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Litters of photosynthetically divergent grasses exhibit differential metabolic responses to warming and elevated CO_2

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Abstract. Climatic stress such as warming would alter physiological pathways in plants leading to changes in tissue chemistry. Elevated CO_2 could partly mitigate warming induced moisture stress, and the degree of this mitigation may vary with plant functional types. We studied the composition of structural and non-structural metabolites in senesced tissues of *Bouteloua gracilis* (C4) and *Pascopyrum smithii* (C3) at the Prairie Heating and CO_2 Enrichment experiment, Wyoming, USA. We hypothesized that *P. smithii* and *B. gracilis* would respond to unfavorable global change factors by producing structural metabolites and osmoregulatory compounds that are necessary to combat stress. However, due to the inherent variation in the tolerance of their photosynthetic pathways to warming and CO_2, we hypothesized that these species will exhibit differential response under different combinations of warming and CO_2 conditions. Due to a lower thermo-tolerance of the C4 photosynthesis we expected *B. gracilis* to exhibit a greater metabolic response under warming with ambient CO_2 (cT) and *P. smithii* to exhibit a similar response under warming combined with elevated CO_2 (CT). Our hypothesis was supported by the differential response of structural compounds in these two species, where cT increased the content of lignin and cuticular-matrix in *B. gracilis*. In *P. smithii* a similar response was observed in plants exposed to CT, possibly due to the partial alleviation of moisture stress. With warming, the total cell-wall bound phenolic acids that cross link polysaccharides to lignins increased in *B. gracilis* and decreased in *P. smithii*, indicating a potentially adaptive response of C4 pathway to warming alone. Similarly, in *B. gracilis*, extractable polar metabolites such as sugars and phenolic acids increased with the main effect of warming. Conversely, in *P. smithii*, only sugars showed a higher abundance in plants exposed to warming treatments indicating that warming alone might be metabolically too disruptive for the C3 photosynthetic pathway. Here we show for the first time, that along with traditionally probed extractable metabolites, warming and elevated CO_2 differentially influence the structural metabolites in litters of photosynthetically divergent grass species. If these unique metabolite responses occur in other species of similar functional types, this could potentially alter carbon cycling in grasslands due to the varying degradability of these litters.

Key words: *Bouteloua gracilis*; climate change; drought; elevated CO_2; environmental stress; infrared spectroscopy; metabolomics; *Pascopyrum smithii*; phenolic compounds; Prairie Heating and CO_2 Enrichment (PHACE); soil carbon; warming.

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Introduction

Responses of plants to changing abiotic and biotic environments could have cascading ecological implications. One such response is the alteration of metabolite profiles in plants exposed to climatic changes, as this response may potentially influence soil carbon storage by altering the chemistry of litter available for decomposition (Tharayil et al. 2011). Although it is well known that the stress adaptation might alter plant metabolic pathways (Dixon and Paiva 1995, Guy et al. 2008, Krasensky and Jonak 2012), most of the studies to date have primarily focused on capturing the compositional changes in metabolite-pools that are readily extractable, and less studied are the climate induced changes in non-extractable structural matrix of plant tissues that forms a major substrate for decomposition. The magnitude of stress response of plants would also depend on the combination of environmental conditions to which they are exposed to, and hence may vary with the physiological adaptation of plant functional types. To date, few studies have evaluated the combined effect of warming and elevated CO_2 on the structural and non-structural metabolites in senesced tissues of photosynthetically divergent species. Understanding these responses could provide a greater insight into potential plant species distributions and soil carbon cycling in a changing world.

The perception and subsequent adaptation of plants to different climatic conditions could differ between species that vary in their photosynthetic carbon assimilation pathways. Generally, climates that are warmer and drier result in partial closure of stomata, which limit the growth of C3 species due to increased photorespiration caused by the lower partial pressure of CO_2 in the vicinity of RUBISCO (Sage and Kubien 2007). However, a concomitant increase in atmospheric CO_2 concentration could allow the RUBISCO to efficiently undertake the carboxylation of RuBP even under the partial closure of stomata, which could partly mitigate the detrimental effect of warmer drier climates (Pearcy and Ehleringer 1984, Sage and Kubien 2007) in C3 species. Conversely, C4 species are less responsive to elevated CO_2 due to the inherent physical separation of carbon assimilation and fixation that reduces the propensity of RUBISCO to undertake oxygenation reactions (Pearcy and Ehleringer 1984, Sage and Kubien 2007). Thus, generally C3 species are found to respond more positively to elevated CO_2 than C4 species with respect to both photosynthesis and biomass production (Smith et al. 1987, Tissue et al. 1995, Wand et al. 1999). For example, in a semi-arid grassland exposed to elevated CO_2 and increased soil moisture, the biomass production of C3 grasses increased under elevated CO_2 while C4 grasses increased their biomass only under increased soil moisture (Dijkstra et al. 2010).

The interactive effect of multiple climatic factors could elicit synergistic, antagonistic or additive plant responses (Dieleman et al. 2012). Although elevated CO_2 facilitates higher plant productivity, this plant response could be altered by accompanying warming or soil moisture stress. Heat stress in general decreases the benefits of elevated CO_2 on photosynthesis by enhancing leaf temperatures in both C3 and C4 species (Lara and Andreo 2011). However, since the stomatal conductance of C4 species is lower than that of C3 species at any given CO_2 concentration, the magnitude of this reduction in conductance is higher in C4 species, resulting in higher leaf temperatures (Hamilton et al. 2008, Wang et al. 2008, Lara and Andreo 2011). Thus, in general, C3 species exhibit a high thermotolerance of photosynthesis under elevated CO_2 (Sage et al. 1995), whereas in C4 species photosynthetic heat tolerance decreases under CO_2 enrichment (Hamilton et al. 2008), resulting in poor performance of C4 species under combinations of warming and elevated CO_2 (Hamilton et al. 2008). For example, a previous study has reported higher biomass production in C4 species when exposed elevated CO_2 combined with ambient temperature, whereas C3 species exhibited a similar response when elevated CO_2 was combined with warming (Hunt et al. 1996).

Grassland ecosystems, which occupy ~30% of the Earth's land area and contain 20% of the Earth's terrestrial soil carbon (FAO 2010, Craine et al. 2013), are dominated by C3 and/or C4 species that exhibit different photosynthetic pathways and capacities to thrive under future climates. Multi-factor climate experiments have shown that C4 grasses flourish under warming

(Morgan et al. 2011). Although the overall responses of the C3 and C4 pathways to various global change factors have been well studied in terms of photosynthetic efficiency and biomass production (Crafts-Brandner and Salvucci 2002, Sage 2002, Lee 2011), remarkably little is known about how the C3 and C4 metabolic response translates to the chemical composition (structural and non-structural) of the senesced tissues, which in turn, could influence litter decomposition and soil carbon storage.

Studies on the effect of climatic stress on plant physiology have generally focused on the metabolite profiles of actively growing green tissues (Du et al. 2011, Rivas-Ubach et al. 2012, Yu et al. 2012). Although elucidating the changes in metabolites in green tissues aids in understanding the underlying physiological mechanisms through which plants adapt to stressful environments, by overlooking the nutrient resorption process that occurs during tissue senescence, such approaches provide little insight into the final composition of the litter that forms the substrate for microbial decomposition. The resorption efficiency for nitrogen and phosphorous compounds exceeds 50% in terrestrial plants (van Heerwaarden et al. 2003), and this remobilization is altered in plants experiencing stressful growing conditions (van Heerwaarden et al. 2003, Kobe et al. 2005). Thus, the metabolite composition of senesced tissues will be significantly different from that of green tissues and might be further altered under climatic stress. Our knowledge of the effects of multiple global change factors, especially warming and elevated CO_2, on the chemical composition of plant litter is still rudimentary, which hinders our efforts to accurately predict the decomposition susceptibility of plant litter produced under future climates.

We used two grass species, *Pascopyrum smithii* (C3) and *Bouteloua gracilis* (C4), collected from the Prairie Heating and CO_2 Enrichment (PHACE) experiment, Wyoming, USA to study the differential responses of the C3 and C4 pathways to elevated CO_2 and warming. These two species contribute to ~50% of the biomass production at PHACE and 90% of the C4 species at the study site is dominated by *B. gracilis* (Dijkstra et al. 2010, Morgan et al. 2011). The main objective of this study was to assess the differences in both structural and non-structural metabolite composition in senesced litters of *P. smithii* (C3) and *B. gracilis* (C4) that were exposed to a factorial combination of warming and elevated CO_2. We hypothesized that the litters of both *P. smithii* and *B. gracilis* that are exposed to unfavorable global change conditions would be abundant in structural metabolites and osmoregulatory compounds that are necessary to combat stress. However, due to the variation in their photosynthetic pathways and differences in their tolerance to warming and CO_2 levels, we hypothesized that *P. smithii* and *B. gracilis* may show the above metabolic-responses under different combinations of warming and CO_2 enrichment. We predicted that warming alone would hamper any metabolic changes in *P. smithii* potentially due to the high moisture stress in this semiarid grassland; whereas warming accompanied by elevated CO_2 would lead to a greater metabolic response in *P. smithii*, potentially due to the partial alleviation of moisture stress. In contrast, because warming combined with elevated CO_2 leads to higher leaf temperatures in C4 species, we expected *B. gracilis* to exhibit a greater metabolic response to warming in the absence of elevated CO_2. Thus, the metabolic responses of *P. smithii* and *B. gracilis* to warming and CO_2 conditions may be modulated by the intensity of the stress perceived by each species under different combinations of warming and CO_2 enrichment.

Methods

Site and experimental design

The plant litter examined in this study was obtained from the Prairie Heating and CO_2 Enrichment (PHACE) experiment located at Cheyenne, Wyoming, USA (latitude 41°11′ N, longitude 104°54′ W). The study site is a semiarid mixed grass prairie where 50% of the vegetation is dominated by the cool-season C3 grass *Pascopyrum smithii* (Rydb.) A. Love and the warm-season C4 grass *Bouteloua gracilis* (H.B.K) Lag. The above ground biomass production of *B. gracilis* and *P. smithii* at PHACE under ambient conditions ranged from 60 to 110 g m^{-2} and from 150 to 170 g m^{-3}, respectively (Morgan et al. 2011). The site exhibits a mean maximum temperature of 17.5°C in July and a minimum of −2.5°C in January, together with mean annual

precipitation of 384 mm. The PHACE experimental plots were subjected to a factorial combination of two levels of CO_2 treatment (385 p.p.m.v. and elevated to 600 p.p.m.v.; abbreviated as *c* and *C*, respectively) and two levels of warming treatment (ambient and +1.5/3.0°C warmer in the day/night; abbreviated as *t* and *T*, respectively). There were five replicates for each treatment in a total of 20 circular plots with a diameter of 3.3 m. Infrared heaters were used to raise the canopy temperature to 1.5°C above ambient temperature during the day and 3°C above ambient temperature during the night. Carbon dioxide was applied using free air CO_2 enrichment (FACE) technology (Dijkstra et al. 2010, Morgan et al. 2011).

In the semiarid PHACE experimental site, soil moisture content varied with different combinations of warming and elevated CO_2. In general, soil moisture increased in elevated CO_2 with ambient temperature treatment while warming with ambient CO_2 treatment resulted in faster and severe soil drying (Morgan et al. 2011, Dijkstra et al. 2012). However, the soil moisture content of warming plus elevated CO_2 treatment was similar to that in the control as the warming induced loss of soil moisture was compensated by lower transpiration rate due to elevated CO_2 (Morgan et al. 2011). The volumetric water content of ambient treatment and CT treatments were 15.5% and 15.6% respectively. Compared to the ambient treatment, the average volumetric soil water content (SWC; 2007–2009) of elevated CO_2 plots increased by 12% whereas warming alone treatment reduced soil moisture content by 16% (Morgan et al. 2011). The productivity of C3 and C4 species in the different treatments also mirrored the changes in soil moisture content indicating that plants experienced moisture stress under different warming and elevated CO_2 treatments. The cool season, C3 species increased productivity (34%) in elevated CO_2 compared to the control, while warming favored C4 species (28% increase in productivity compared to control; Morgan et al. 2011).

Litter collection and processing

We collected senesced leaf litter of *B. gracilis* and *P. smithii* from the experimental plots during the fall of 2010. The litter was collected just after complete senescence while the leaves were still attached to the plant. The litter was collected as part of the biomass harvest done at the end of the growing season. The litter was air-dried (<35°C) and about five grams from each plot were ground to a fine powder with a ball mill.

The samples were first extracted with aqueous methanol, and the polar metabolites such as amino acids, phenolic acids, organic acids, sugars and sugar alcohols (Table 1) in this extract were subjected to targeted metabolomics analysis to identify changes in the composition of non-structural compounds in the senesced litter that are often highly susceptible to microbial degradation. The methanol extracted litter was further subjected to mild-alkaline hydrolysis using 1N NaOH to release the metabolites that were ester linked to the cell-wall, and the hydrolysates were analyzed for phenolic compounds (Table 2). We further used diffuse reflectance infra-red Fourier transform (DRIFT) spectroscopy to characterize the structural chemistry of both the non-extracted and methanol extracted tissues.

Litter chemistry analyses

Extractable polar metabolites.—Metabolic profiling of plants is a powerful tool that provides unbiased insight into plant tissue chemistry (Fiehn et al. 2000, Meyer et al. 2007, Tohge and Fernie 2010). The polar metabolites were extracted from the ground samples as per Lisec et al. (2006) and Kind et al. (2009), with slight modifications. Briefly, 50–100 mg samples were extracted with 1.5 ml of methanol containing ribitol (10 μg) as internal standard at 50°C for 15 minutes in 2 ml-microcentrifuge tubes. The tubes were centrifuged (12,000*g* for 5 minutes), and the supernatants were transferred to 8-ml glass culture tubes containing 1 ml of water and cooled to 4°C, after which the non-polar compounds from the extracts were partitioned with 500 μl of chloroform at 4°C in a rotary shaker. Next, the tubes were centrifuged at 1,500 *g* for 10 minutes, and the top, aqueous methanol phase was drawn into glass vials. Subsamples (100–150 μl) were transferred to vials with glass inserts (250 μl) and dried completely under nitrogen. Five microliters of a fatty acid methyl ester standard mixture (C4-C30, even carbon) in hexane (100 μg ml^{-1}) and 5 μl of d27-myristic acid in hexane (1 mg ml^{-1}) were added to the vials, which were then dried completely under

Table 1. List of solvent extractable polar metabolites identified in the senesced tissues of *B. gracilis* and *P. smithii*.

No.	Metabolite	Fragment ions (m/z)†	Retention time‡
Common to both			
1	L-alanine	116, 117, 190	0.409
2	allo-inositol	318, 217, 305	1.160
3	D-allose	205, 160, 217	1.034
4	Benzoic acid	179, 77, 135	0.547
5	Ferulic acid	338, 323, 308	1.164
6	Fructose	103, 217, 307	1.027
7	Fumaric acid	245, 246, 143,	0.634
8	Galactose	205, 319, 217	1.064
9	Gluconic acid	333, 292, 205	1.104
10	L-glutamic acid	246, 128, 156	0.849
11	Glyceric acid	189, 292, 133	0.614
12	Glycerol	205, 117, 103	0.564
13	Glycine	174, 248, 86	0.595
14	Glycolic acid	66, 177, 205,	0.384
15	4-hydroxycinnamic acid	219, 293, 308	1.072
16	4-hydroxybenzoic acid	276, , 223, 193	0.858
17	4-hydroxybenzaldehyde	223, 176, 208	0.756
18	2-hydroxybutyric acid	131, 132, 205,	0.431
19	Hydroquinone	239, 254, 240	0.697
20	DL-isoleucine	158, 218, 147	0.583
21	Lactobionic acid	204, 217, 191	1.499
22	Linoleic acid	67, 81, 55, 129	1.235
23	D-lyxosylamine	103, 217, 307	0.887
24	D-lyxose	103, 217, 307	0.873
25	Maleic acid	245, 75, 67	0.503
26	Malonic acid	66, 233, 133	0.503
27	Palmitic acid	117, 313, 132, 278	1.137
28	L-proline	70, 75, 103	0.480
29	L-pyroglutamic acid	156, 157, 230,	0.775
30	L-serine	204, 218, 100	0.640
31	Stearic acid	117, 341, 132, 145	1.252
32	Succinic acid	75, 247, 129	0.603
33	Tagatose	103, 217, 307	1.018
34	D-threitol	217, 103, 205	0.760
35	Tyrosine	218, 219, 280	1.069
36	L-valine	144, 73, 218	0.514
37	4-hydroxy-3-methoxybenzoic acid	197, 267, 312, 253	0.954
38	Xylitol	217, 103, 205	0.908
Detected only in *P. smithii*			
1	Trans-aconitic acid	229, 211, 285	0.942
2	Citraconic acid	259, 103, 89	0.633
3	Coniferyl alcohol	324, 293,204	1.072
4	3,4-dihydroxybenzoic acid (Protocatechuic acid)	193, 370, 355	0.992
5	2′-deoxyguanosine	280, 295, 281, 103	1.518
6	L-glutamine	246, 128, 247	0.849
7	Itaconic acid	259, 215, 74	0.627
8	L-leucine	158, 159, 232	0.565
9	Methyl-beta-D-galactopyranoside	204, 217, 133	1.283
10	Melibiose	204, 361, 217	1.530
11	Palatinose	361, 204, 217	1.555
12	Quinic acid	345, 255, 346	1.014
13	Salicylic acid	267, 135, 268	0.767
14	Thymine	255, 270, 113	0.676
15	L-tryptophan	202, 203, 291	1.236
16	Uracil	241, 99, 255	0.623
17	Urea	189, 66, 98	0.537
18	4-hydroxy-3-methoxybenzyl alcohol	209, 298, 268	0.864
19	DL-4-hydroxy-3-methoxymandelic acid	297, 298, 371	1.026
Detected only in *B. gracilis*			
1	Aspartic acid	232, 100, 218	0.772
2	Citric acid	273, 347, 75	0.987
3	4-guanidinobutyric acid	174, 304, 75	0.782
4	Lactic acid	117, 191, 191	0.369
5	Maltitol	361, 204, 217, 103	1.548
6	Mannose	205, 319, 160, 217	1.038
7	Phosphoric acid	299, 314, 211	0.563
8	Pipecolic acid	156, 157, 230,	0.647

Table 1. Continued.

No.	Metabolite	Fragment ions (m/z)†	Retention time‡
9	Porphine	184, 134, 285	0.618
10	Raffinose	361, 217, 204	1.853
11	Threonine	117, 218, 291	0.662

† All compounds produce ion fragments of m/z = 73 (base peak) that corresponds to [$(CH_3)_3$ SiOH], and m/z = 147 that corresponds to [$(CH_3)_3$ SiOSi$(CH_3)_2$] which are characteristics for MSTFA derivatization.
‡ Relative to myristic acid.

nitrogen. The samples were further methoxylaminated via reaction with 20 μl of methoxylamine hydrochloride (20 mg ml^{-1}) in pyridine at 40°C for 90 minutes. Additionally, the acidic protons in the compounds were silylated via reaction with 80 μL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) for 40 minutes at 40°C. The samples were finally stored at 4°C and were subjected to gas chromatography-mass spectrometry (GC-MS) analysis within 10 hrs of derivatization.

The samples were analyzed using an Agilent 7980A GC system coupled with a 5975 C series mass detector. The separation of metabolites was achieved in a DB-5 MS capillary column (30 m length × 0.25 mm internal diameter × 0.20 μm film thickness) using split (1:10 and 1:100) injection (1 μl) with the following temperature program: 60°C for 1 minute, followed by ramping at 10°C per minute to 315°C, with a 10-minute hold at 315°C prior to cool down. The carrier gas (He) was maintained at a constant pressure of 10.7 psi; the injection port and the MS interphase were maintained at 270°C; the MS quad temperature was maintained at 150°C; and the MS source temperature was set at 240°C. The electron multiplier was operated at a constant gain of 10 (EMV = 1478 V), and the scanning range was set at 50–600 amu, achieving 2.66 scans sec^{-1}. The mass spectra were further processed using the Automatic Mass Spectral Deconvolution and Identification System (AMDIS, v2.71, NIST) with the following deconvolution parameters: match factor, 75%; resolution, high; sensitivity, medium; shape requirements, medium. The compounds were positively identified based on an in-house retention index mass spectral library supplemented with Fiehn Lib (Agilent Technologies, Wilmington, Delaware, USA, G1676AA), which contains the retention indices and mass fragment information for 768 plant metabolites, and the NIST11 mass spectral library. All positive matches were confirmed by manual curation, and the integrated area with reference to internal standard was used for further statistical analysis. The extractable polar metabolites were grouped into amino acids, organic acids, phenolic acids, sugars and sugar alcohols for statistical analyses.

Table 2. List of major ester-bound phenolic compounds identified in *B. gracilis* and *P. smithii*.

Sl. no.	Phenolic compounds
1	Acetovanillone
2	Ferulic acid
3	4-Hydroxybenzoic acid
4	4-Hydroxybenzaldehyde
5	*m*-coumaric acid
6	*p*-coumaric acid
7	Sinapic acid
8	Syringic acid
9	Vanillin
10	Vanillic acid
11	4-hydroxy acetophenone
12	Syringaldehyde
13	Acetosyringone
14	Sinapaldehyde

Cell wall bound phenolics (ester-bound phenolics).—This extraction procedure removes the ester-bound phenolics from cell wall tissue via mild alkaline hydrolysis (Martens 2002). Briefly, 200 mg of leaf litter that was prior extracted with methanol (used for metabolomics analysis) was combined with 6 ml of 1 M NaOH for alkaline hydrolysis and incubated at 25° ± 2°C for 15 hours on an orbital shaker at 26 rpm. The supernatant was incubated on a heating block for two hours at 90°C to release the conjugated phenolics. Next, the solution was acidified (pH < 1.5) using 50% HCl and centrifuged at 2,000 rpm for 4 minutes. The supernatant was volumetrically transferred to glass tubes, and the phenolic compounds in the solution were recovered through liquid-liquid partitioning with 2 ml of ethyl acetate on an orbital shaker at 4°C. The percent recovery of all phenolic compounds

using this method was >75% (Tharayil et al. 2013). A subsample of the ethyl acetate fraction was evaporated to dryness under N_2 and redissolved in 50:50 (v/v) methanol:water for analysis via high performance liquid chromatography (HPLC).

HPLC analysis of the ester fractions.—Phenolics were separated in an Onyx C18 column (monolithic silica, 130A^0; 100 mm; 4.6 mm I.D.; Phenomenex, Torrance, CA) via binary gradient elution using 5% acetonitrile containing 0.2% acetic acid (mobile phase A) and 50% acetonitrile (mobile phase B), with a linear increase in the strength of the mobile phase from 100% A at 0 min to 76% at 17 min and a flow rate of 0.8 ml min^{-1}. These settings provided a minimum peak resolution (Rs) of 1.8. The limit of detection and quantitation was defined as a signal-to-noise ratio greater than 8 and 25, respectively. The reported values are based on the peak area at 272 nm. The compounds in the extracts were identified and quantified by comparison of the obtained retention times and UV spectra to those of an in-house library of 42 plant phenolic compounds (Tharayil et al. 2013).

DRIFT spectroscopy.—Senesced litter that was not subjected to solvent extraction and the residual litter samples remaining after methanol extraction were used in the DRIFT analyses. The samples were dried at 50°C to remove any moisture and further powdered to <10 μm particle size. The DRIFT spectra of the litter samples were collected in transmission mode using a spectrometer (Perkin-Elmer Spectrum One DRIFT) equipped with a deuterated triglycine sulfate detector. The finely powdered litter samples were mixed with spectral grade KBr at a ratio of 1:50. The mixture was carefully packed into a macro-cup sampling accessory, and the spectra were recorded from 4,000 to 650 cm^{-1} at a 4 cm^{-1} resolution. For each sample, we collected 40 interferograms, which were averaged and corrected against the background spectrum of pure KBr. The spectra were baseline corrected and transformed with the Kubelka-Munk function using ACD Spec Manager (Advanced Chemistry Development, Ontario, Canada). Spectral assignment was performed based on pure standards and according to Silverstein et al. (2005), and peak interpretation was based on Lammers (2008), Lammers et al. (2009), and Movasaghi et al. (2008). We selected 17 identifiable peaks that corresponded to major functional groups for statistical analysis. We calculated the relative peak heights of the functional groups in each sample for comparison across samples from different climate treatments. The relative peak heights were computed as the ratio of the intensity of each peak to the sum of the intensities of all selected peaks (Haberhauer and Gerzabek 1999). We conducted a principal component analysis (PCA) of the relative peak heights to interpret the DRIFT spectra (Suseela et al. 2013).

Data analysis

The different extractable polar metabolite groups such as amino acids, phenolic acids, organic acids, sugars and sugar alcohols were grouped by adding the concentration of individual compounds that were normalized with ribitol. The data were range scaled for further analyses. The metabolomics data of each species were first analyzed using multivariate analysis of variance (MANOVA) which permitted the analyses of all variables in a single statistical method and the evaluation of main and interactive effects of treatments within the experimental design (Johnson et al. 2007, Rivas-Ubach et al. 2012). The significant effects ($P < 0.05$) from MANOVA were further analyzed using univariate analysis of variance (ANOVA). The univariate analyses were used to identify the metabolite groups that caused differences among treatments (Rivas-Ubach et al. 2012). The main and interactive effects of warming and CO_2 on ester-bound phenolics were analyzed using PROC MIXED. Extractable polar metabolite data, DRIFT spectroscopy and ester-bound phenolics data were analyzed via principal component analysis (PCA; Tharayil et al. 2011). In all statistical analyses differences among individual treatments was determined using Tukey's HSD multicomparison test. All analyses were done using SAS (SAS version 9.2, SAS Institute, Cary, North Carolina, USA).

Results

Extractable polar metabolites

Multivariate analysis of variance showed significant effects of treatments on the different

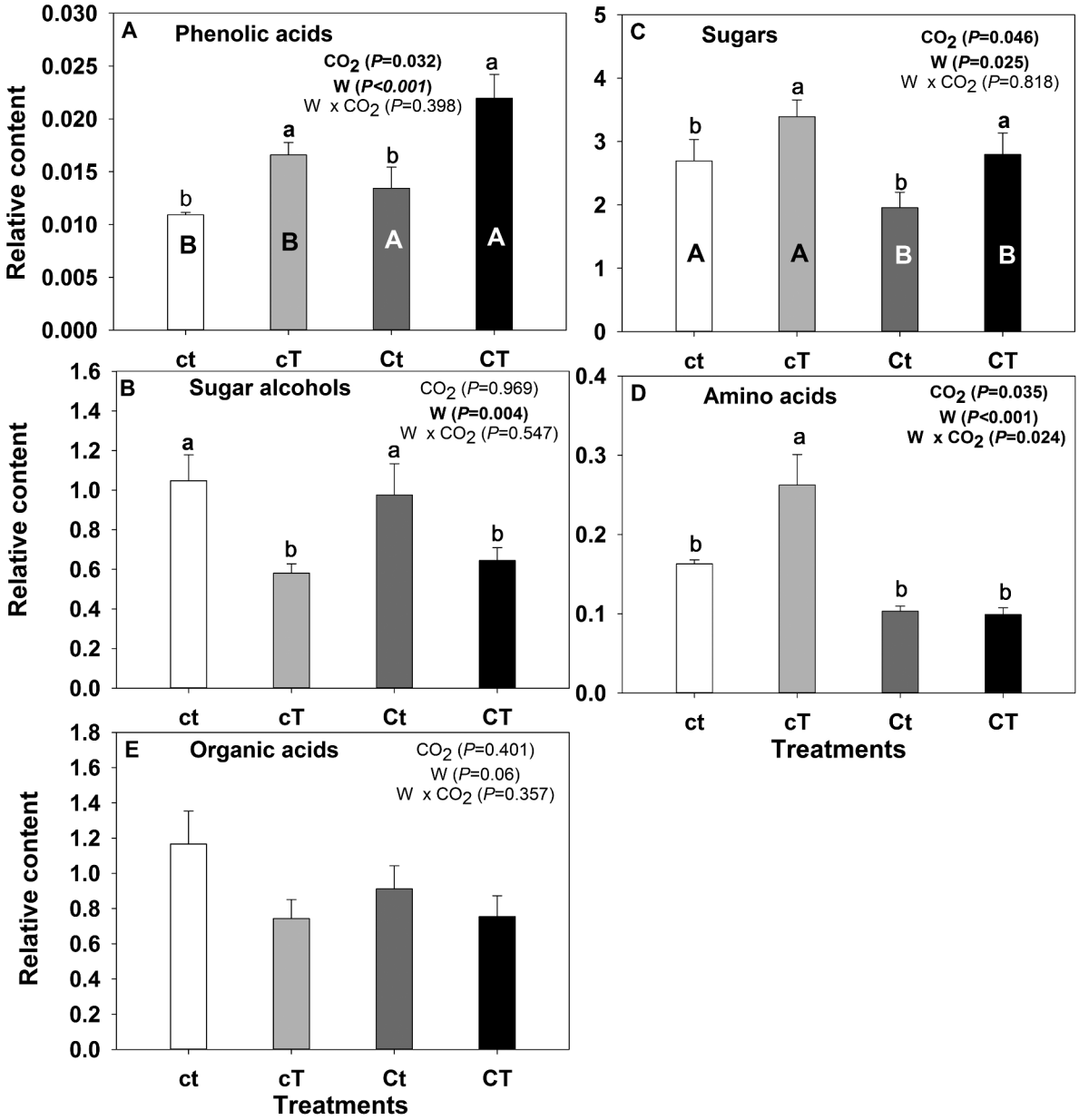
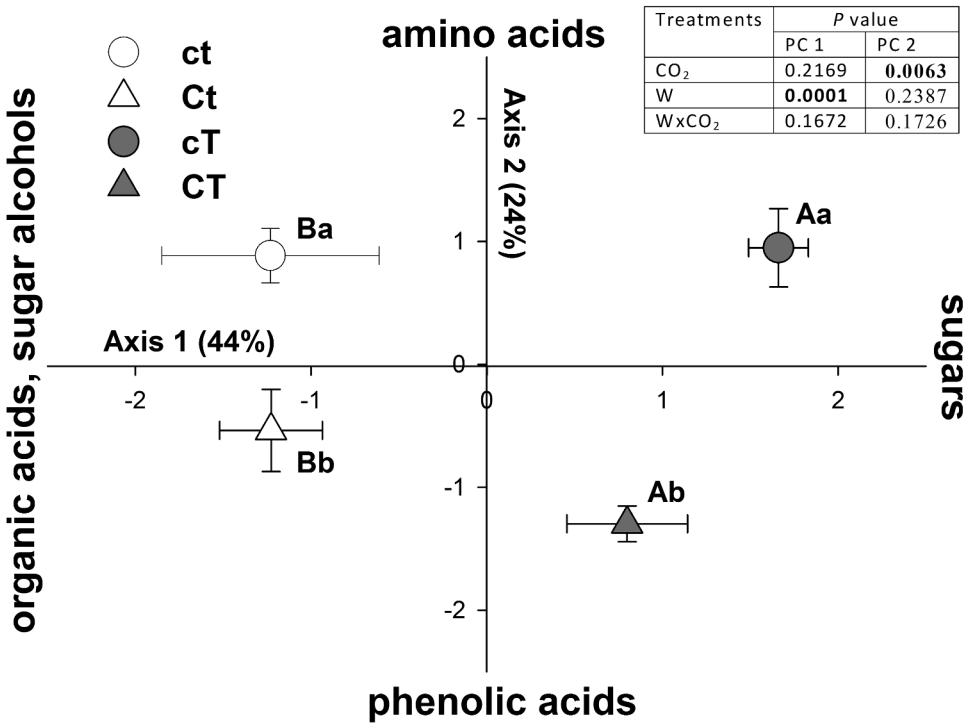


Fig. 1. Effect of warming and CO_2 treatments on the relative contents of (A) phenolic acids, (B) sugars, (C) sugar alcohols, (D) amino acids and (E) organic acids in *B. gracilis*. Values represent means ± SE (n = 5). Letters 'a' and 'b' indicate Tukey's difference between warming treatments and letters 'A' and 'B' between CO_2 treatments.

groups of extractable polar metabolites in *B. gracilis* exposed to different warming and CO_2 treatments ($P < 0.001$). The important metabolite groups such as phenolic acids, sugar alcohols, sugars, and amino acids responded differently to warming and elevated CO_2 (univariate ANOVA; Fig. 1A–D) while organic acids marginally differed between the warming treatments ($P = 0.06$; Fig. 1E). Warming increased the relative amounts of phenolic acids ($P < 0.001$; Fig. 1A) and sugars ($P = 0.025$; Fig. 1B) but decreased the contents of sugar alcohols



Treatments	*P* value	
	PC 1	PC 2
CO_2	0.2169	**0.0063**
W	**0.0001**	0.2387
W x CO_2	0.1672	0.1726

Fig. 2. Principal component analysis of the relative intensities of the extractable polar metabolite groups (amino acids, organic acids, phenolic acids, sugars and sugar alcohols) of *B. gracilis* subjected to different climatic treatments (ct, ambient CO_2 +ambient temperature; cT, ambient CO_2 +warming; Ct, elevated CO_2 +ambient temperature; CT, elevated CO_2 +elevated temperature). The first and second PC axes explained 68.4 % of the variance in the data. Significant treatment effects (*P* values from ANOVA of principal component analysis) for PC1 and PC2 axes are shown as inset. Letters 'A' and 'B' indicate Tukey's difference between treatments separated by PC1 axis and 'a' and 'b' between treatments separated by PC2 axis.

($P = 0.004$; Fig. 1C). The *B. gracilis* exposed to the elevated CO_2 treatment showed a higher relative phenolic acid content ($P = 0.032$; Fig. 1A) and a lower sugar content (Fig. 1B; $P = 0.046$). The relative amount of amino acids responded to the interactive effect between the warming and CO_2 treatments, where plants exposed to warming plus ambient CO_2 treatment exhibited the highest relative content of amino acids ($P = 0.024$; Fig. 1D). These results were further supported by the PCA of extractable metabolite groups (Fig. 2). The PC 1 axis, which explained 44% of the variance in the data, statistically separated warming treatments from the unwarmed treatments. *B. gracilis* exposed to warming with ambient CO_2 had a higher relative abundance of sugars and those exposed to warming with elevated CO_2 had a higher abundance of phenolic acids. However, the abundance of sugar alcohols and organic acids were found to be associated with the unwarmed treatments. Along PC 2 axis, which explained 24% of the observed variance, *B. gracilis* exposed to elevated CO_2 treatments were separated from ambient CO_2 treatments. The elevated CO_2 treatments had a higher relative abundance of phenolic acids.

In *P. smithii*, MANOVA showed significant effect of treatments on the different metabolite groups ($P = 0.01$). However, only sugars and sugar alcohols varied with different treatments where the levels of sugar alcohols decreased with warming (univariate ANOVA; $P = 0.039$; Fig. 3B), while those of sugars increased with warming ($P = 0.002$) and decreased under elevated CO_2 ($P = 0.009$; Fig. 3C). Organic acids, phenolic acids and amino acids did not differ between the treatments ($P > 0.05$; Fig. 3). The PCA of the

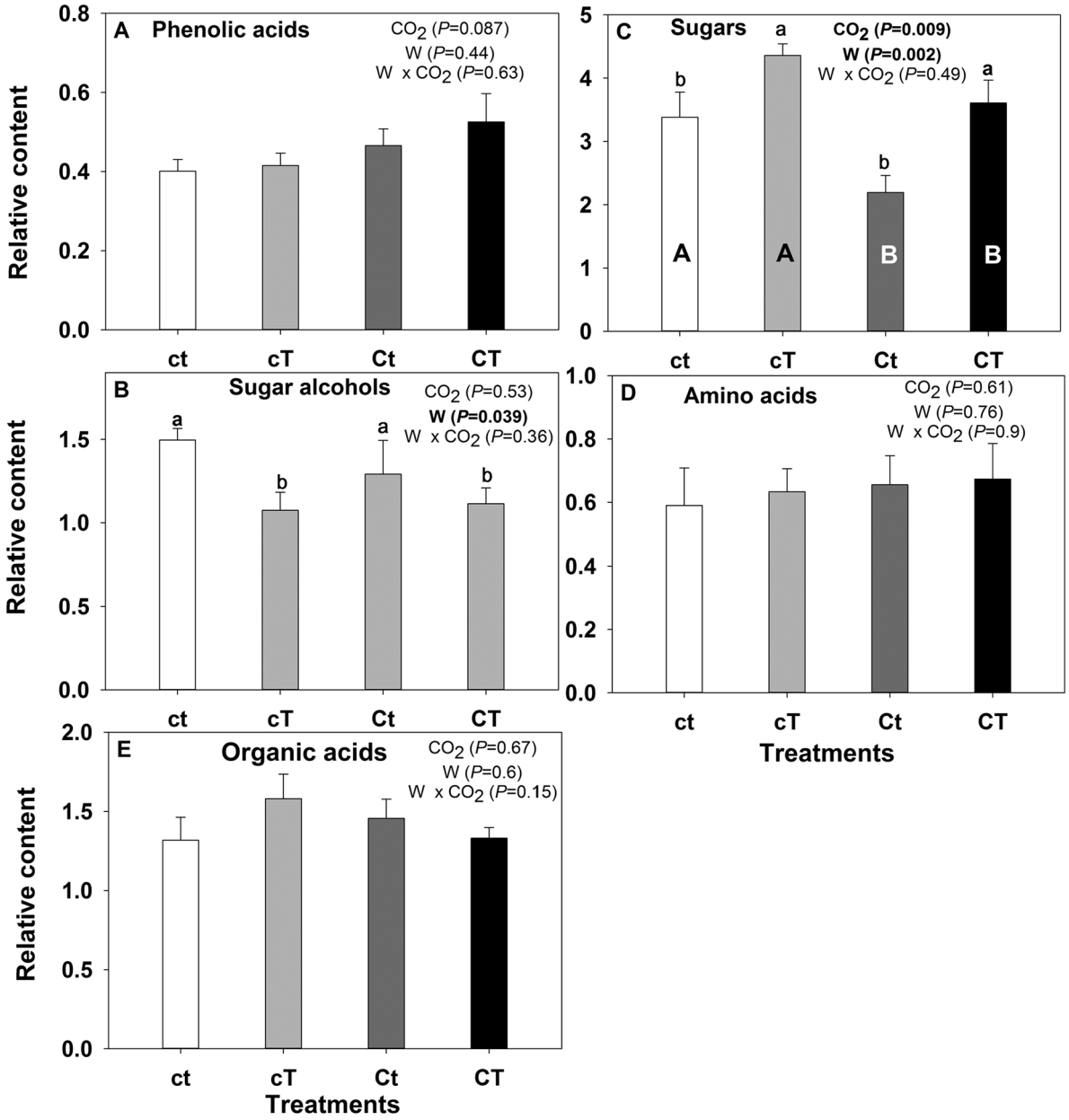
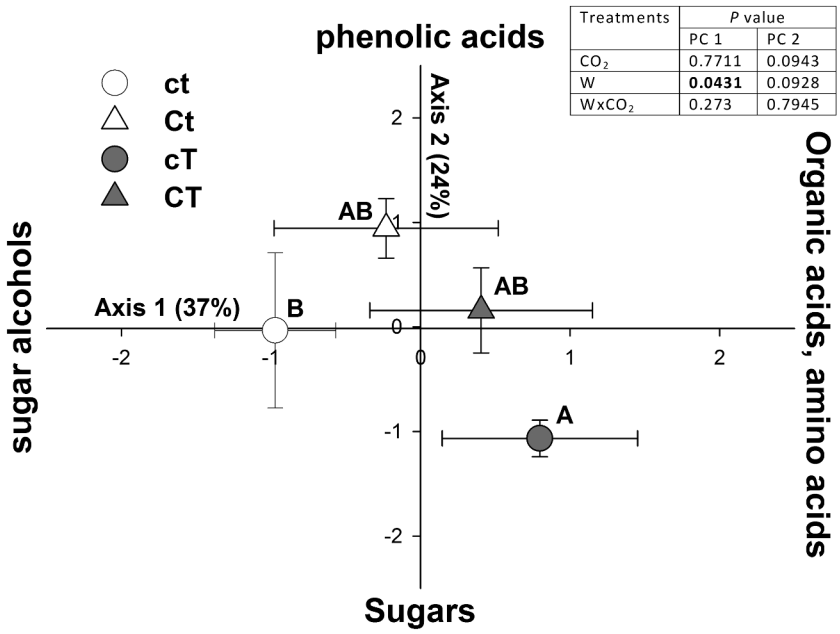


Fig. 3. Effect of warming and CO_2 treatments on the relative contents of (A) phenolic acids, (B) sugars, (C) sugar alcohols, (D) amino acids and (E) organic acids in *P. smithii*. The presented values represent the means ± SE of the treatment replicates (n = 5). Letters 'a' and 'b' indicate Tukey's difference between warming treatments and letters 'A' and 'B' between CO_2 treatments.

extractable polar metabolite groups of *P. smithii* that explained 61% of the variance in the data revealed that warming with ambient CO_2 increased the relative abundance of sugars separating this treatment from the control (ct) treatment that had a higher relative abundance of sugar alcohols (Fig. 4).

Ester-bound phenolic acids

Out of more than 20 phenolic compounds detected, ferulic acid and *p*-coumaric acid represented >70% of the total phenolic compounds in both species (Fig. 5). The total phenolic acid content in *B. gracilis* increased with the main



Treatments	*P* value	
	PC 1	PC 2
CO_2	0.7711	0.0943
W	**0.0431**	0.0928
W x CO_2	0.273	0.7945

Fig. 4. Principal component analysis of the relative intensities of the extractable polar metabolite groups (amino acids, organic acids, phenolic acids, sugars and sugar alcohols) of *P. smithii* subjected to different climatic treatments (ct, ambient CO_2 +ambient temperature; cT, ambient CO_2 +warming; Ct, elevated CO_2 +ambient temperature; CT, elevated CO_2 +elevated temperature). The first and second PC axes explained 57.1 % of the variance in the data. Significant treatment effects (P values from ANOVA of principal component analysis) for PC1 and PC2 axes are shown as inset. Letters 'A' and 'B' indicate Tukey's difference between treatments separated by PC1 axis.

effect of warming (11%; $P = 0.001$; Fig. 5A) and with the main effect of elevated CO_2 ($P = 0.009$), while in *P. smithii*, total phenolic acid content decreased with warming (5%; $P = 0.022$; Fig. 5B) and increased with elevated CO_2 treatment (7%; Fig. 5B). In *B. gracilis*, compared to the control, the concentration of ferulic acid increased by 14% with warming under ambient CO_2 ($P = 0.011$; Fig. 5C), while in the senesced tissues of *P. smithii*, the concentration of ferulic acid increased with the main effect of elevated CO_2 treatments (8%; $P = 0.038$; Fig. 5D). Warming treatments did not affect the concentration of ferulic acid in *P. smithii*.

In *B. gracilis*, the concentration of *p*-coumaric acid was highest in the warmed plots with elevated CO_2 (CT) compared to treatments involving warming under ambient CO_2 (cT; 46%; $P = 0.004$), elevated CO_2 and ambient temperature (Ct; 27%; $P < 0.0001$), or ambient CO_2 and ambient temperature (ct; 35%; $P = 0.002$; Fig. 5E). However, in *P. smithii*, the main effect of warming decreased the concentration of *p*-coumaric acid by 16% relative to plants in the ambient temperature treatments ($P < 0.001$; Fig. 5F) and elevated CO_2 increased the concentration of *p*-coumaric acid (5%; $P = 0.026$; Fig. 5F) compared to ambient CO_2 treatments.

The results of the principal component analysis of the relative concentrations of ester-bound phenolic acids varied between the species. For *B. gracilis*, the PC 1 axis, which explained 53% of the observed variance, statistically separated plants exposed to warming with elevated CO_2 from all other treatments ($P < 0.001$; Fig. 6A) and was associated with a higher relative abundance of 4-hydroxybenzoic acid, syringic acid and *p*-coumaric acid. Along PC axis 2, which explained 17% of the variance in the data, the *B. gracilis* in the warmed plots under ambient CO_2 were significantly separated from all other treatments ($P = 0.014$) and showed a higher relative abundance of ferulic acid, *m*-coumaric acid and vanillin.

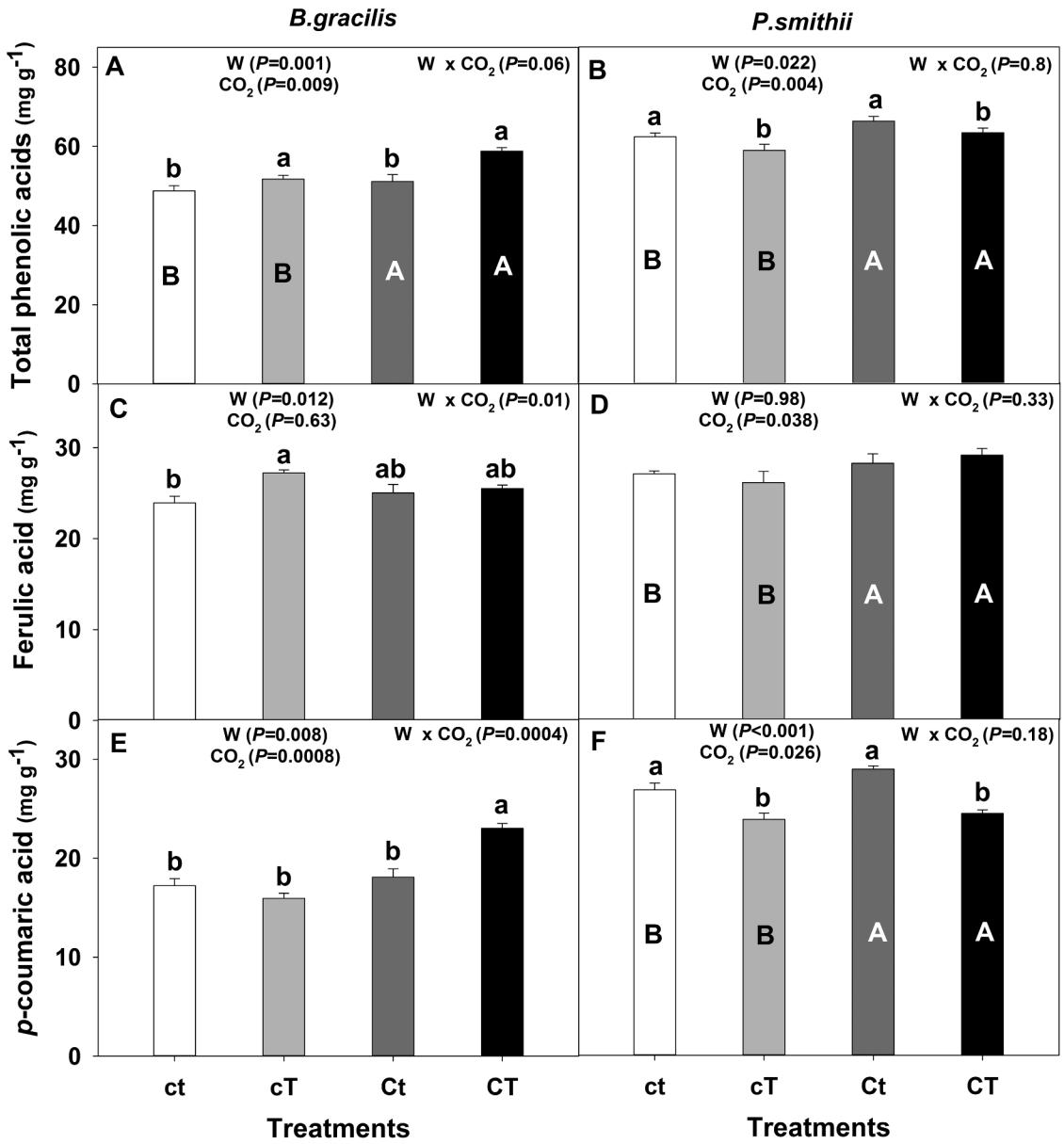


Fig. 5. Effect of warming and CO_2 treatments on the concentration of total ester-bound phenolic acids, ferulic acid and *p*-coumaric acid in *B. gracilis* (A, C, E) and *P. smithii* (B, D, F). The presented values represent the means ± SE of the treatment replicates (n = 5). Letters 'a' and 'b' represents difference (Tukey's HSD) between the main or interactive effect of warming and CO_2 treatments.

For *P. smithii*, PC axes 1 and 2 explained 35 and 20% of the variance in the data, respectively (Fig. 6B). The PC 1 axis statistically separated plants in warmed plots exposed to both ambient (cT) and elevated CO_2 (CT) from the ambient temperature treatments (ct, Ct; $P < 0.001$). The warmed treatments were associated with relatively higher levels of sinapic acid and syringic acid, and the plants in the ambient temperature treatments were characterized by an abundance of acetovanillin and *p*-coumaric acid.

DRIFT analyses

The initial DRIFT analysis of non-extracted litter of *B. gracilis* and *P. smithii* did not reveal any treatment effects; however the DRIFT analysis of

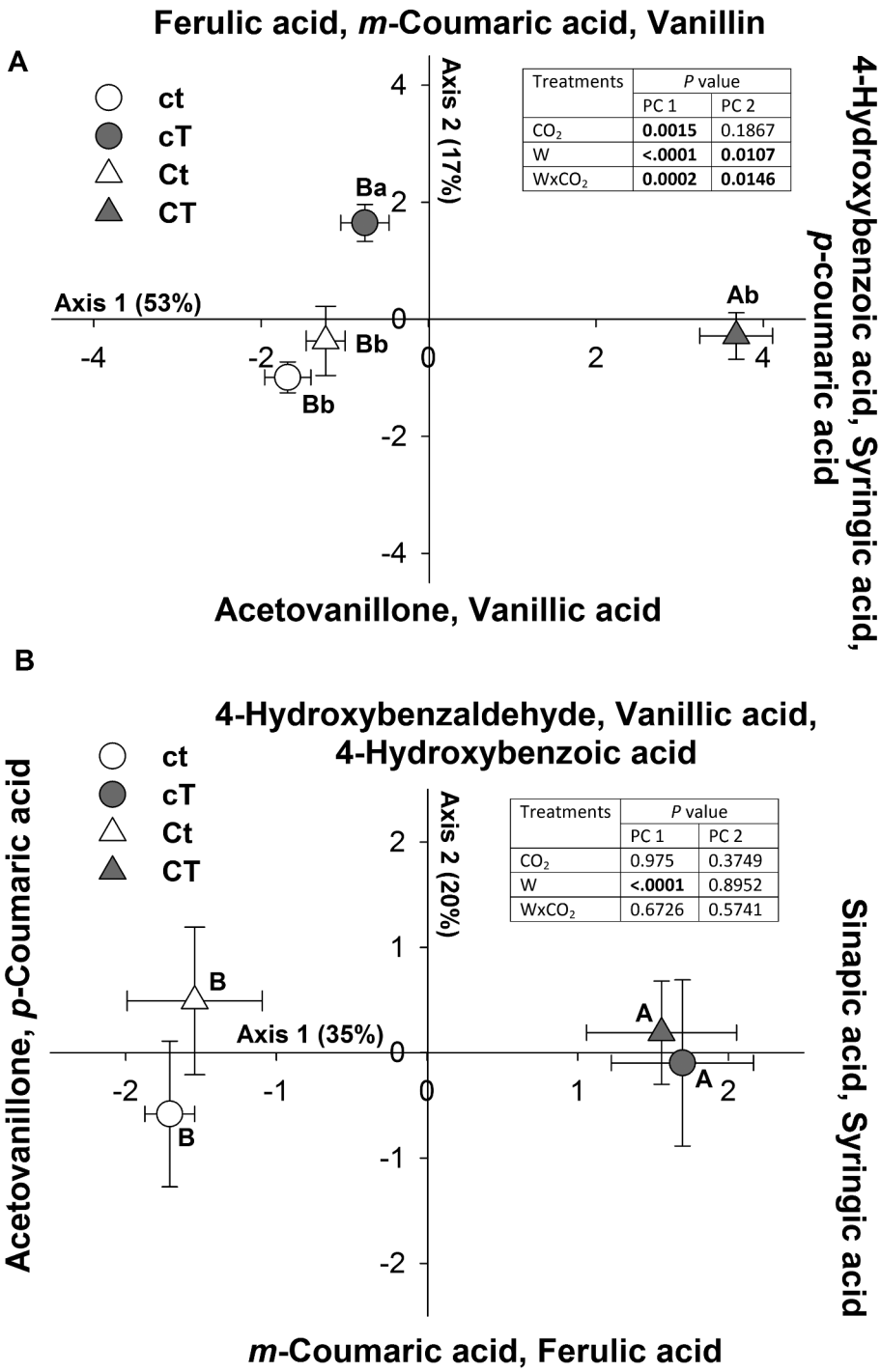


Fig. 6. Principal component analysis of the relative intensities of the ester-bound phenolic acids in (A) *B. gracilis* (letters 'A' and 'B' indicate Tukey's difference between treatments separated by PC1 axis and 'a' and 'b' between treatments separated by PC2 axis) and (B) P. smithii (letters 'A' and 'B' indicate Tukey's difference between treatments separated by PC1 axis) from different climatic treatments. The phenolic compounds in the litter with the highest eigenvector loadings are listed on each principal component axis. Each point represents a mean of five replicates. Significant treatment effects (P values from ANOVA of principal component analysis) for PC1 and PC2 axes are shown as inset.

the solvent-extracted litter (residual litter after metabolomics analysis) showed significant treatment effects. DRIFT analysis targets vibrational motions in covalent bonds, and along with the cell wall components, a large portion of the extractable phenolics, other aromatic compounds and carbohydrates present in the plant tissue are expected to contribute to these molecular vibrations. Thus, removing the extractable metabolites from the litter matrix helped us to identify the effect of climate on the compositional differences of the non-extractable structural matrix of the litter.

Principal component analysis of the relative intensities of the DRIFT peaks in the residual litter obtained after solvent extraction revealed the effect of warming and CO_2 on the non-extractable structural components of the litter (Fig. 7). For *B. gracilis*, PC axes 1 and 2 explained 62 and 21% of the variance in the data, respectively (Fig. 7A). Along PC axis 1, the *B. gracilis* exposed to warming with ambient CO_2 (cT) differed significantly from those in the ambient temperature treatments exposed to either ambient CO_2 (ct) or elevated CO_2 (*Ct*; $P < 0.005$). The *B. gracilis* exposed to warming together with elevated CO_2 also differed significantly from those exposed to elevated CO_2 alone. Warming increased the relative abundance of alkyl compounds from waxes and cuticular-matrix (2851 cm^{-1} [CH_2 asymmetric stretch], 1424 cm^{-1} [C-H deformation]) and lignin (1515 cm^{-1} [aromatic C-C stretch]) and decreased the abundance of carbohydrates (1106, 1059, 1160 cm^{-1} [combination of C–O stretching and O–H deformation]).

For *P. smithii*, PC axes 1 and 2 explained 62 and 15% of the variance in the data, respectively (Fig. 7B). The combined effects of warming and elevated CO_2 increased the relative abundance of alkyl carbon (2918 cm^{-1}, 1424 cm^{-1}, 2897 cm^{-1}) and lignin (1515 cm^{-1}, $P = 0.003$) compared with the other treatments. The *P. smithii* that experienced elevated CO_2 without warming (Ct) were characterized by a higher relative abundance of carbohydrates (1059, 1106, 1160 cm^{-1}).

Discussion

In response to environmental stress, plants modify the pathways that regulate the biosynthesis and resorption of metabolites, resulting in an altered chemical composition of senesced tissues. Given that grasslands are important sinks for soil carbon and that the majority of them contain a mixture of C3 and C4 species, obtaining a better understanding of the changes in litter chemistry associated with these functional types due to warming and elevated CO_2 is critical to predict carbon storage in grasslands under future climatic conditions. Here, we report that *P. smithii* (C3) and *B. gracilis* (C4) in a semiarid grassland exhibit differential litter chemistry when exposed to a factorial combination of warming and elevated CO_2 potentially due to warming induced reduction in soil moisture. The response of extractable compounds did not exhibit a clear relationship with climate treatments, possibly due to the concomitant effect of climate on production and resorption of these mobile metabolites. In *B. gracilis* the total cell-wall bound phenolic acids increased with the main effect of warming, ferulic acid increased with cT treatment and *p*-coumaric acid increased with CT treatment and structural compounds such as lignins, waxes and cuticular-matrix showed greater abundance with warming in the absence of elevated CO_2. Thus in general, warming treatments elicited greater physiological response in C4 species. Conversely, *P. smithii* (C3) in warmed treatments had lower total cell-wall bound phenolic acids and *p*-coumaric acid, and only warming accompanied by an increase in CO_2 resulted in an increased concentration of structural components such as lignins, waxes and cuticular-matrix, indicating a better physiological response of *P. smithii* to moderately stressful climates.

Response of extractable polar metabolites to warming and elevated CO_2

In *B. gracilis* the relative abundance of extractable polar metabolites such as sugars and free phenolic acids increased with the main effect of warming, and amino acids increased under warming with ambient CO_2 (Fig. 1). High temperature and the accompanying moisture stress (25% reduction in soil volumetric water content compared to elevated CO_2; Morgan et al. 2011) often translate into osmotic stress in plants (Szabados et al. 2011), and can induce several types of physiological and biochemical changes,

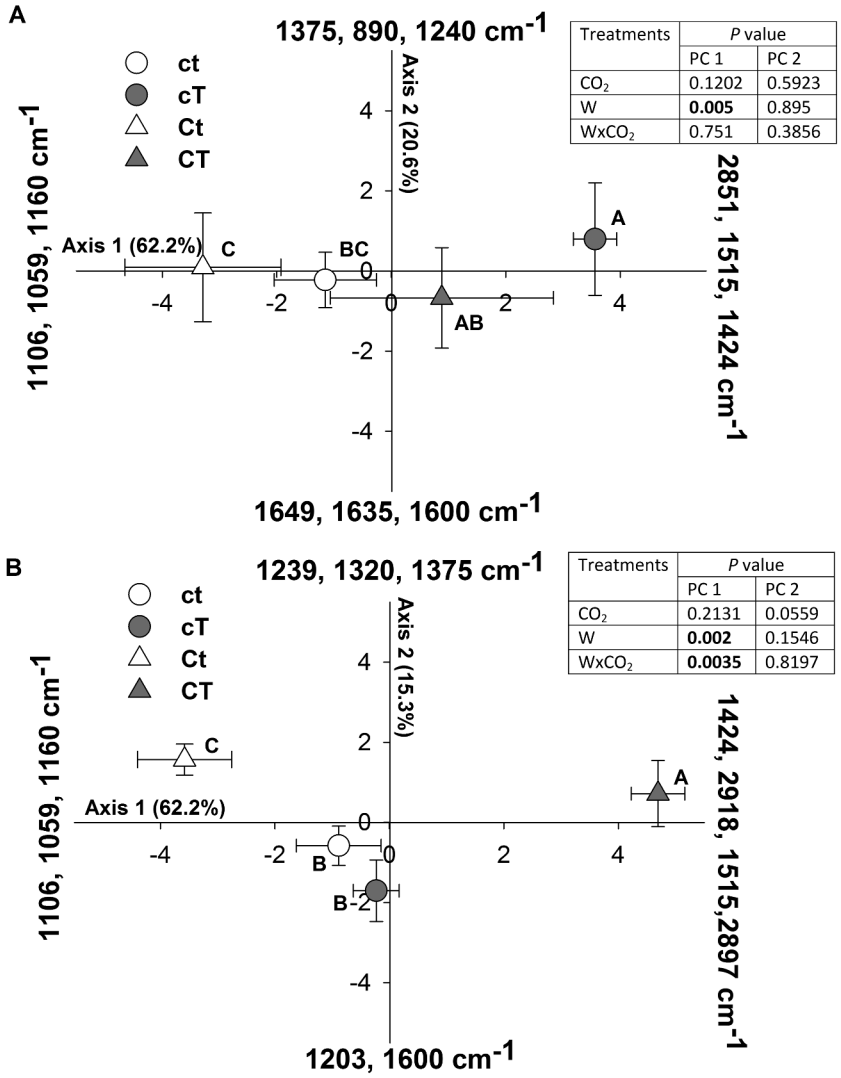


Fig. 7. Principal component analysis of the relative intensities of the dominant DRIFT peaks of leaf litter of (A) *B. gracilis* and (B) *P. smithii* from different climatic treatments. The wave numbers (carbon functional groups in the litter corresponding to different compounds) with the highest eigenvector loadings are listed on each principal component axis. Each point represents a mean of three replicates. Significant treatment effects (P values from ANOVA of principal component analysis) for PC1 and PC2 axes are shown as inset. Letters 'A', 'B' and 'C' indicate Tukey's difference between treatments separated by PC1 axis.

resulting in the altered production of metabolites (Ahuja et al. 2010). Plants subjected to heat and drought stress have been shown to accumulate sugars (Guy et al. 2008) that act as osmoregulants (Rivas-Ubach et al. 2012) and phenolic compounds that function as antioxidants (Urquiaga and Leighton 2000). We interpret the observed changes in extractable polar metabolites in *B. gracilis* as an adaptive response, as previous investigations performed at the same site as the present study have shown 23% higher biomass production of this species under warming treatments (Morgan et al. 2011).

In *P. smithii*, only sugars showed a greater relative abundance in response to the main effect of warming (Fig. 3). Thus, the response of extractable metabolites in both the species did not confirm our initial hypothesis. The relative

change in abundance of different polar metabolites in the litter of *B. gracilis* and *P. smithii* observed in the present study (Figs. 1 and 2) could be a function of the climatic effect on both production and resorption. Also soil moisture availability, a critical factor determining the resorption of nutrients during senescence, (del Arco et al. 1991) would potentially influence the observed metabolite profiles in the senesced grass tissues.

Response of ester-bound phenolics and structural components to warming and elevated CO_2

Among the total phenolic compounds in plants, those associated with cell wall (ester-bound compounds) exhibit a better protective function than those stored in vacuoles (Antonelli et al. 1998, Fischbach et al. 1999) and are not affected by resorption. Ester-bound hydroxycinnamates and their dimers provide structural stability in the cell walls of grasses (Harris and Trethewey 2010), act as initiation sites for lignin and cross-link polysaccharides to lignin (Carpita 1996, Hatfield et al. 1999). In general, in *B. gracilis* (C4), we observed an increase in the total ester-bound phenolic contents, ferulic acid and *p*-coumaric acid in the warmed treatments (Fig. 5A, C, E). These cell wall-bound phenolics have been found to increase in plants exposed to drought, thus increasing resistance to moisture stress (Hura et al. 2011). In addition, by acting as a physical barrier against fungal penetration, cell wall-bound phenolics confer improved disease resistance (de Ascensao and Dubery 2003, Santiago et al. 2009). Similarly, due to their high UV absorption capacity, cell wall-bound phenolics can attenuate UV-induced oxidative damage to the photosynthetic apparatus (Landry et al. 1995, Schweiger et al. 1996, Hura et al. 2009), to which plants are more prone under drought (Garcia-Plazaola and Becerril 2000). Thus, climatic stress may have led to the greater production of cell wall-bound phenolics in *B. gracilis*, which in turn, could protect this species against water stress, irradiation and pest and pathogen attack. Thus, *B. gracilis* exhibited consistency in responding to warming treatments, and was abundant in both structural (Fig. 5A, C, E) and non-structural (Fig. 1) metabolites and also exhibited a 23% higher biomass production under warming (Morgan et al. 2011). However, in *P. smithii* the total cell wall-bound phenolics, ferulic and *p*-coumaric acids only increased with the main effect of CO_2 and total cell wall-bound phenolics and *p*-coumaric acid decreased with the main effect of warming (Fig. 5B, D, F), indicating a metabolic response of this C3 species only under elevated CO_2.

The DRIFT spectra obtained for *B. gracilis* and *P. smithii* captured the differential responses of structural compounds in litter of these two species to the climatic treatments. The *B. gracilis* plants exposed to warming exhibited a higher relative abundance of lignin, waxes and cuticular-matrix compared to plants exposed to the ambient temperature treatment, which showed a greater relative abundance of carbohydrates (Fig. 7A). Additionally, in combination with elevated CO_2, the warming treatment produced a marginally higher relative abundance of lignin, waxes and cutin in *B. gracilis*. Warming in conjunction with elevated CO_2 is thought to pose a greater stress for C4 plants due to the high leaf temperatures caused by the partial closure of stomata (Hamilton et al. 2008). Along with cutin, which forms a water-impermeable layer on leaf surfaces, increased lignin contents, especially the stress lignin formed from acylated monolignols (del Rio et al. 2007), could represent an adaptation to moisture stress (Bargel et al. 2006, Lee et al. 2007, Kosma et al. 2009).

In *P. smithii*, a similar relative abundance of lignin and cuticular materials was observed only in plants exposed to warming in conjunction with elevated CO_2 (Fig. 7B). Because *P. smithii* is a cool-season grass, the greater heat/drought stress experienced by this C3 grass when subjected to warming alone might have been overly disruptive to physiology and could have suppressed any metabolic response. This scenario is supported by the observation that in C3 species the metabolic inhibition occurs when the relative water content in the leaves falls below 70%, and CO_2 assimilation rates fail to recover even after removal of water stress (Lawlor 2002, Flexas et al. 2004). Previous studies from the same site have reported similar response of biomass in C3 species to climate treatments, where their biomass production increased by 34% only in elevated CO_2 treatments, but did not respond to warming (Morgan et al. 2011). Thus, the increase in relative abundance of structural

metabolites in *P. smithii* could be construed as a response that occurs only when warming is supplemented with elevated CO_2, and could partially be attributed to low moisture stress in these treatments (25% higher soil volumetric water content than warming alone treatments; Morgan et al. 2011).

Overall, under a warmer climate, *B. gracilis* (C4) responded by allocating more carbon for the production of non-structural osmoregulatory compounds, bound phenolics and protective structural components, including lignins, waxes and cutin, whereas a similar response in structural components (lignins, waxes and cutin) in *P. smithii* (C3) was initiated only when warming was supplemented with elevated CO_2. Because the described responses of C3 and C4 species clearly coincide with higher biomass production in these species in the respective climates at this study site (Read and Morgan 1996, Morgan et al. 2011), these changes in structural and non-structural components could represent a potential adaptive response to the respective climates. Thus, our observations regarding the variation in the chemical composition of leaf litter provide a novel, finer, metabolite-level, explanation for a widely known plant-level response that is traditionally quantified by biomass production.

Implications of metabolic changes on the decomposability of tissues

The organismal-level responses to environmental stresses have generally been elucidated by profiling extractable polar metabolites in previous studies (Du et al. 2011, Riikonen et al. 2012, Yu et al. 2012). However, only <10% of the compounds in plant litter could be extracted by solvents. A major proportion of carbon (~50% of dry mass) resides in the structural cellular components (Vogel 2008), which also undergo quantitative and qualitative changes in response to biotic and abiotic cues. Thus, the present study, which examined both structural and nonstructural components of tissues obtained after sequential extraction, provides an unprecedented, comprehensive understanding of the overall metabolite-level response of plants to climatic changes.

Our analyses of *P. smithii* and *B. gracillis* exposed to warming and CO_2 treatments revealed the climatic stress-induced changes in plant structural and non-structural metabolites that could have significant implications for the digestibility and decomposability of these tissues. The higher concentration of extractable polar metabolites observed under warming in *B. gracillis* and *P. smithii* could make these tissues more palatable. However, the abundance of ester-bound phenolic compounds, and the structural components such as lignins, cutins and waxes could affect the overall nutritive quality, as phenolic compounds are toxic to microbes in the rumens of animals (Akin 1982). In grasses, the major phenolic acids such as ferulic acid and *p*-coumaric acid are ester and/ or ether linked to lignin and these cell wall bound phenolics also cross-link polysaccharides to lignin that provides structural integrity to cell walls (Carpita 1996, Hatfield et al. 1999) making the litter more resistant to decay (Grabber et al. 2004, Suseela et al. 2014). Hence, the higher relative abundance of the cell wall-bound phenolics may also decrease the rate of litter decomposition. Thus, even though physiological adaptation might enable *B.gracillis* and *P. smithii* to produce higher biomass under future climates, because of the metabolite level changes brought about by the physiological responses, the overall nutritive quality and decomposition susceptibilities of this biomass could be significantly different. Although current models predict higher productivity in grasslands under elevated CO_2 alone, and combined warming and elevated CO_2 scenarios (Parton et al., 2007), our results suggests that in the long-term, concurrent increase in plant structural compounds and cell-wall bound phenolics would reduce the rate of litter decomposition and nutrient cycling leading to a negative feedback to productivity. To our knowledge, this is the first study that captures the differential response of plant metabolites in the senesced tissues of C3 and C4 exposed to factorial combinations of warming and elevated CO_2.

Although our study reveals the change in litter quality due to warming and CO_2 enrichment, the direct impact of these changes on soil carbon cycling in these grassland ecosystems could be influenced by complex organismal interactions. For example, although litter chemistry is a major driver of soil carbon cycling in ecosystems in the intervening period (Tamura and Tharayil 2014), the soil microbial community could adapt to any

climate induced changes in litter chemistry over the long-term through compositional shift. Thus, higher inputs of relatively recalcitrant litter could shift the microbial community to more fungal dominated than a bacterial dominated system that could further alter soil carbon cycling (McGuire and Treseder 2010, Suseela et al. 2013). Changes in litter chemistry at the molecular level may also influence carbon accrual (Tamura and Tharayil 2014) in different SOM pools, as observed in an Australian grassland ecosystem exposed to warming and elevated CO_2, where higher soil organic carbon and particulate organic carbon contents were observed under C4 species exposed to warming, but not under C3 vegetation (Pendall et al. 2011). Our study suggests that if the unique metabolic responses that we observed in *B. graciis* (C4) and *P. smithii* (C3), occur in other species of similar functional types, this could also potentially alter the carbon cycling and species distributions in grassland ecosystems.

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