, and Carol Goranson, Hollie Emery, Susanne Höppner, Brita Jessen, and the many other people who helped to build and maintain the BACE. The authors also thank Dr Richard Norby and two anonymous reviewers for their constructive comments in improving the quality of the manuscript. The BACE was supported by grants to J.S.D. from NSF (grant DEB-0546670) and the US Department of Energy's Office of Science (BER), through the Northeastern Regional Center of the National Institute for Climatic Change Research. This research article is Technical Contribution No. 5899 of the Clemson University Experiment Station.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Total reducing capacity of *Acer* litter as affected by various precipitation treatments.

Table S1 Relationship between the litter quality parameters and their protein precipitation capacity determined by forward stepwise multiple regression analysis

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