Global Change Biology

Global Change Biology (2015), doi: 10.1111/gcb.13033

# Warming and drought differentially influence the production and resorption of elemental and metabolic nitrogen pools in *Quercus rubra*

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## Abstract

The process of nutrient retranslocation from plant leaves during senescence subsequently affects both plant growth and soil nutrient cycling; changes in either of these could potentially feed back to climate change. Although elemental nutrient resorption has been shown to respond modestly to temperature and precipitation, we know remarkably little about the influence of increasing intensities of drought and warming on the resorption of different classes of plant metabolites. We studied the effect of warming and altered precipitation on the production and resorption of metabolites in *Quercus rubra*. The combination of warming and drought produced a higher abundance of compounds that can help to mitigate climatic stress by functioning as osmoregulators and antioxidants, including important intermediaries of the tricarboxylic acid (TCA) cycle, amino acids including proline and citrulline, and polyamines such as putrescine. Resorption efficiencies (REs) of extractable metabolites surprisingly had opposite responses to drought and warming; drought treatments generally increased RE of metabolites compared to ambient and wet treatments, while warming decreased RE. However, RE of total N differed markedly from that of extractable metabolites such as amino acids; for instance, droughted plants resorbed a smaller fraction of elemental N from their leaves than plants exposed to the ambient control. In contrast, plants in drought treatment resorbed amino acids more efficiently (>90%) than those in ambient (65–77%) or wet (42–58%) treatments. Across the climate treatments, the RE of elemental N correlated negatively with tissue tannin concentration, indicating that polyphenols produced in leaves under climatic stress could interfere with N resorption. Thus, senesced leaves from drier conditions might have a lower nutritive value to soil heterotrophs during the initial stages of litter decomposition despite a higher elemental N content of these tissues. Our results suggest that N resorption may be controlled not only by plant demand, but also by climatic influences on the production and resorption of plant metabolites. As climate–carbon models incorporate increasingly sophisticated nutrient cycles, these results highlight the need to adequately understand plant physiological responses to climatic variables.

*Keywords:* climate change, climatic stress, drought, metabolic pathways, metabolomics, nutrient resorption, tannins

*Received 16 March 2015; revised version received 5 May 2015 and accepted 28 May 2015*

## Introduction

The functioning of terrestrial ecosystems can be broadly summarized as the exchange of elements between the atmosphere and different trophic levels of the biosphere (Mulder *et al.*, 2013). In an era of intense anthropogenic environmental changes, alterations in the cycling of elements such as carbon and nitrogen and feedbacks from these cycles to climate change can affect ecosystem functions (Penuelas *et al.*, 2013). In the terrestrial carbon cycle, the major pathway of carbon flow from the atmosphere to soils occurs through carbon fixation by plants, and the major return pathway results from the subsequent mineralization of this assimilated carbon by soil heterotrophs (Cotrufo *et al.*, 2013; Prescott, 2010). Thus, the chemical composition of senesced plant tissues that partly drives the soil heterotrophic metabolism forms one of the critical factors that regulate the storage of fixed carbon in soil. Climate regulates the chemical makeup of plant litter not only through its direct influence on the biosynthesis of metabolites during growth (Tharayil *et al.*, 2011), but also through the subsequent resorption of metabolites during tissue senescence (Fig. 1). Thus, an in-depth understanding of how climatic conditions affect the production–resorption dynamics of metabolites would enable us to better predict how terrestrial biogeochemical cycles will respond to climate change.

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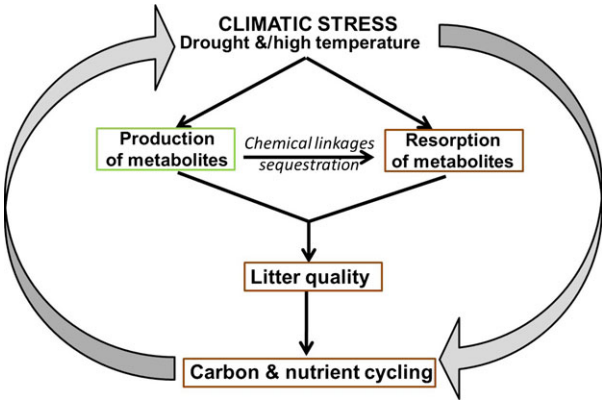


**Fig. 1** Conceptual diagram illustrating the effect of climatic stress on the production and resorption of plant metabolites leading to altered litter quality and biogeochemical cycling that can feed back to climate change. Along with the direct effect of climate on production and resorption, some of the compounds produced under climatic stress can interfere with the remobilization of metabolites during senescence. This in turn could affect the nutrient reserve of perennial plants. The altered resorption could also change the soil carbon and nutrient cycling by altering the quality of the plant litter.

Nutrient resorption, an important resource conservation strategy in perennial plant species (Chapin & Kedrowski, 1983; Franklin & Agren, 2002; Freschet *et al.*, 2010), not only contributes to plant fitness, but also regulates terrestrial nutrient cycling. Globally, resorption of nitrogen and phosphorus from senescing plant tissues is estimated to be 62% and 65%, respectively (Vergutz *et al.*, 2012). This resorption pattern varies with plant functional types (Aerts, 1996; Vergutz *et al.*, 2012) and soil nutrient status (Kobe *et al.*, 2005; Rejmankova, 2005; Yuan *et al.*, 2005; Vergutz *et al.*, 2012) and is broadly regulated by climatic parameters such as temperature and precipitation (Yuan & Chen, 2009; Han *et al.*, 2011; Reed *et al.*, 2012). Yet, surprisingly, we know little about the interactive effects of multiple climatic factors on the patterns of nutrient resorption. Most studies to date have quantified nutrient resorption in an elemental context (McGroddy *et al.*, 2004; Yuan & Chen, 2009), which provides accurate measurement regarding the overall recycling of elements in ecosystems. However, due to the non-specificity of the measured parameter, the elemental approach is less robust in tracking the identity of compounds that are being preferentially resorbed during leaf senescence. Also this approach provides less information about the composition of metabolites remaining in the senesced tissues that subsequently fuel microbial metabolism during initial stages of litter decomposition. Thus the knowledge about the metabolic regulations underlying nutrient resorption patterns could potentially improve our predictive understanding about plant growth and terrestrial nutrient cycling under future climates.

Climate warming and drought can affect plant phenology, which could potentially alter nutrient resorption through several mechanisms (Estiarte & Peñuelas, 2015). For example, warming can advance the length of growing season (Penuelas & Filella, 2001; Norby & Luo, 2004), subsequently disrupting N resorption by delaying the disassembly of chloroplasts (Collier & Thibodeau, 1995; Proietti, 1998). Delayed senescence of leaves increases the risk that an early autumnal frost could damage leaves, reducing nutrient recovery (Norby *et al.*, 2003; Schreiber *et al.*, 2013). Thus, warming that delays leaf senescence could potentially decrease nutrient resorption. Warming-induced heat stress can also increase plant respiration and alter conformation of proteins and membranes (Crafts-Brandner & Law, 2000; Crafts-Brandner & Salvucci, 2000; Kim & Portis, 2005), which could influence both production and resorption of metabolites. Moisture stress due to drought would lead to an early onset of senescence (Rivero *et al.*, 2007) that could potentially increase resorption of nutrients (Lajtha, 1987), particularly the extractable metabolites. However, under severe moisture stress, the phloem loading of photosynthates is impaired, which significantly reduces the resorption efficiency (RE) of nutrients (Pugnaire & Chapin, 1992).

Although many studies have focused on plant responses to individual stress factors, few have explored the interactive effects of drought and heat stress on the production and resorption of nutrients, which could be different from those that are elicited by either factor alone. For example, the transcriptome profiling of *Arabidopsis* exposed to a combination of drought and warming revealed a unique response of >770 transcripts which were not altered by either warming or drought alone (Rizhsky *et al.*, 2004). Similarly, drought and warming together altered the allocation of nutrients and metabolites to shoots and roots in a way not predictable by the effects of either factor alone (Gargallo-Garriga *et al.*, 2015). Under combinations of drought and heat stress, stomatal closure may lead to lower transpiration, subsequent increase in leaf temperature (Mittler, 2006; Vile *et al.*, 2012), decrease in photosynthesis and an increase in respiration rate (Rizhsky *et al.*, 2002). Warming and drought could also accelerate the production of reactive oxygen species that damage proteins, lipids, carbohydrates and DNA (Gill & Tuteja, 2010). As leaf senescence is an active process that involves the coordinated expression of multiple genes (Buchananwollaston, 1997), the heightened oxidation stress under the combined influence of warming and drought may disrupt senescence and subsequent resorption of nutrients.

Plants reprogram their metabolic pathways to circumvent or adapt to climatic stresses leading to changes in the upstream production and/or downstream utilization of metabolites (Cramer *et al.*, 2011; Baerenfaller *et al.*, 2012). For example, recent extensive and elegant metabolomic approaches have revealed that plants exposed to climatic stresses invest in the production of important metabolites such as amino acids, organic acids, phenolic acids, polyamines and lipids that partially mitigate stress by acting as osmoregulators, antioxidants and defense compounds (Dauwe *et al.*, 2012; Rivas-Ubach *et al.*, 2012, 2014; Penuelas *et al.*, 2013). Climatic stress due to warming and drought could also alter the content and composition of leaf proteins, leading to changes in the proportion of structural and soluble proteins. The dynamic process of resorption of nitrogenous compounds is further structured by the potential interference from polyphenolic compounds (Fig. 1). For example, despite greater production of soluble proteins under climatic stress (Pugnaire & Chapin, 1992), the concomitant abundance of polyphenols produced under this environment (Pizzi & Cameron, 1986; Moore *et al.*, 2000; Tharayil *et al.*, 2011; Ahmed *et al.*, 2014) could complex and precipitate proteins, resulting in lower RE of nitrogen.

Thus, nutrient conservation in plants and the chemical composition of plant litter are regulated both by the initial production and the subsequent resorption of metabolites, with climate differentially influencing both these processes (Fig. 1). However, we still lack a predictive understanding of the main and interactive effects of warming and drought on the production and resorption of plant metabolites. The main objectives of this study were to assess (i) the effect of climatic stress (warming and drought) on the production and resorption of specific plant metabolites and (ii) whether the resorption of easily extractable metabolites follows the general pattern of resorption estimated in terms of elemental content. We hypothesized that combinations of drought and warming would elicit unique responses in the production of plant metabolites that are different from responses to either drought or warming alone. As warming and drought induce moisture stress in plants, we hypothesized that these climatic stresses would decrease the RE of extractable metabolites. To test these hypotheses, we exposed *Quercus rubra* (northern red oak), a dominant deciduous tree species in North American temperate forest ecosystems, to six different combinations of warming and precipitation at the Boston-Area Climate Experiment. Although most of the North American *Quercus* species are fairly tolerant to drought, red oak is generally considered as a mesophytic species (Abrams, 2003). Thus, assessing the metabolic response of *Q. rubra* to drought and heat stress would provide valuable insights into the performance and distribution of this dominant species under future climates, which in turn may affect the structure and function of ecosystems they occupy.

## Materials and methods

### *Study site and species*

The Boston-Area Climate Experiment (BACE), Waltham, MA (42°23.1 N, 71°12.90 W), subjects square, 4 $m^2$ plots to factorial combinations of four levels of warming [ambient, +~0.8°C (low), +~2.4°C (medium) and +~4°C (high warming)] and three levels of precipitation [ambient, drought (−50%) and wet (+50% during the growing season)]. The site has a loamy topsoil and gravelly sandy loam subsoil. Warming treatments were administered using different wattage infrared heaters (200 W for low, 600 W for medium and 1000 W for high warming) installed at the four corners of each plot (Auyeung *et al.*, 2013; Suseela & Dukes, 2013). Precipitation treatments were achieved using rain out shelters, sprinkler systems and storage tanks. There were three experimental blocks with three replicate plots per treatment. The treatments started in July 2008. The study site has an annual average temperature of 10.3 °C and mean annual precipitation (MAP) of 1063 mm (for further site details see Suseela *et al.*, 2012, 2014a). In 2013, the study site received ~840 mm of rainfall (Jan-Dec; NOAA National Climate Data center station ID 190535, Bedford, MA, USA) which corresponds to the rainfall received in the ambient precipitation treatment. In 2013, the dry and wet precipitation treatments received ~420 mm and ~1089 mm (+50% during the growing season), respectively. In the eastern US, *Q. rubra* is distributed across a wide climatic envelope, with MAP ranging from 512 to >2000 mm (Iverson *et al.*, 1999, Landscape change research group 2014). In 2013, we collected mature, nonshaded green and senesced leaves of *Q. rubra* exposed to six climatic treatments: unwarmed (0) and high warming (H) plots within the ambient (A), wet (W) and drought (D) precipitation treatments (referred to hereafter as A0, AH, W0, WH, D0 and DH). Two green leaves of similar size from 2 to 3 apical whorls were collected from each of the three *Q. rubra* trees per plot during the first week of September. During the second week of October, we collected two completely senesced (still attached to stem) leaves of similar size from each apical whorl (three trees per plot). Each harvest resulted in at least 12 leaves per plot. Immediately after harvest, the leaves for the metabolomic analysis were flash-frozen in dry ice and kept at −80 °C. Leaves from three trees within each replicate plot were pooled to obtain a composite sample, and the three treatment replicates were maintained for all analyses.

### *Extraction and analysis of metabolites*

We removed the leaf petioles before finely grinding the samples in dry ice. We extracted the polar metabolites from both green and senesced tissues of *Q. rubra* exposed to the six climatic treatments. We followed the procedure described in

Lisec *et al.* (2006) and Kind *et al.* (2009), with slight modifications. About 600 mg of each freshly ground sample was extracted twice with 50 ml of 80% methanol. Four milliliters of the supernatant was then transferred to prechilled 8-ml glass tubes, and 1 ml of chilled chloroform was added and cooled at 4 °C. The extracted metabolites were then fractioned into polar and nonpolar phases with addition of 2 ml of chilled water, wherein the polar metabolites fractionated into the top aqueous-methanol phase. After centrifugation (2500 *g* for 10 min), we transferred 200 μl of the aqueous-methanol phase to a vial with a glass insert (250 μl) and dried the sample completely under nitrogen. To this vial, we then added 5 μl of ribitol in methanol (500 μg $ml^{-1}$) to serve as the internal standard and 5 μl of d27-myristic acid in hexane (1 mg $ml^{-1}$) as the retention time lock compound for the subsequent chromatographic analysis. The vials were again dried completely under nitrogen. The samples were methoxylaminated via reaction with 20 μl of methoxylamine hydrochloride (20 mg $ml^{-1}$) in pyridine at 40 °C for 90 min to protect carbonyl moieties from enolization through the formation of corresponding oxime derivatives. Further, the acidic protons in the compounds were silylated via reaction with 90 μL of N-methyl-N-(trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane for 40 min at 40 °C. The derivatized samples were analyzed using gas chromatography–mass spectrometry (GC-MS) within 10 h of derivatization (Suseela *et al.*, 2014b).

We analyzed the samples using an Agilent 7980A GC system coupled with a 5975 C series quadrupole mass analyzer, and separation of metabolites was attained on a DB-5 MS capillary column (30 m length × 0.25 mm internal diameter × 0.20 μm film thickness). We used split (1 : 10 and 1 : 100) injection (1 μl) with the temperature program: 60 °C for 1 min, followed by ramping at 10 °C per minute to 315 °C, with a 10-min 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 set at 270 °C; the MS quad temperature was maintained at 150 °C; and the MS source temperature at 240 °C. The electron multiplier was operated at a constant gain of 2 (EMV = 1478 V). We set the scanning range at 50–600 amu, achieving 2.66 scans $s^{-1}$. We used the Automatic Mass Spectral Deconvolution and Identification System (AMDIS, v2.71; NIST, Gaithersburg, MD, USA) to process the mass spectra with the following deconvolution parameters: match factor – 75%; resolution – high; sensitivity – medium; shape requirements – medium. We used an in-house retention index mass spectral library supplemented with Fiehn Lib (G1676AA; Agilent Technologies, Wilmington, DE, USA), and the Wiley 9th/NIST08 mass spectral library to positively identify the compounds based on derivatized molecular ion, mass fragment pattern and retention time/index (Table 1). We then confirmed all positive matches by manual curation, and the integrated area with reference to the internal standard and representative standards for the major metabolite groups was used for statistical analysis. We broadly grouped the extractable metabolites into amino acids, organic acids, sugars, sugar alcohols, polar fatty acids, phenolic compounds, polyamines, polyols, phosphorous compounds, other nitrogen compounds, polar lipids and other compounds (Table 1).

### *Protein analysis*

We used a BCA protein assay kit to quantify the proteins as per the manufacturer-recommended protocol (BCA protein assay kit; Pierce Chemical Co., Rockford, IL, USA). About 50 mg of the green leaves from each replicate was treated with phosphate buffer (pH 5.6). The mixture was placed in an ice bath, sonicated for 60 s and centrifuged at 12 000 rpm for 5 min. The supernatant was transferred to centrifuge tubes to measure the extractable proteins. The pellet remaining after centrifugation was treated with 1.5 ml of 1% sodium dodecyl sulfate (SDS) to obtain the detergent-extractable proteins. This mixture was put in an ice bath for 10 min and vortexed for 5 min, and the step was repeated four times. The mixture was centrifuged, and the supernatant was used for measuring the detergent-extractable proteins. The pellet remaining after centrifugation was dried at 60 °C and used for measuring elemental carbon and nitrogen content. The nitrogen remaining in the pellet was considered to be the nonextractable nitrogen. The working reagent in the BCA protein assay kit was prepared using reagent A and reagent B in the ratio 1 : 50 (reagent A: sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide; reagent B: 4% cupric sulfate) and added to supernatant obtained after buffer and SDS extraction. The buffer-extractable and detergent-extractable proteins were quantified by measuring absorbance using a UV/VIS spectrophotometer at 562 nm. The amount of protein in each sample was quantified using bovine serum albumin (BSA) as standard.

### *Carbon and nitrogen analyses*

Subsamples of the leaf tissues (green, senesced and tissues remaining after protein extraction) were dried at 60 °C and finely powdered using a genogrinder (Spex sample Prep, Matuchen, NJ, USA). The samples were then used for percent carbon and nitrogen analyses using an elemental analyzer (Carlo Erba NA 1500 Elemental Analyzer; Thermo Scientific, Lakewood, NJ, USA).

### *Resorption efficiency*

Resorption efficiencies of individual metabolite groups and elemental N were calculated as the proportion of each metabolite group or elemental nitrogen (depicted as nutrient) that was taken back by the plant during senescence (Van Heerwaarden *et al.*, 2003):

$$\mathrm{RE} = \frac{[\mathrm{Nutrient}]_{\mathrm{green}} - [\mathrm{Nutrient}]_{\mathrm{senesced}}}{[\mathrm{Nutrient}]_{\mathrm{green}}} \times 100$$

As the metabolomic analysis necessitated the instantaneous cessation of all enzymatic activities, the sampled leaves were immediately flash-frozen with dry ice upon harvest. This precluded the calculation of the specific leaf

**Table. 1** Extractable plant metabolites identified in the green tissues of *Quercus rubra* exposed to different climatic treatments classified into broad groups

| Sl No | Compound | Abbreviation | *q*-value (FDR corrected *P* value)‡ | *m/z** | Retention time† |
|---|---|---|---|---|---|
| | Amino acids | | | | |
| 1 | L-alanine | Ala | 5.89E-05 | 116, 190, 100 | 0.420 |
| 2 | Aspartic acid | Asp | 5.89E-05 | 160, 130, 117 | 0.774 |
| 3 | L-asparagine | Asn | 5.89E-05 | 116, 231, 132, 188 | 0.886 |
| 4 | *β*-alanine | Bala | 7.07E-05 | 174, 248, 290 | 0.701 |
| 5 | Citrulline | Cit | 9.06E-05 | 157, 256, 142 | 0.995 |
| 6 | L-glutamic acid | Glu | 7.07E-05 | 156, 230, 258 | 0.775 |
| 7 | L-glutamine | Gln | 5.89E-05 | 156, 245, 128 | 0.960 |
| 8 | Glycine | Gly | 5.89E-05 | 174, 248, 86 | 0.599 |
| 9 | DL-isoleucine | Ile | 5.89E-05 | 158, 218, 232 | 0.587 |
| 10 | L-leucine | Leu | 0.000169 | 86, 188, 103 | 0.468 |
| 11 | L-lysine | Lys | 0.000165 | 174, 317, 156, 230, | 1.060 |
| 12 | L-methionine | Met | 8.11E-05 | 176, 128, 219 | 0.773 |
| 13 | Phenylalanine | Phe | 5.89E-05 | 120, 146, 130 | 0.797 |
| 14 | L-proline | Pro | 6.94E-05 | 142, 216, 170 | 0.591 |
| 15 | L-serine | Ser | 6.31E-05 | 204, 218, 100, 188 | 0.644 |
| 16 | L-threonine | Thr | 6.31E-05 | 218, 117, 101 | 0.666 |
| 17 | L-tryptophan | Trp | 6.31E-05 | 202, 291, 218 | 1.237 |
| 18 | L-tyrosine | Tyr | 6.31E-05 | 218, 280, 100, 179 | 1.071 |
| 19 | L-valine | Val | 5.99E-05 | 144, 218, 100 | 0.519 |
| | Phenolic compounds | | | | |
| 20 | Vanillic acid | VanilA | 5.89E-05 | 297, 267, 223 | 0.954 |
| 21 | Vanillylmandelic acid | VanilMA | 5.89E-05 | 297, 371, 399 | 1.111 |
| 22 | 4-hydroxybenzoic acid | 4HBA | 7.07E-05 | 267, 223, 193 | 0.857 |
| 23 | 4-hydroxycinnamic acid | 4HCA | 7.07E-05 | 219, 293, 308 | 1.072 |
| 24 | Benzoic acid | BenA | 0.000129 | 197, 135, 105 | 0.549 |
| 25 | Caffeic acid | CaffA | 5.89E-05 | 219, 396, 191 | 1.191 |
| 26 | Epicatechin | Catec | 0.00025 | 368, 355, 267 | 1.573 |
| 27 | Gallic acid | GallA | 5.89E-05 | 281, 179, 133 | 1.081 |
| 28 | Phloroglucinol | Phloro | 0.00019 | 342, 327, 133 | 0.868 |
| 29 | Pyrogallol | Pyrgall | 7.07E-05 | 239, 342, 133 | 0.791 |
| | Sugars | | | | |
| 30 | D-allose | Allose | 7.07E-05 | 319, 205, 160 | 1.054 |
| 31 | Cellobiose | Cello | 7.66E-05 | 204, 217, 103, 129 | 1.493 |
| 32 | Fructose | Fruc | 7.07E-05 | 103, 217, 307 | 1.027 |
| 33 | D-glucose | Glucos | 5.89E-05 | 319, 205, 160 | 1.044 |
| 34 | Isomaltose | Isomal | 5.89E-05 | 813, 204, 217 | 1.600 |
| 35 | Lactose | Lacto | 5.89E-05 | 204, 361, 217 | 1.500 |
| 36 | Leucrose | Leuc | 0.006125 | 361, 204, 217 | 1.537 |
| 37 | D-lyxose | Lyso | 0.000118 | 103, 307, 189 | 0.876 |
| 38 | Maltose | Maltos | 9.57E-05 | 361, 204, 217, 103 | 1.507 |
| 39 | D-mannose | Mann | 5.89E-05 | 205, 319, 160 | 1.039 |
| 40 | Sucrose | Sucro | 5.89E-05 | 361, 217, 271, 169 | 1.457 |
| 41 | Melibiose | Melib | 0.0020803 | 204, 217, 129, 103, 191 | 1.577 |
| 42 | Raffinose | Raffi | 5.89E-05 | 361, 217, 204, 129 | 1.733 |
| 43 | Ribose | Ribos | 5.89E-05 | 103, 217, 307, 189 | 0.892 |
| 44 | Tagatose | Taga | 0.000642 | 103, 217, 307 | 1.032 |
| 45 | Talose | Talose | 0.000728 | 319, 205, 103 | 1.056 |
| | Sugar alcohols | | | | |
| 46 | Allo-inositol | Alloino | 0.000979 | 318, 217, 305, 191 | 1.126 |
| 47 | Galactinol | Galcnol | 6.94E-05 | 204, 217, 129 | 1.608 |
| 48 | Glycerol | Glycerol | 5.89E-05 | 205, 117, 103, 218 | 0.570 |

 

**Table 1** (continued)

| Sl No | Compound | Abbreviation | *q*-value (FDR corrected *P* value)‡ | *m/z** | Retention time† |
|---|---|---|---|---|---|
| 49 | Maltitol | Maltit | 0.002791 | 361, 204, 217, 117 | 1.564 |
| 50 | Maltotriitol | Maltotr | 0.000149 | 204, 361, 217 | 1.464 |
| 51 | D-sorbitol | Sorbi | 0.003518 | 205, 319, 217, 307 | 1.074 |
| 52 | D-threitol | Threit | 5.89E-05 | 217, 103, 205, 189 | 0.765 |
| 53 | Xylitol | Xylit | 5.89E-05 | 217, 103, 307, 319 | 0.924 |
| | Organic acids | | | | |
| 54 | 3-dehydroshikimic acid | DshikA | 5.99E-05 | 296, 386, 208 | 0.970 |
| 55 | DL-3-aminoisobutyric acid | AminobutA | 5.89E-05 | 102, 176, 218 | 0.726 |
| 56 | L-ascorbic acid | AscorA | 0.000173 | 332, 205, 117 | 1.075 |
| 57 | Chlorogenic acid | ChlorA | 5.89E-05 | 345, 307, 219 | 1.727 |
| 58 | Citric acid | CitA | 5.99E-05 | 273, 347, 133 | 0.991 |
| 59 | Dehydroascorbic acid | DHAsA | 0.000264 | 173, 157, 245, 316 | 1.010 |
| 60 | Fumaric acid | FumA | 5.99E-05 | 245, 115, 217 | 0.635 |
| 61 | Galacturonic acid | GlacuroA | 0.000745 | 333,160, 217 | 1.069 |
| 62 | Gluconic acid | GlucA | 5.89E-05 | 205, 333, 292, 319 | 1.107 |
| 63 | Glyceric acid | GlyceA | 5.89E-05 | 189, 292, 133, 103 | 0.618 |
| 64 | Glycolic acid | GlycoA | 0.00612 | 177, 161, 117 | 0.397 |
| 65 | Lactobionic acid | LacBA | 0.000221 | 204, 191, 217 | 1.503 |
| 66 | Lactic acid | LactA | 0.00282 | 117, 191, 219 | 0.383 |
| 67 | Maleic acid | MaleiA | 5.89E-05 | 245, 133, 115 | 0.595 |
| 68 | D-malic acid | MalicA | 5.89E-05 | 133, 233, 245 | 0.748 |
| 69 | Malonic acid | MaloA | 5.89E-05 | 66, 233, 131 | 0.508 |
| 70 | Mucic acid | MucicA | 0.000173 | 333, 292, 305 | 0.994 |
| 71 | O-acetylsalicylic acid | Oasaly | 5.89E-05 | 267, 135, 91 | 0.766 |
| 72 | Oxalic acid | OxA | 5.89E-05 | 117, 133, 219 | 0.444 |
| 73 | D-saccharic acid | SaccharA | 5.99E-05 | 333, 292, 189 | 1.137 |
| 74 | Shikimic acid | Shikim | 5.89E-05 | 204, 133, 255 | 0.983 |
| 75 | Succinic acid | SuccA | 5.89E-05 | 247, 129, 172 | 0.604 |
| 76 | Tartaric acid | TarA | 7.07E-05 | 292, 219, 189 | 0.862 |
| 77 | Uric acid | UricA | 5.89E-05 | 367, 172, 158 | 1.167 |
| | N compounds | | | | |
| 78 | Acetylserine | AceSeri | 9.06E-05 | 174, 116, 218 | 0.671 |
| 79 | *β*-cyano-L-alanine | BCAlan | 5.89E-05 | 141, 202, 130 | 0.652 |
| 80 | L-3,4-dihydroxyphenylalanine | LDOPA | 6.31E-05 | 218, 267, 179 | 1.167 |
| 81 | N-acetyl-L-leucine | Naceleu | 5.99E-05 | 86, 128, 158 | 0.707 |
| 82 | Porphine | Proph | 5.89E-05 | 184, 134, 285, 174 | 0.621 |
| 83 | Purine riboside | PuRibo | 0.000133 | 217, 245, 230 | 1.320 |
| 84 | L-pyroglutamic acid | Pyroglu | 0.001339 | 156, 230, 258 | 0.774 |
| 85 | Urea | Urea | 0.00612 | 189, 171, 66 | 0.540 |
| | P compounds | | | | |
| 86 | D-glucose-6-phosphate | DG6Phos | 5.89E-05 | 299, 217, 160 | 1.293 |
| 87 | Glycerol-1-phosphate | Gly1P | 0.000213 | 299, 211, 129 | 0.951 |
| 88 | O-phosphocolamine | Pcolo | 7.07E-05 | 299, 315, 174 | 0.967 |
| 89 | Phosphoric acid | PhosA | 0.00612 | 299, 314, 211 | 0.568 |
| | Fatty acid | | | | |
| 90 | 4-guanidinobutyric acid | 4GBA | 5.89E-05 | 174, 304, 246 | 0.783 |
| 91 | Palmitic acid | PalA | 9.06E-05 | 313, 117, 129, 145 | 1.138 |
| 92 | Stearic acid | SteaA | 0.000107 | 117, 341, 132, 145 | 1.253 |
| | Polyamines | | | | |
| 93 | Putrescine | Putre | 8.11E-05 | 174, 214, 200 | 0.938 |
| 94 | D-lyxosylamine | Lysosa | 5.99E-05 | 217, 307, 189 | 0.881 |
| | Polar lipids | | | | |
| 95 | DL-dihydrosphingosine | DiHsping | 0.000172 | 204, 414, 115 | 1.368 |

**Table 1** (continued)

| Sl No | Compound | Abbreviation | *q*-value (FDR corrected *P* value)‡ | *m*/*z** | Retention time† |
|---|---|---|---|---|---|
| 96 | D-sphingosine | Dspingo | 7.07E-05 | 204, 129, 103 | 1.362 |
| | Polyol | | | | |
| 97 | Quinic acid | Quini | 5.89E-05 | 345, 255, 204 | 1.018 |
| | Other compounds | | | | |
| 98 | 2-ketogulonic acid | 2KGluA | 5.89E-05 | 204, 147, 437 | 1.078 |
| 99 | Gluconic acid lactone | GluALAc | 7.07E-05 | 217, 205, 244 | 1.049 |
| 100 | Methyl-*β*-D-galactopyranoside | MBDP | 7.07E-05 | 204, 217, 191 | 1.277 |

*All compounds produce ion fragments of $m/z$ = 73 (base peak) that corresponds to $[(CH_3)_3SiOH]$, and $m/z$ = 147 that corresponds to $[(CH_3)_3\ SiOSi(CH_3)_2]$ which are characteristics for MSTFA derivatization.
†Relative to retention time of myristic acid. For compounds that contribute to more than one chromatographic peak due to multiple trimethylsilyl derivatives, the relative retention time of the most abundant derivative is represented in this table.
‡Expected proportion of false positives incurred when calling that feature significant.

area of leaves used for metabolomic analyses. Hence, the RE in this study is reported on a mass basis. A subset of green and senesced leaves collected during the respective harvest times was used for the calculation of specific leaf area. The specific leaf area was similar across all treatments and between the green and senesced leaves within each treatment (Figure S1), indicating that in this study the RE on an leaf area basis would also follow a similar trend as that observed on a leaf mass basis.

### *Data analysis*

The metabolomic data were autoscaled to satisfy the assumptions of normality. To evaluate the main and interactive effects of warming and precipitation treatments, we first analyzed the metabolomic data with permutational multivariate analysis of variance (PERMANOVA; Primer 7 PERMANOVA+, version 7.0.5; Primer-E Ltd, Plymouth, UK) using the Euclidean distance (Rivas-Ubach *et al.*, 2012). Occurrence of Type-1 error due to multiple comparison across metabolites were controlled by false discovery rate (FDR). The 100 metabolites that had a FDR-adjusted *P* value (*q* value) of <0.05 (delta = 3.0) were utilized in the subsequent multivariate analyses. The extractable metabolite data were further analyzed with partial least squares discriminant analyses (PLS-DA; MetaboAnalyst 3.0; Gargallo-Garriga *et al.*, 2015). The cross-validation of PLS-DA model (k-fold cross-validation) had a model goodness of fit ($R^2$) and a cross-validation $R^2$ ($Q^2$; model predictive ability) of >0.8. The robustness of the class discrimination was verified through permutation testing of separation distance based on the ratio of the between-group sum of the squares and the within-group sum of squares and had $P < 0.0005$ over 2000 iterations. A principal component analysis (PCA) was also conducted on the metabolome and separately on classes of compounds (Table 1) using the sums of the individual compounds in each class. Each of the metabolites in the important compound classes such as amino acids, organic acids and sugars that separated the climatic treatments in the PCA was analyzed individually using mixed-model restricted maximum likelihood ANOVA (PROC MIXED; SAS version 9.2; SAS Institute, Cary, NC, USA), with precipitation and warming treatments as fixed factors and block as a random factor. A heatmap was generated (MetaboAnalyst 3.0; Xia *et al.*, 2012) to better visualize the responses of 100 metabolites across different climatic treatments (Angelcheva *et al.*, 2014). Hierarchical clustering analysis was performed based on both the treatments and metabolites. Percentage of elemental N, protein fractions and nonextractable N in green tissues, and RE of metabolite groups and RE of elemental N was analyzed using PROC MIXED. The differences among individual treatments were assessed using Tukey's HSD multicomparison test.

## Results

### *Abundance of extractable metabolites in green tissues*

The metabolomic approach adopted in this study is focused toward identification of polar metabolites in the leaf extracts. However, a subset of relatively polar compounds such as flavonoid glycosides that are less amenable to gas chromatographic analysis was characterized using liquid chromatography–tandem mass spectrometry and is not presented here. The total ion chromatogram of GC-MS processed using AMDIS parameters specified in the method section identified 625–650 component peaks with unique fragmentation and retention time. Further based on the mass fragmentation and retention time/index matches with the mass spectral libraries, we established the molecular identity of 100 components across all climate treatments that had an FDR corrected *P* value (*q*) of <0.05 (Table 1). As one of the objectives of the study was to elucidate the climate-induced metabolic regulations in plants, only those components with a confirmed molecular identity were used for the analyses. The cumulative peak area of the identified metabolites constituted more than 80% of the total peak area of the total ion chromatogram in all samples.

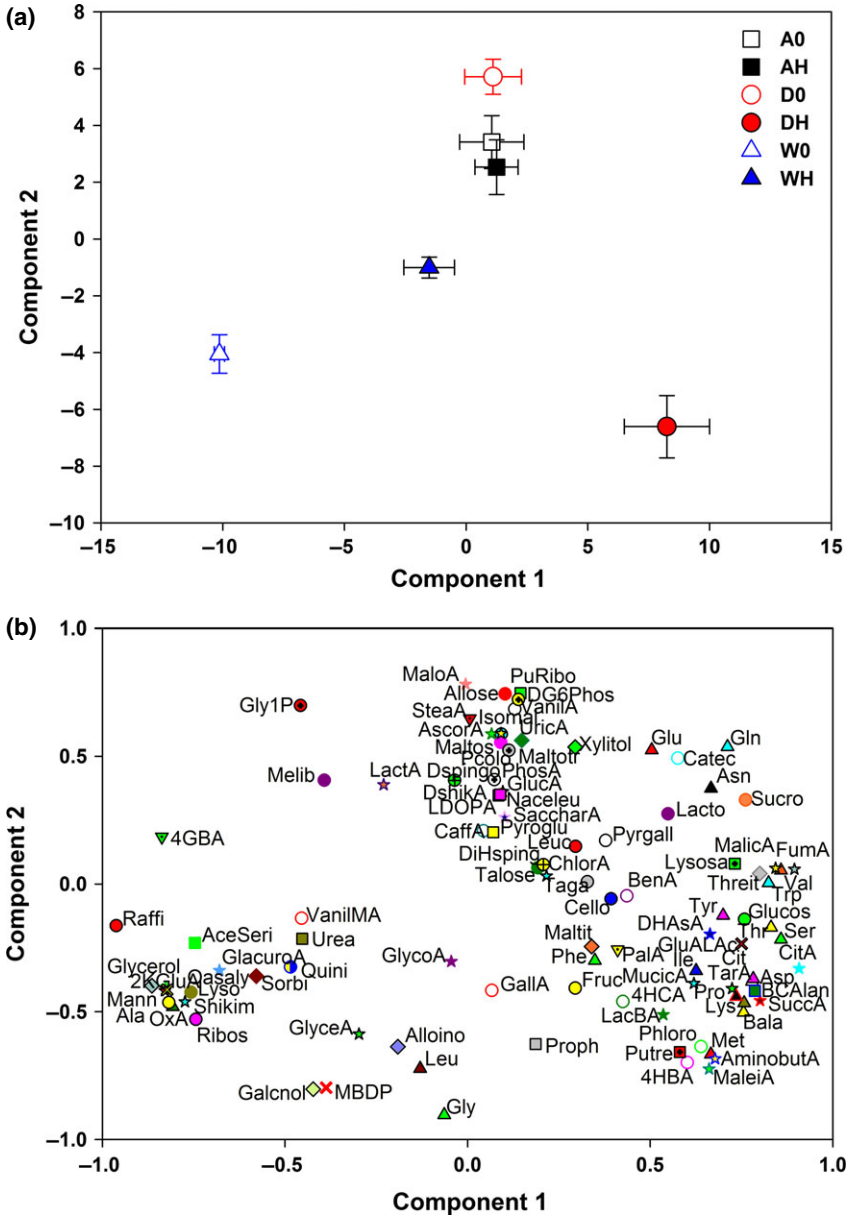


**Fig. 2** Partial least squares discriminant analysis (PLS-DA) of the relative intensities of 100 plant metabolites of *Quercus rubra* exposed to six different climate treatments. (a) Sample categorized score (mean ± SD; $n = 3$) plot between component 1 and component 2 (key: A0, ambient precipitation + no warming; AH, ambient precipitation + high warming; W0, wet precipitation + no warming; WH, wet precipitation + high warming; D0, dry precipitation + no warming; and DH, dry precipitation + high warming). (b) Loading plot between component 1 and component 2 of the metabolite variables (see Table 1 for metabolites).

The PERMANOVA analysis of the responses of the entire metabolome of *Q. rubra* to different climate treatments identified an interaction between warming and precipitation treatments (pseudo-$F$ 15.17; $P < 0.001$). The results of the PLS-DA of the metabolome were consistent with the interaction effect identified by PERMANOVA: the effect of warming on metabolite composition varied by precipitation treatments (Fig. 2). Principal component analysis of the relative intensities of all extractable metabolites identified from the green tissues of *Q. rubra* revealed that the climate treatments affected metabolite compositions, with the effect of warming depending on the precipitation treatment (Figure S2; warming × precipitation, $P < 0.001$). PC axes 1, 2 and 3 explained 68% of the variation in the data, with PC axis 1 separating DH from all other treatments in terms of metabolite composition. A hierarchical clustering of the metabolites clearly revealed the close grouping of climate treat-

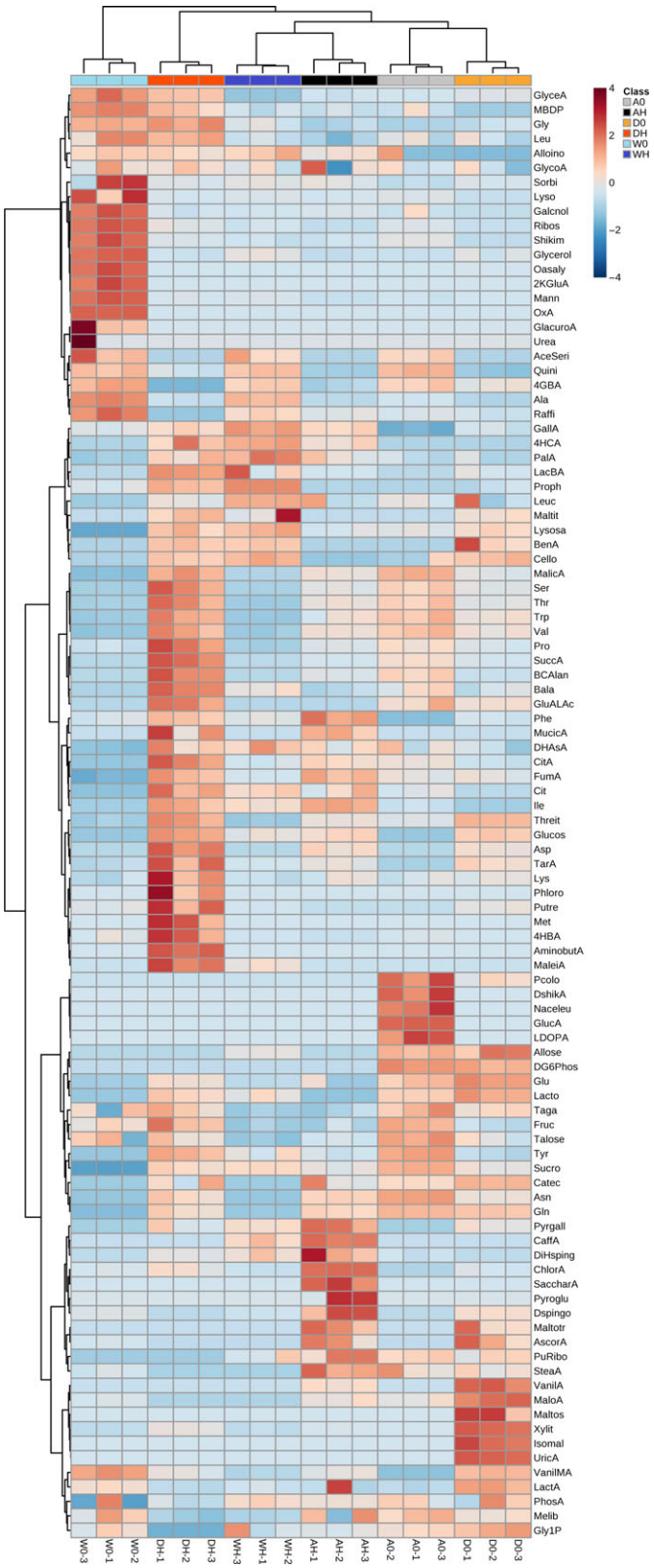


**Fig. 3** Heatmap and two-way hierarchical clustering of metabolomic data of green leaves of *Quercus rubra* exposed to different climate treatments. Each column represents a sample from a climate treatment, and each row represents a positively identified metabolite (Table 1) based on mass spectral fragmentation pattern and retention indices. Key as in Fig. 2.

 

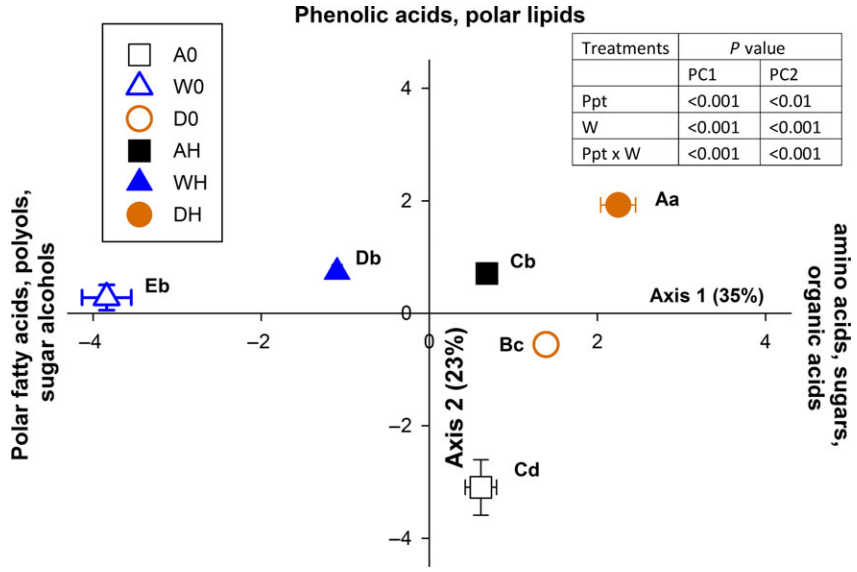


**Fig. 4** Principal component analysis of the relative intensities of the broad metabolite groups (amino acids, phenolic compounds, organic acids, sugars, sugar alcohols, polyols, polyamines, polar fatty acids, polar lipids, N compounds, P compounds and other compounds) of *Quercus rubra* exposed to different climate treatments. Key as in Fig. 2. Letters 'A to E' indicate Tukey's difference between treatments separated by PC axis 1 and 'a to c' between treatments separated by PC axis 2. Graphed values represent means ($n$ = 3) ± SD.

ments based on the abundance of various metabolites (Fig. 3). The metabolite composition of the unwarmed ambient precipitation treatment (A0) grouped with the unwarmed drought (D0) treatment. Similarly, the warmed treatments receiving ambient and wet precipitation (AH, WH) grouped together. The DH and W0 treatments, the two extremes of the stressed and nonstressed treatments, respectively, did not group with any of the other climate treatments.

Climate treatments also appeared as separate groups in a principal component analysis of the sum of relative intensities of metabolite groups in green tissue (Fig. 4). PC axes 1 and 2 explained 58% of the variation in the data and showed that precipitation treatments affected responses to warming ($P < 0.001$). PC axis 1 statistically distinguished all treatments except A0 and AH. Leaves from the combination of drought and warming (DH) had higher relative abundances of amino acids, sugars, and organic acids than leaves from other treatments. Leaves from the wet precipitation (W0) had the greatest abundances of polar fatty acids, polyols and sugar alcohols. As the abundance of major compound classes such as amino acids, organic acids and sugars varied between the stressed (DH) and the nonstressed (W0) treatments, we analyzed the individual metabolites within these compound classes separately.

*Amino acids.* A total of 19 amino acids were identified in the green leaves of *Q. rubra* exposed to different climate treatments, of which 11 had greater abundance in plants exposed to warming, drought or their combination (Fig. 5). The concentration of amino acids in the ambient (A0) treatment was 12.1 ± 1.2% of total leaf N. The effect of warming and precipitation treatments on the abundance of amino acids interacted for all amino acids except tyrosine ($P < 0.05$; Fig. 5). Tyrosine content varied only with precipitation, such that drought and ambient treatments had greater abundance of tyrosine than the wet treatment ($P = 0.002$). Warming also increased the abundance of tyrosine ($P < 0.001$). Abundances of proline, β-alanine, aspartic acid, threonine, methionine, serine and lysine were greater in the DH treatment than in all other treatments (Fig. 5). Warming increased the abundance of citrulline and β-alanine across all precipitation treatments. Tryptophan and valine had greater abundance in AH and DH treatment compared to the A0 treatment. Among the 19 amino acids identified, 10 had lower abundance in W0 and WH treatments ($P < 0.05$).

*Organic acids.* Among the 24 organic acids identified in green leaves of *Q. rubra* exposed to combinations of warming and precipitation treatments (Table 1), the organic acid intermediaries in the TCA cycle such as citric acid and succinic acid had higher abundance in the DH treatment than in the A0 treatment ($P < 0.001$; Fig. 5). Fumaric acid, an organic acid intermediary in the TCA cycle, exhibited higher abundance in the

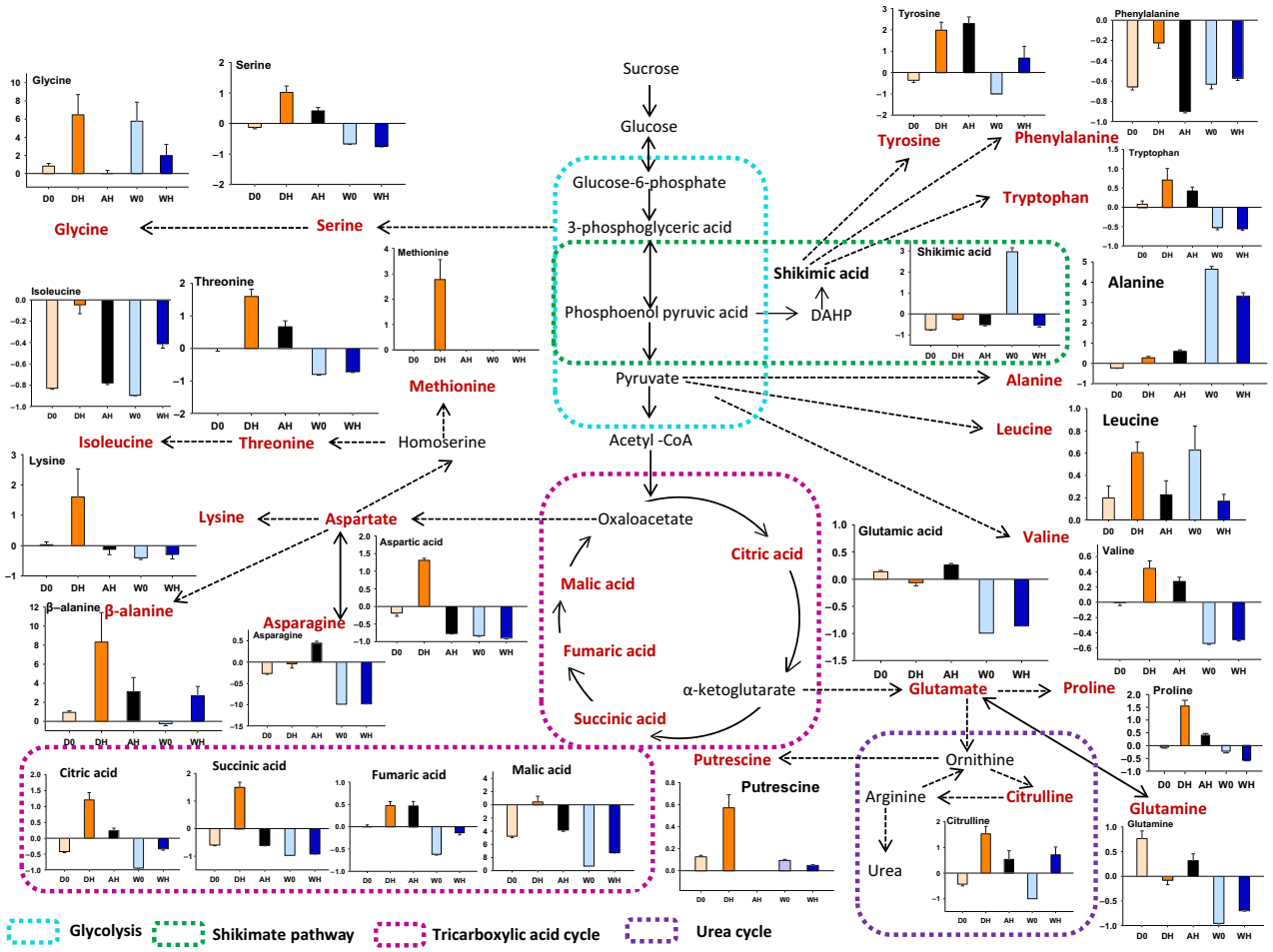


**Fig. 5** Response of individual metabolites in representative metabolic pathways to climate treatments measured as the fold difference between individual treatments and that in the unwarmed ambient precipitation (control) treatment. Bars in graphs represent means ($n$ = 3) ± SE. Y axis of the bar graphs represents fold change of individual metabolites from the ambient unwarmed treatment. Key as in Fig. 2.

warming treatment than in the unwarmed treatment ($P < 0.001$; Fig. 5). Fumaric acid also had higher abundance in the ambient and drought precipitation treatments than in the wet treatment ($P < 0.001$: Fig. 5). The abundance of nine organic acids (citric acid, dehydroascorbic acid, fumaric acid, lactobionic acid, maleic acid, malic acid, mucic acid, succinic acid, and tartaric acid) was higher in *Q. rubra* exposed to DH compared to the W0 treatment ($P < 0.05$). On the other hand, shikimic acid (Fig. 5), *O*-acetylsalicylic acid and glyceric acid were higher in W0 treatment than in the DH treatment ($P < 0.05$).

*Sugars.* A total of 16 sugars were identified in green leaves (Table 1) of which sucrose, glucose, tagatose and fructose were the most abundant carbohydrates. Sucrose was the most abundant sugar and its concentration was higher in DH than in WH ($P < 0.001$; Fig. 3). Lactose also exhibited a similar pattern of response, with a higher concentration in DH than in W0 ($P < 0.001$). Ribose, raffinose and lyxose reached higher abundance in W0 than in DH ($P < 0.01$; Fig. 3).

*Polyamine.* Putrescine, an important polyamine, was more abundant in the DH treatment than in all other treatments ($P < 0.01$; warming × precipitation interaction; Fig. 5).

### *Resorption efficiency of extractable metabolites*

Climate treatments differentially affected the RE of various metabolite groups such as amino acids, organic acids, sugars and compounds containing phosphorus (Fig. 6). The RE of amino acids decreased as precipitation increased, and generally decreased with warming,

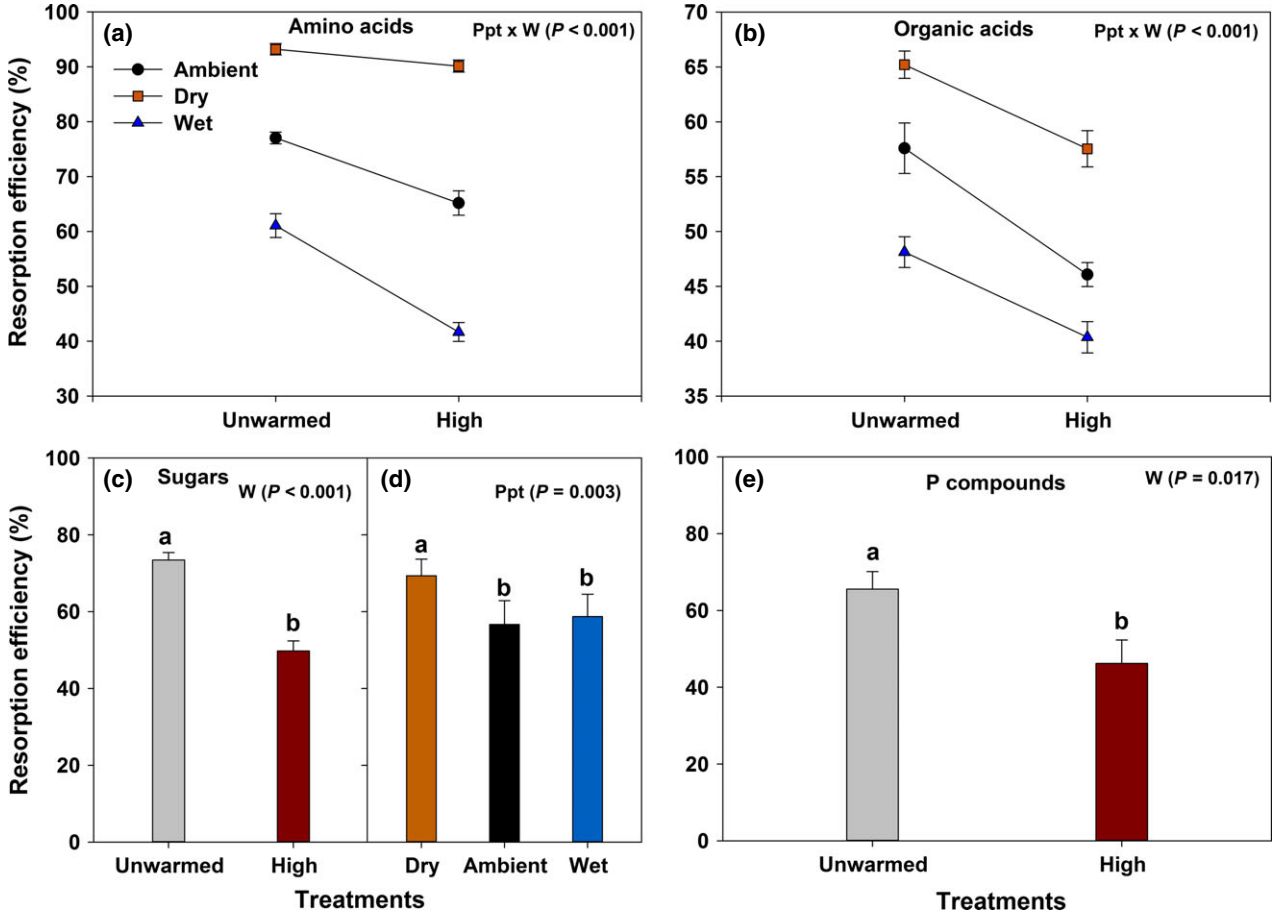


**Fig. 6** Resorption efficiency of (a) amino acids [$n = 3$], (b) organic acids [$n = 3$] (c and d) sugars [$n = 9$ and $n = 6$, respectively] and (e) phosphorus compounds [$n = 9$] in *Quercus rubra* exposed to different climate treatments. In (c, e), different letters identify differences in means (Tukey's honestly significant difference (HSD)) among warming treatments and in (d) among precipitation treatments.

with the strongest effects of warming in the wettest treatments. The droughted unwarmed (D0) and warmed (DH) treatments had the highest REs of amino acids (~90%; $P < 0.01$; Fig. 6a). Plants exposed to the wet, warmed treatment had the lowest RE of amino acids (40%; $P < 0.001$). Warming decreased RE of amino acids in the ambient (12%) and wet precipitation (20%) treatments compared to their respective unwarmed treatments (Fig. 6a).

Similar to amino acids, the RE of organic acids decreased with increasing precipitation, and with warming, with an interaction between warming and precipitation treatments ($P < 0.001$; Fig. 6b); in this case, the effect of warming was greatest under ambient precipitation. The RE of sugars responded similarly to warming and precipitation treatments, again decreasing with warming ($P < 0.001$; Fig. 6c) and with increasing precipitation ($P = 0.003$; Fig. 6d), but there was no interaction among treatments and no difference in RE of sugars between the ambient and wet precipitation treatments (Fig. 6d). Warming decreased the RE of phosphorus compounds ($P < 0.017$; Fig. 6e), but precipitation treatments had no effect.

### *Nitrogen dynamics*

*Abundance in green leaves.* Leaves exposed to warming or drier soils had lower total N concentration in green tissues, with warming having the strongest effect in the wettest treatment (warming × precipitation: $P = 0.002$; Fig. 7a). Thus, the wet, unwarmed treatment had the highest N concentration and the DH treatment had the lowest.

*Extractable Proteins and nonextractable nitrogen in green leaves.* The responses of total soluble proteins to the treatments were nearly opposite to those of N concentration. In this case, plants exposed to drier soils (precipitation: $P < 0.001$; Fig. 7b) or warming ($P < 0.001$; Fig. 7c) had higher concentrations of soluble pro-

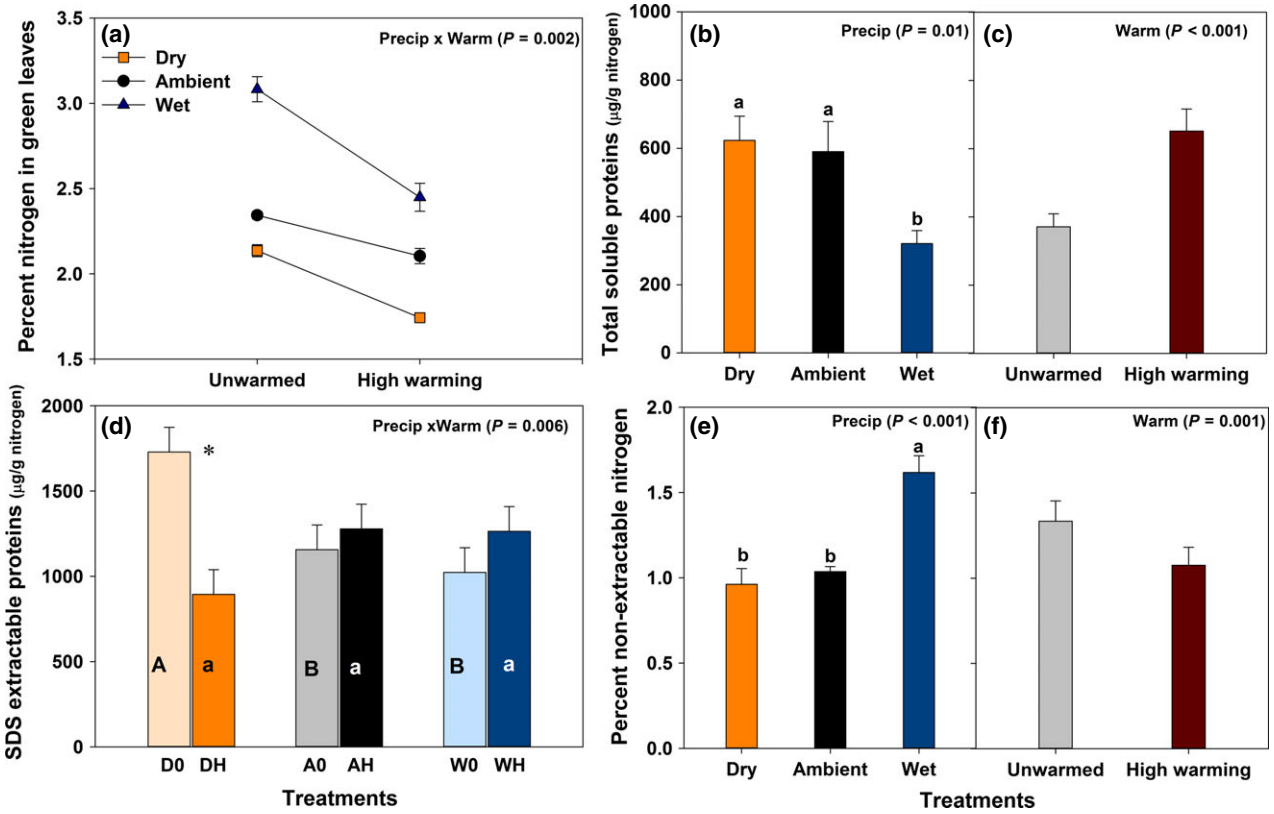


**Fig. 7** Response of (a) percent nitrogen [$n$ = 3], (b and c) total soluble proteins [$n$ = 6; $n$ = 9, respectively], (d) SDS extractable proteins [$n$ = 3] and (e and f) percent nonextractable nitrogen ($n$ = 6; $n$ = 9, respectively) in green leaves of *Quercus rubra* exposed to different climate treatments. In (b, e), different letters identify differences in means (Tukey's honestly significant difference (HSD)) among precipitation treatments. In (d) along the unwarmed treatments bars with the same upper case letter indicate no difference between the treatments and along the warmed treatments bars with the same lower case letter indicate no difference between the treatments. Asterisk indicate difference between the unwarmed and the high warming treatments within a precipitation treatment. Values represent means ± SE.

teins in green leaf tissue, with no interaction between treatments. Ambient and drought treatments increased total soluble proteins by 94% and 85%, respectively, compared to the wet treatment. Similarly, warming increased total soluble proteins by 75% (Fig. 7b and c). Detergent-extractable proteins increased in the drought treatment, but only when unwarmed (D0; Fig. 7d). The N remaining in the leaf tissues after protein extraction (non-extractable N) responded similarly to total N concentration, and nearly opposite to the total soluble proteins (Fig. 7e and f). Non-extractable N was greatest in the wet treatment ($P$ < 0.001; Fig. 7e) and decreased with warming ($P$ = 0.001; Fig. 7f).

*Resorption efficiency of elemental nitrogen.* Warming suppressed RE of elemental N, although wet treatment partially ameliorated the suppressive effect of the warming (warming × precipitation: $P$ < 0.001; Fig. 8). The relatively high RE of elemental N in control (A0) treatment (60%) decreased to 30% with warming (AH) and less than 20% in the drought treatments (DH).

## Discussion

Our results show that climatic factors strongly affect both the production and resorption of plant metabolites, which could potentially influence plant growth and soil nutrient cycling. Drought and warming additively increased the abundances of amino acids and organic acids in green tissues. However, these climatic parameters differentially influenced the RE of extractable metabolites, where RE increased under drought, but decreased under warming. Although extractable metabolites that are routinely identified in metabolomic studies represent only <10% of the initial tissue mass, this metabolite pool could disproportionately influence tissue decomposition because of the rapid bioavailability to soil heterotrophs. Interestingly, the RE of elemen-

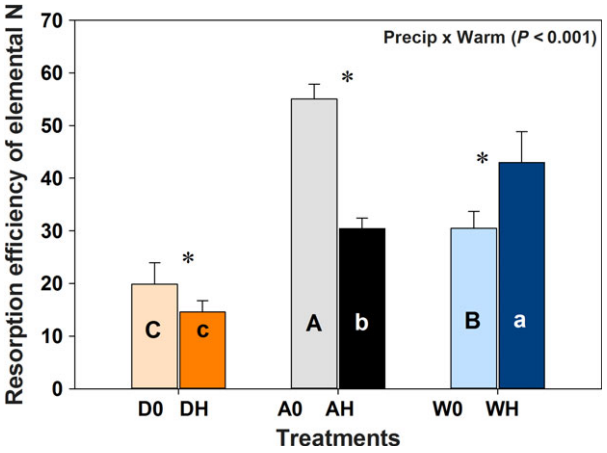


**Fig. 8** Resorption efficiency of total elemental nitrogen in *Quercus rubra* exposed to different climate treatments (mean ± SE; $n = 3$). Along the unwarmed treatments bars with the same upper case letter indicate no difference (Tukey's honestly significant difference (HSD)) between the treatments and along the warmed treatments bars with the same lower case letter indicate no difference between the treatments. Asterisk indicate difference between unwarmed and high warming treatments within a precipitation treatment.

tal N responded oppositely to that of the extractable N metabolites – for instance, RE of elemental N was <20% under drought, while RE of amino acids was >90% under drought treatments. This decline in RE of elemental N implies a reduced recapture of N by plants under drought, which could hinder their growth in the following year. Concomitantly, the lower abundance of labile N pool (amino acid fraction) in the senesced tissue formed under drought treatment could result in a lower rate of decomposition of this litter, despite their greater element N content. Thus, apart from the direct effect of moisture stress, plant survival might be challenged under drought conditions not only because of a lower tissue reserve of N, but also due to slower nutrient cycling in soils.

### *Warming and drought differentially influence production and resorption of extractable metabolites*

The pattern of metabolite abundance observed in our study resembles that reported by Gargallo-Garriga *et al.* (2015), where plants exposed to a combination of drought and warming produced a different metabolic profile than would have been expected from the responses of plants to the individual factors (Figs 2 and 3). In our study, *Q. rubra* leaves had greater abundances of amino acids and organic acid intermediaries of the TCA cycle when exposed to the combination of warming and drought (DH; Figs 4 and 5) than when exposed to either drought or warming alone. Intermediaries of the TCA cycle such as succinic acid, citric acid and malic acid provide carbon skeletons and reduction equivalents to a majority of plant biochemical pathways (Meyer *et al.*, 2007). A similar increase of many metabolites in the TCA cycle has been reported in plants subjected to moisture stress (Urano *et al.*, 2009). As drought induces stomatal closure and concomitant warming leads to high leaf temperatures, plants exposed to the combination of drought and warming experience higher cellular respiration (Vile *et al.*, 2012). Thus, the abundance of TCA intermediaries in the DH treatment (Fig. 5) suggests greater cellular respiration during combined heat and drought stress. Although previous studies have reported increase in citric acid due to heat stress alone (Du *et al.*, 2011), we did not observe a similar increase in warmed treatments (AH and WH; Fig. 5), plausibly due to the greater availability of moisture in wet and ambient precipitation treatments, which could have partially alleviated the heat stress. The above response parallels the suppression of citric acid in plants exposed to heat stress in combination with elevated $CO_2$, compared to those exposed to heat stress under ambient $CO_2$ (Yu *et al.*, 2012), potentially due to the lower moisture stress under elevated $CO_2$. The increased mitochondrial activity under DH (Fig. 5) could provide carbon skeletons required for the biosynthesis of amino acids (Vasquez-Robinet *et al.*, 2008). This observation was supported by coordinated increases in the abundance of amino acids such as proline, and citrulline, and polyamine such as putrescine derived from α-ketoglutarate (Fig. 5), an intermediate in the TCA cycle. These metabolites function as signaling compounds and osmoregulators, maintain protein and enzyme integrity and scavenge hydroxyl radicals, thus helping plants to tolerate abiotic stress (Galston, 1991; Akashi *et al.*, 2001; Matysik *et al.*, 2002; Kaplan *et al.*, 2004; Alcazar *et al.*, 2006; Groppa & Benavides, 2008; Alcazar *et al.*, 2010; Szabados & Savoure, 2010; Wang *et al.*, 2014). As proline protects subcellular structures and maintains the structural integrity of macromolecules including proteins under dehydration, biosynthesis of proline is upregulated and its catabolism is repressed under moisture stress (Szabados & Savoure, 2010). Similar increases in proline and several other secondary metabolites such as GABA, choline and glycine betaine were reported in grasses exposed to combined warming and drought stress, whereas drought alone reduced levels of amino acids, sugars and nucleosides associated with energy and growth metabolism pathways (Gargallo-Garriga *et al.*, 2015). In our study, *Q. rubra* exposed to DH also had greater concentrations of aspartate, lysine, methionine and threonine derived from oxaloacetate (TCA cycle intermediate; Fig. 5).

Cellular homeostasis is maintained not only by biosynthesis, but also through utilization, catabolism and inter-cellular/inter-organ transport of metabolites. Thus, the observed abundance of amino acids could also result from the degradation of proteins that could act as energy sources specifically under reduced photosynthesis (Guy *et al*., 2008; Caldana *et al*., 2011) or could be the result of a bottleneck in the effective utilization of these amino acids in the downstream metabolic pathway (Rizhsky *et al*., 2002). Conversely, plants exposed to favorable climatic conditions that promote active photosynthesis such as the wet treatment could effectively utilize the amino acids for protein synthesis (Morot-Gaudry *et al*., 2001). Thus, the lower abundance of many amino acids in plants exposed to the wet treatment (Fig. 5), particularly the important amide amino acids such as glutamine, glutamate and aspartate and metabolites in the TCA cycle could result from a faster plant growth and accompanying greater demand for these compounds in the downstream pathway for the synthesis of cellular components (Meyer *et al*., 2007).

Our study revealed that REs of metabolite groups increased with drought treatments but decreased with warming (Fig. 6). While we did not determine the mechanisms behind these contrasting responses, we speculate that they could be attributed to differential effects of high temperature and drought on plant phenology, physiology and metabolism. Warming could delay leaf senescence (Bauerle *et al*., 2012) resulting in lower RE, while drought could enhance tissue senescence (Rivero *et al*., 2007) leading to greater RE of extractable metabolites. Plants also produce a cascade of signaling responses upon perception of heat stress including changes in protein and peptide structures, and enzyme conformation and activities (Ruelland & Zachowski, 2010) that may have affected the RE. It should be noted that the metabolic perturbances in plants is a function of the physiological stress they perceive in a given environment, rather than being a response to an absolute temperature or drought regimen. Hence plants that are less adapted to environmental stress would be more metabolically stressed than those that are acclimatized to aberrant climate conditions.

### *Drought and warming altered total nitrogen and the proportion of soluble proteins in green leaves*

As nitrogen is the most limiting nutrient in many ecosystems, understanding plant N dynamics under changing climates is critical to predict both plant growth and soil biogeochemical cycling, each of which may feed back to climate change. In general, warming decreased green leaf N in all precipitation treatments (Fig. 7a). Similar decreases in green leaf N with warming have been recorded in other tree species (Tjoelker *et al*., 1999; Weih & Karlsson, 2001), in shrubs (Sardans *et al*., 2008b) and in grasses (An *et al*., 2005). Despite a similar specific leaf area, the total elemental N in green leaves was highest in *Q. rubra* exposed to the most favorable climatic treatment (W0), while the DH treatment resulted in the lowest N content in green tissues (Fig. 7a). This pattern could be due to the influence of climatic stresses on plant physiology and soil N mineralization. For example, the combination of drought and warming would reduce photosynthesis and lower the sink strength (Llorens *et al*., 2004; Sardans *et al*., 2008a, b), while warming- and drought-induced limitation in soil moisture decreases the mineralization and diffusion of soil N (Emmett *et al*., 2004; Sardans *et al*., 2008a; Auyeung *et al*., 2013) together decreasing N in green leaves exposed to DH.

Probing deeper into the N dynamics of green tissues, our study revealed that the allocation of N to different protein fractions also varied with climate treatments (Fig. 7b–f). The higher amount of total soluble proteins with heat and moisture stress observed in this study (Fig. 7b) is indicative of plants allocating more N to the photosynthetic apparatus, as Rubisco typically comprises 50% of total soluble proteins (Feller *et al*., 2008). Also, plants exposed to heat and drought stress may increase the production of heat-shock proteins (Huang & Xu, 2008; Gechev *et al*., 2013). Concurrently, the non-extractable N increased in green tissues produced under nonstressed climatic conditions such as the wet and unwarmed treatments (Fig. 7e–f). In mesic environments, wetter conditions generally favor nitrogen uptake, leading to faster plant growth and higher nutrient concentrations in tissues (Mulder *et al*., 2013; Sardans & Penuelas, 2013).

### *Climatic treatments differentially influence the resorption of elemental and metabolic pools of nitrogen*

Surprisingly, the pattern of RE of elemental N was opposite to that of RE of extractable N metabolites such as amino acids. In drought treatments (D0, DH) RE of amino acids was ~90% (Fig. 6a). However, the RE of elemental nitrogen in drought treatments was <20% (Fig. 8). Although the solvent-extracted physiological amino acid pool contributes to a lower fraction of total leaf N (12.1 ± 1.2% of total leaf N in A0 treatment), this labile pool of N could be important in facilitating tissue decomposition and soil nutrient cycling (Top & Filley, 2014). An impairment of phloem-loading capacity in moisture-stressed plants could contribute to the lower resorption of total N in drought treatments (D0 and

DH; Hill, 1980; Pugnaire & Chapin, 1992). In general, environments that are limiting in nutrients but sufficient in moisture favor greater RE from senescing tissues (Arco *et al.*, 1991; Pugnaire & Chapin, 1992). As xylem water potential plays a critical role in the phloem transport of photosynthates and in phloem loading (Holtta *et al.*, 2006), moisture stress tends to limit RE irrespective of the nutrient availability in soils (Pugnaire & Chapin, 1992). Thus, the pattern of RE of elemental N observed in our study could be partly attributed to the moisture stress in drought treatments, which directly interferes with the physiology of nutrient resorption in plants. A similar pattern of lower elemental C : N ratios has been observed in senesced leaves of other tree species exposed to drought treatments at the BACE site (J.S. Dukes, unpublished results). Protein catabolism predominantly yields amino acids (Vierstra 1993), and hence the differential catabolism of proteins between the wet and drought treatments during leaf senescence could also influence the observed RE of amino acids. Although plants adapted to dry climates generally exhibit higher nutrient resorption than plants adapted to tropical climates (Vergutz *et al.*, 2012), the decrease in RE of elemental N under drought observed in this study indicates that plants that are less acclimatized to moisture stress might suffer under future drier and warmer climates due to a greater loss of assimilated N during senescence.

In addition to the moisture availability, the quantitative and qualitative changes in certain polyphenolic defense compounds in plant tissues can interact with other cellular metabolites, leading to the changes in the resorption potential (Chapin & Kedrowski, 1983). Plants experiencing moisture stress can upregulate the acetate and shikimate pathway, leading to the enhanced production of tannins (Bussotti *et al.* 1998, Tharayil *et al.*, 2011; Zhang *et al.*, 2012), which are polyphenols that can complex with proteins. In our study, shikimic acid, the precursor of the phenylpropanoid pathway, was lowest in plants exposed to climatic stress (Fig. 5), potentially indicating a greater utilization of shikimate for the production of phenylpropanoid protective compounds such as flavonoids, lignins and tannins (Xu & Huang, 2012; Yu *et al.*, 2012; V. Suseela, unpublished results). In general, trees grown in drought treatments at the BACE had higher concentrations of tannins than those grown in wet precipitation treatments (Tharayil *et al.*, 2011). Tannins can not only complex with cellular proteins, preventing their hydrolysis, but also can deactivate proteases that facilitate proteolysis during senescence, thus limiting the RE of N. This argument is supported by a robust negative correlation of the concentration of tannins in green leaves of *Q. rubra* sampled for the present study (S.M. Top, unpublished results) and the observed RE of total N (Fig. 9). Thus, the concomitant abundance of tannins and soluble proteins in climatically stressed trees could enhance tannin–protein complexation, which interferes with the resorption of N. In addition, tannins can complex with, and inactivate soil microbial enzymes that facilitate litter decomposition (Triebwasser *et al.*, 2012). Although solvent-extractable metabolites represent a smaller pool compared to the structural matrix in litter (Berg & McClaugherty, 2008; Suseela *et al.*, 2014b), the extractable metabolite pool would have a disproportionate influence on tissue decomposition because of their rapid bioavailability to soil heterotrophs (Chapin & Kedrowski, 1983; Norby *et al.*, 2000; Suseela *et al.*, 2013). Thus, the disparity in senesced tissues of the DH treatment between the substantially depleted pool of extractable amino acids and the relatively high abundance of tissue elemental N may indicate that these tissues might have a lower initial decomposition rate, in spite of their lower elemental C/N ratio. This pattern of an initial lower rate of decomposition of litter from drought treatment sampled for the present study was confirmed through lab (S.M. Top, unpublished results), and field (V. Suseela, unpublished) experiments.

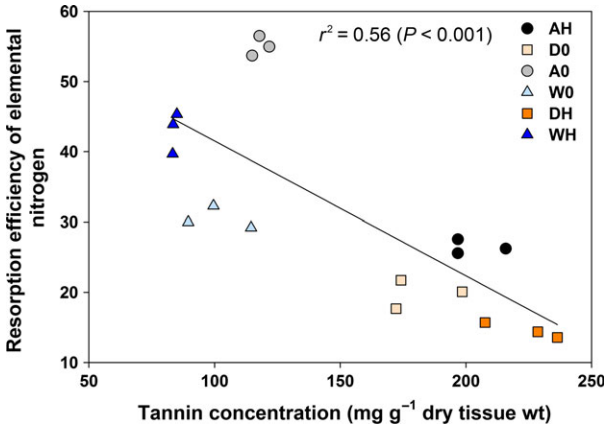


**Fig. 9** Relationship between the resorption efficiency of total elemental nitrogen and the tannin concentration in green leaves of *Quercus rubra* across the six different climate treatments. Each point represents values from a single experimental plot.

### *Production and resorption of plant metabolites under climatic stress: implications for climate–carbon feedbacks*

The retranslocation of N from senesced tissues is an important parameter in the land components of some Earth system models (e.g., the Community Land

Model 4.5; CLM; Oleson *et al*., 2013). In CLM, the pool of retranslocated N from senescing tissues can support new growth and is assumed to be proportional to plant N demand, implying that plants with high N demand also have high N resorption efficiency. Our results suggest that this pool of retranslocated N is also modulated by the climatic stress experienced by the plants. Although the metabolic demand for N would be higher in the wet treatments to meet the high growth demand, compared to the ambient (A0) treatment, we generally observed a lower RE of N in the wet treatments both with regard to extractable N metabolites (amino acids) and elemental N. Our results also indicate that the RE of N would be tightly regulated by the climatic stress-induced production of biopolymeric defense compounds, with climatic stress resulting in lower resorption. Our study thus indicates that the differential response of climatic stresses on both production and resorption of plant metabolites would ultimately affect plant growth and the quality of litter available for microbial decomposition. This could further influence nutrient availability and soil fertility, which could in turn feed back to climate change. Further in our study, the combination of drought and warming increased the abundance of many metabolites in the green tissues in a manner that would not have been predicted from responses to either factor alone. Our study thus emphasizes that the interactive effects of combined stresses such as warming and drought on plant metabolomics cannot be extrapolated from responses to individual stress factors. Adequate simulation of these changes in climate–carbon models could help to better forecast responses of terrestrial ecosystems to future climates and their feedback to warming. As multiple climatic stresses are becoming more common under natural settings, understanding plant physiological and metabolic responses to combined stress factors would enable us to better predict ecosystem functions and processes in a warmer world.

## Acknowledgements

The authors are grateful to Dr. Jordi Sardans for valuable and thoughtful comments. We thank Carol Goranson for maintaining the BACE site. We also thank Georgianna Scott and Amith Maroli for help with protein extraction and Sara Top for tannin analysis. This research was supported by the National Science Foundation grant (DEB-1145993) to N. T., B. X. and J. S. D. and the NSF Postdoctoral Research Fellowship in Biology (DBI-1306607) to V. S. The BACE infrastructure was constructed with support from NSF (DEB-0546670), and the project has been maintained with support from the U.S. Department of Energy's Office of Science (BER), through the Northeastern Regional Center of the National Institute for Climatic Change Research, and through the Terrestrial Ecosystem Sciences program. This research article is Technical Contribution No. 6371 of the Clemson University Experiment Station and the Purdue Climate Change Research Center (No: 1517).

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Specific leaf area of green and senesced leaves of *Quercus rubra* exposed to six different climatic treatments. (A0, ambient precipitation + no warming; AH, ambient precipitation + high warming; W0, wet precipitation + no warming; WH, wet precipitation + high warming; D0, dry precipitation + no warming; DH, dry precipitation + high warming).
**Figure S2.** Principal component analysis of the relative intensities of 100 plant metabolites of *Quercus rubra* exposed to six different climatic treatments (A0, ambient precipitation + no warming; AH, ambient precipitation + high warming; W0, wet precipitation + no warming; WH, wet precipitation + high warming; D0, dry precipitation + no warming; DH, dry precipitation + high warming).