Warming Alters Plant Chemical and Nutrient Compositions by Affecting Metabolites in Cunninghamia lanceolata (Lamb.) Hook

Qiufang Zhang ¹,², Zhijie Yang ¹,², Tingting Chen ¹,², Xiaoying Gong ¹,², Decheng Xiong ¹,², Wangmin Ye ¹,², Yuehmin Chen ¹,²,³, * and Yusheng Yang ¹,²,³

¹ State Key Laboratory for Subtropical Mountain Ecology of the Ministry of Science and Technology and Fujian Province, Fujian Normal University, Fuzhou 350007, China
² School of Geographical Sciences, Fujian Normal University, Fuzhou 350007, China
³ Institute of Geography, Fujian Normal University, Fuzhou 350007, China

* Correspondence: ymchen@fjnu.edu.cn; Tel.: +86-591-83465013; Fax: +86-591-83465397

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Abstract: Research Highlights: Warming alters the chemical composition of Cunninghamia lanceolata (Lamb.) Hook, resulting in increased production of macromolecular compounds that protect against heat stress. Background and Objectives: Low latitude forests are experiencing obvious climatic warming; however, the plant physiological responses to warming are not well understood. As warming induces moisture stress, we hypothesized that warming activates metabolites (i.e., lipids, phenolic compounds, amino acids) and causes damage to the leaves, exemplified by the increased concentrations of reactive oxygen species. Materials and Methods: We conducted a warming experiment in a C. lanceolata plantation. Plant physiological traits associated with nutrient status, reactive oxygen species, antioxidant enzymes species, and metabolites were measured. Results: Warming altered the chemical composition of C. lanceolata as it increased C:N ratios of leaves and roots. In particular, the concentrations of N and P in leaves and roots were significantly decreased under the warming condition, which might be related to the biomass production, namely, a dilution effect. Under the warming condition, most of the phospholipid compounds and proteins significantly increased. Leaf C, carbohydrates, amino acids, organic acids, flavonoids, and phenolic compounds were identified to have significantly lower concentrations under the warming treatment than those under the control treatment. These results suggested that moisture stress under the warming treatment may drive C deficiency and metabolic restriction in plants. Conclusions: Under the warming condition, C. lanceolata changed its energy utilization strategy and invested more resources to produce macromolecular compounds for protecting against heat stress. Warming in sub-tropical forests alters plant chemical properties, and thus may have an important consequence for nutrient cycling and soil C sequestration.

Keywords: climate change; warming; metabolites; sub-tropical forest; nutrient concentration

1. Introduction

Low latitude forests serve as one of the most active biogeochemical engines on Earth [1]. Despite only covering 15% of the planet’s terrestrial surface, more than two thirds of the global biomass is produced in low latitude regions [2,3]. Climate models predict drastic changes in weather patterns in low latitude regions over the next 20 years [4–6]. Given the large amount of carbon (C) stored and cycled in low latitude forests, investigations on the response of these systems to environmental drivers are crucial to fully understand and thus accurately predict future global climate effects on biogeochemical cycles [7].
Temperature is an important factor in terrestrial ecosystems and has implications for plant growth. Some studies reported that warming promotes plant growth [8,9]. However, an increasing number of studies have demonstrated that the effects of warming are not always promotional for productivity. For example, Bruhn et al. [10] found that *Eucalyptus pauciflora* (Sieber ex Sprengel) respiration acclimated to long-term nocturnal warming, and warming had no significant effect on *E. pauciflora* growth. Using thermal infrared radiation to experimentally increase ambient temperature, the height and total biomass of *Abies faxoniana* (Rehd. et Wils) were found to significantly decrease [11]. In addition, it was reported that the physiological metabolism of plants was affected by water availability, temperature, and other factors [12–14]. Generally, warming alone can increase forest moisture stress [15]. McDowell et al. [16] postulated three possible mechanisms by which moisture stress could lead to the mortality of trees [15]: (1) Hydraulic failure: Extreme drought and warming kill trees through cavitation of water columns within the xylem; (2) C starvation: Protracted moisture stress drives plant C deficiency and metabolic restriction that lead to C starvation; (3) biotic agent demographics: Extended warming during drought drives changes in the demographics of mortality agents (i.e., insects and fungi), which subsequently drives forest mortality. Although the above hypotheses are gradually being confirmed [17], our knowledge of physiology is still insufficient to predict regional plant growth patterns under climate warming.

Under climatic stress, plants will allocate relatively more energy and resources to the production of compounds that allow them to better acclimatize to the stresses [14,18]. Extractable metabolites make up to 30% of the dry weight of plants [19,20]. Analysis of metabolites offers a potential approach to gain insights into the metabolic pathways involved in the efficient use of nutrients [21]. In particular, reprogramming of stress-induced plant metabolic pathways resulted in the up- or down-regulation of several compounds, such as amino acids, organic acids, carbohydrate, phenolic compound, polyamines, and phospholipids [22–24]. Many of these metabolites function as osmotic regulators, scavenging free radicals, and maintaining the structural integrity of proteins, enzymes, and other macromolecules [25–27]. Changes in metabolites and their levels are thought to be the ultimate response of plants to environmental pressures [28,29]. For instance, Gargallo-Garriga et al. [26] revealed that warming promoted the accumulation of proline in shoots, increased the concentration of amino acids, and protected the grasses *Holcus lanatus* (Linn.) and *Alopecurus pratensis* (Linn.) against moisture stress. A warming experiment conducted by Johnson and Hartley [12], showed that there was a defensive trade-off as silicon and phenolic concentrations were negatively correlated with each other. Moreover, both of these defenses were negatively correlated with plant mass and compatible with the growth defense balance [12]. Zhao et al. [30] reported that soil and air temperature elevated by about 2 °C did not induce oxygen toxicity or alteration of most of the antioxidant enzymes in *Pinus tabulaeformis* (Carr.). Long et al. [31] explained that a warmer climate with increased nutrient availability will likely reduce defenses of subarctic tundra vegetation. However, most of these studies have mainly focused on herbaceous plants [12,26,31]. In general, tropical areas are warmer than temperate areas [1], and this may enable plants in tropical forests to allocate more resources to plant defenses. Modifications of metabolites in forests in response to warming have not been systematically investigated in low latitude forests.

To assess the effect of climate warming on the plant physiological traits in low latitude forests, a warming experiment was performed on *Cunninghamia lanceolata* (Lamb.) Hook. This species was selected due to its wide distribution and great economic importance: It covers over 9.11 million ha in southern China and accounts for 18% of forest plantations in China and 5% worldwide [32,33]. Climate warming was simulated by the use of heating cables. Plant nutrient status, reactive oxygen species, antioxidant enzymes species, and metabolites were measured. As warming induces moisture stress, we hypothesized that warming activates metabolites (i.e., phenolic compounds, lipids, amino acids) and causes damage in the leaves, exemplified by the increased concentrations of reactive oxygen species.
2. Methods

2.1. Study Site and Experimental Setup

The study was conducted at the Chenda Observation Study Site of Sanming Forest Ecosystem and Global Change Research Experiment (26°19′55″ E, 117°36′53″ N, 300 m above sea level), Fujian Province, China. The study site has a subtropical monsoon climate, annually receives ~1750 mm of rainfall, with a mean annual temperature of 19.1 °C. The climate data are annual averages for the period from 1974 to 2014 and are from the Sanming Meteorological Station which is 9.4 km from the study site. The soil was classified as Oxisols according to the USDA Soil Taxonomy.

*C. lanceolata* seedlings were subjected to two levels of temperature treatments: Control (CT, ambient temperature) and warming (W). Warming treatments were administered continuously using heating cables (TXLP/1, Nexans, Norway) buried at a soil depth of 10 cm, and at intervals of 20 cm. The outputs of the heaters were set at 17 W m\(^{-1}\) at 230 V, which resulted in a 5 °C temperature increase [34]. In total, the design consisted of ten plots (2 m × 2 m) with five replicates of each temperature treatment. Each plot was divided into four 1 m × 1 m subplots and one-year-old *C. lanceolata* seedlings were planted in each subplot in November 2013. In total, 40 one-year-old *C. lanceolata* seedlings were selected from a local nursery; the seedlings were uniform in plant height and basal diameter. The average height and basal diameter of seedlings were 25.7 ± 2.5 cm and 3.4 ± 0.4 mm, respectively [34].

2.2. Sampling and Processing of Leaves and Roots

Tree height was measured in the third year of warming. Leaves were sampled in July 2017 and were collected within a short time period (i.e., between 11:00 and 14:00) to minimize the change of metabolite concentration [21]. Ten fully-expanded current-year leaves were harvested from the top of the trees from each tree and were then mixed to make a composite sample from each plot, flash frozen using liquid nitrogen, and stored at −80 °C for the metabolite analyses [35]. We also sampled the fine-roots (< 2 mm) of *C. lanceolata* from each plot at five random points using a 5 cm diameter stainless steel drill to obtain the soil cores. All soil cores were taken at a depth of 10 cm.

2.3. Carbon, Nitrogen, and Phosphorus Analyses

Plant samples (leaf and root) were oven-dried at 65 °C for 1 week and ground to fine powder using a pulverizer. The concentrations of C and N were analyzed using a CN auto analyzer (Vario Max CN, Germany). To determine the P concentration, samples were first digested with H\(_2\)SO\(_4\):HClO\(_4\) (4:1) and passed through a 0.45 µm glass fiber filter (Q/IEF J01-1997, Shanghai). Plant P concentration was then determined using a continuous flow analyzer (Skalar san++, Netherlands).

2.4. Determination of the Concentrations of Reactive Oxygen Species and Osmotic Adjustment Substances

We analyzed the rate of production of superoxide anion radicals (O\(_2^−\)), following the method of Brawn and Fridovich [36], by monitoring the rate of nitrite formation from hydroxylamine in the presence of O\(_2^−\). First, 500 mg frozen leaves were homogenized with 1.5 mL of the 50 mM phosphate buffer (pH 7.4). The homogenate was centrifuged at 8000×g for 15 min at 4 °C. Second, we placed 0.5 mL supernatant into a centrifuge tube, added 0.45 mL 50 mM phosphate buffer and 0.5 mL 10 mM hydroxylamine hydrochloride, and incubated at 25 °C for 20 min. Then 8.5 mM aminobenzene sulfonic acid and 3.5 mM alpha naphthylamine were added to the tube. Last, the absorbance was measured at 550 nm (UV-2450/2550, Shimadzu, Japan) after 20 min of color development at 25 °C. The supernatant for determining hydrogen peroxide (H\(_2\)O\(_2\)) concentration was extracted from frozen leaves with ice acetone. In order to form precipitate, 2 mL 5% titanium sulfate and 5 mL concentrated ammonia was added to the supernatant. After being centrifuged at 8000×g for 10 min, leaving the precipitate in the tube, 5 mL 2 M H\(_2\)SO\(_4\) was added to dissolve the precipitate, then the solution was transferred to a 10-mL volumetric flask, and the absorbance value was determined at 405 nm with a scanning spectrophotometer [37]. We determined the malondialdehyde (MDA) concentration...
by the thiobarbituric acid (TBA) reaction, according to the method described by Dhindsa et al. [38]. The reaction mixture contained 50 µL 0.1 M phosphate buffer, 0.75 mL 10% trichloroacetic acid, and 0.25 mL 0.6% TBA solution. Using bovine serum as the standard, the concentration of soluble protein (stained with Coomassie Brilliant Blue) was measured with a scanning spectrophotometer at 595 nm absorbance [39]. In order to determine free proline concentration, 500 mg of fresh leaves was homogenized using a pestle and mortar under ice-cold conditions with 5 mL of 3% sulfosalicylic acid. After centrifugation at 8000×g for 10 min, 2 mL of the supernatant was pipetted and incubated with 2 mL distilled water, 2 mL glacial acetic acid, and 4 mL 2.5% acidic ninhydrin at 100 °C for 60 min. After cooling down, 4 mL of toluene was added to the mixture and the absorbance of the chromophore containing toluene was recorded at 520 nm [40].

2.5. Determination of the Activities of Antioxidant Enzymes

Under freezing conditions, 500 mg of frozen leaves was homogenized and 4.5 mL of 0.1 M phosphate buffer (pH 7.4) were added. The homogenate was centrifuged at 8000×g for 15 min at 4 °C. The supernatant was then used to determine the activities of antioxidant enzymes. Superoxide dismutase (SOD) activity was determined as the percentage reduction of nitrobluetetrazolium with a scanning spectrophotometer at 550 nm absorbance [41]. The reaction system contained 1.0 mL of 0.1 M phosphate buffer, 0.1 mL of 0.1 mol L⁻¹ hydroxylamine hydrochloride solution, 0.1 mL of 75 mmol L⁻¹ xanthine solution, and 0.1 mL of 0.037 U L⁻¹ xanthine oxidase. The determination of peroxidase (POD) activity was based on the measurement of guaiacol oxidation by H₂O₂ at 420 nm absorbance [42]. The reaction system contained 2.4 mL of 0.1 M phosphate buffer, 0.1 mL of 2% H₂O₂, 100 µL of 40-fold diluted enzyme extract, and 1 mL of 50 mmol L⁻¹ guaiacol. We measured the catalase (CAT) activity following the method described by Trevor et al. [43], i.e., by determining the decrease in absorbance at 240 nm. The reaction mixture contained 1 mL of 0.1 M potassium phosphate buffer (pH 7.4), 50 µL of the enzyme extract, and 0.1 mL of 0.1 M H₂O₂. Following the method described by Nakano and Asada [44], we determined the decrease in absorbance at 290 nm to measure the reduction in ascorbic acid. The ascorbate peroxidase (APX) activity was measured after ascorbic acid was oxidized in the presence of H₂O₂. The method described by Dinis [45] was used to determine the total antioxidant capacity (T-AOC)—leaf blade extracts were mixed with FeCl₂·4H₂O and ferrozine, and the absorbance of the mixture was determined at 562 nm after 10 min.

2.6. Metabolite Extraction for LC-MS

Approximately 50 mg of frozen leaves was used in the metabolite extraction procedure. This procedure initially involved extraction with 800 µL of methanol, followed by the addition of 10 µL of internal standard (2.9 mg mL⁻¹, DL-o-chlorophenylalanine). All samples were ground to a fine powder using a grinding mill at 65 Hz for 120 s. The samples were then ultrasonicated at 40 KHz in an ice bath for 30 min, and centrifuged at 8000 g at 4 °C for 15 min. The resulting extracts were used in the metabolite analyses.

Supernatants (200 µL) were transferred to vials for LC-MS analysis. Analyses were performed using a high-performance liquid chromatography system coupled with a mass spectrometer equipped with a Quadrupole Time-of-Flight Mass Analyzer (Agilent, 1290 Infinity LC, 6530 UHD and Accurate-Mass Q-TOF/MS). Chromatography was performed on a reversed-phase gold column (100.0 × 2.1 mm; 1.8 µm; Agilent, Shanghai, China) at 40 °C. Mobile phase A included water and 0.1% formic acid; mobile phase B included acetonitrile and 0.1% formic acid. The injection volume of the samples was 4 µL; the elution gradient of the mobile phase is shown in Table 1. The automatic injector temperature was set at 4 °C.
Table 1. Gradient of the mobile phase.

| Time (min) | Flow Rate (mL min$^{-1}$) | Pressure Limit (bar) | Solv Ratio B (%) |
|------------|---------------------------|----------------------|------------------|
| 0          | 0.35                      | 800                  | 5                |
| 1          | 0.35                      | 800                  | 5                |
| 6          | 0.35                      | 800                  | 20               |
| 9          | 0.35                      | 800                  | 50               |
| 13         | 0.35                      | 800                  | 95               |
| 15         | 0.35                      | 800                  | 95               |

All samples were injected twice, once with the electrospray ionization (ESI) operating in positive ionization mode (ESI+) and once in negative ionization mode (ESI−). For ESI+ detection, the instrument settings of capillary voltage, sampling cone voltage, source temperature, desolvation temperature, cone gas flow, desolvation gas flow, and extraction cone voltage were 4 kV, 35 kV, 100 °C, 350 °C, 50 L h$^{-1}$, 600 L h$^{-1}$, and 4 V, respectively. ESI− acquisition was conducted with the following modifications: Capillary voltage was 3.5 kV, sampling cone voltage was 50 kV, and desolvation gas flow was 700 L h$^{-1}$. Centroid data were collected over the 50–1000 m/z range with a scan time of 0.03 s and interscan delay of 0.02 s.

2.7. Statistical Analyses

The effects of warming on the concentrations of plant C, N, P, leaf reactive oxygen species, and osmotic adjustment substances, and the activities of antioxidant enzymes were analyzed with an independent samples t test using SPSS 20.0 software. Results were considered as statistically significant at $p < 0.05$ in all cases. The metabolite data were subjected to feature extraction, preprocessed with a ‘XCMS’ package in R 3.5.2 software (www.r-project.org), and then normalized and edited into a two-dimensional data matrix using Excel 2016 software. The matrix included the retention time (RT), mass-to-charge ratio (MZ), observations (samples), and peak intensity. After editing, the data were assessed with a multivariate analysis using SIMCA-P+13.0 software (Umetrics AB, Umea, Sweden). Multivariate ordination principal component analyses (PCAs) were performed to detect patterns of sample ordination in the metabolites. Before analysis by orthogonal projections to latent structures via partial least-squares-discriminant Analysis (PLS-DA), all data were mean-centered, univariate-scaled, and divided into the two treatment groups: Control and warming. Combinations of VIP (variable importance in the projection) statistics (threshold > 1) of the PLS-DA model and t-test ($p < 0.05$) were used to quantify the differences in metabolites between the two treatments.

3. Results

3.1. Tree Height and Plant Nutrients

Compared to the control treatment, the plant height of *C. lanceolata* was slightly higher under the warming treatment (~4.4%, not significant) (Table 2). The concentrations of C and P in the leaves decreased significantly, by ~1.7% and ~26.3%, respectively, under the warming treatment (Table 2). The concentrations of C, N, and P in the roots decreased significantly by ~6.4%, ~28.2%, and ~25.7% under the warming treatment, whereas the ratio of C:N in the roots significantly increased by ~29.2% (Table 2).
Table 2. The effects of warming on tree height and concentrations of leaf and root C, N, and P in Cunninghamia lanceolata.

| Index              | Control                   | Warming                   |
|--------------------|---------------------------|---------------------------|
| Tree height (m)    | 4.28 ± 0.15 a             | 4.47 ± 0.10 a             |
| Leaf C concentration (mg g⁻¹) | 457.49 ± 4.46 a         | 449.86 ± 3.23 b         |
| Leaf N concentration (mg g⁻¹) | 6.21 ± 0.99 a         | 5.47 ± 0.47 a         |
| Leaf P concentration (mg g⁻¹) | 0.95 ± 0.17 a         | 0.70 ± 0.12 b         |
| Leaf C:N ratio     | 74.97 ± 10.35 a           | 82.78 ± 7.01 a           |
| Root C concentration (mg g⁻¹) | 462.30 ± 7.20 a       | 432.76 ± 10.45 b       |
| Root N concentration (mg g⁻¹) | 8.22 ± 1.26 a         | 5.90 ± 0.66 b         |
| Root P concentration (mg g⁻¹) | 0.35 ± 0.03 a         | 0.26 ± 0.04 b         |
| Root C:N ratio     | 57.28 ± 8.38 b           | 74.00 ± 6.87 a           |

Values are the means of five replicates ± standard deviation. Different lowercase letters within rows indicate significant (p < 0.05) differences among treatments (n = 5).

3.2. The Concentrations of Reactive Oxygen Species and Osmotic Adjustment Substances, and the Activities of Antioxidant Enzymes

The concentrations of O₂⁻, H₂O₂, and MDA did not differ between the control and warming treatments (Figure 1). Compared to the control treatment, the warming treatment increased the activities of POD, APX, and glutathione peroxidase (GSH-PX) by ~28.4%, ~6.8%, and ~9.1%, respectively, but these increases were not significant (Figure 2). In the warming treatment, the SOD, CAT, and T-AOC activities decreased by ~6.7%, ~7.3%, and ~4.3% respectively, compared with the control treatment, but these decreases were not significant (Figure 2). Warming significantly decreased leaf proline concentration by about 24.1% (p < 0.05) (Figure 3a). The concentration of soluble protein in the warming treatment was significantly higher (by 2.9%) than that of the control treatment (Figure 3b).

Figure 1. The concentrations of reactive oxygen species in Cunninghamia lanceolata under the control (CT) and warming (W) treatments (mean ± standard deviation, n = 5). (a) Superoxide anion (O₂⁻), (b) hydrogen peroxide (H₂O₂), (c) malondialdehyde (MDA).
3.3. Metabolite Profiling

In total, we measured 1156 features using the ESI+ ion mode and 760 features using the ESI− ion mode. Multivariate analysis using the PLS-DA modeling method revealed a clear separation of metabolite samples between the control and warming treatments ($R^2X = 0.699$, $R^2Y = 0.995$, $Q^2 = 0.847$).
in ESI+ ion mode; \( R^2_X = 0.667, R^2_Y = 0.974, Q^2 = 0.708 \) in ESI– ion mode) (Figure 4). A total of 41 different features were identified in the leaves between the control and warming treatments according to the VIP (threshold > 1) and the t-test (Table 3). Among them, 37 features were measured using the ESI+ ion mode and 4 features were measured using the ESI– ion mode (Table 3; Figure 5).

**Figure 4.** Partial least squares discriminant analysis (PLS-DA) score plots of the metabolites in *Cunninghamia lanceolata* under the control (CT) and warming (W) treatments (\( n = 5 \) replicates each). Each data point represents one flag leaf sample of *C. lanceolata*, and the distance between points indicates the significant difference between samples. (a) ESI+ mode, (b) ESI– mode.
Table 3. Extractable plant metabolites identified in the leaf of *Cunninghamia lanceolata* under the control (CT) and warming (W) treatments, broadly classified into groups.

| No | Compound | Abbreviation | RT (min) | Molecular Weight | VIP  | t test | Fold Change (CT/W) | Mode |
|----|----------|--------------|----------|------------------|-----|--------|--------------------|------|
| 1  | DG(14:0/15:0/16:0) | DG1 | 16.65 | 526.4597 | 1.89 | 0.031 | −0.97 | ESI+ |
| 2  | DG(14:1(9Z)/15:0/16:0) | DG2 | 17.60 | 524.4441 | 1.99 | 0.020 | −0.43 | ESI+ |
| 3  | DG(14:1(9Z)/22:2(13Z,16Z)/0:0) | DG3 | 16.64 | 618.5223 | 1.87 | 0.034 | −0.52 | ESI+ |
| 4  | DG(18:3(6Z,7Z,10Z,13Z,16Z)/17:3(9Z,10Z)) | DG4 | 16.10 | 664.5067 | 2.06 | 0.014 | −0.53 | ESI+ |
| 5  | DG(18:3(6Z,7Z,10Z,13Z,16Z)/17:3(9Z,10Z)) | DG5 | 16.09 | 662.4910 | 2.29 | 0.004 | −0.87 | ESI+ |
| 6  | PC(18:2(9Z,12Z)/P-18:1(11Z)) | PC1 | 13.59 | 767.5829 | 2.20 | 0.006 | 1.22 | ESI+ |
| 7  | PC(18:3(9Z,12Z,15Z)/22:5(4Z,7Z,10Z,13Z,16Z)) | PC2 | 15.87 | 829.5622 | 2.27 | 0.004 | −0.75 | ESI+ |
| 8  | PC(22:4(7Z,10Z,13Z,16Z)/24:1(15Z)) | PC3 | 15.84 | 919.7030 | 1.96 | 0.023 | −0.99 | ESI+ |
| 9  | lysoPC(20:2(11Z,14Z)) | PC4 | 15.13 | 547.3638 | 2.14 | 0.009 | −0.24 | ESI+ |
| 10 | PC(15:0/18:1(11Z)) | PC5 | 10.83 | 745.5622 | 3.44 | 0.000 | 2.07 | ESI− |
| 11 | PE(20:3(5Z,8Z,11Z)/22:6(4Z,7Z,10Z,13Z,16Z)) | PE1 | 15.69 | 813.5309 | 2.50 | 0.000 | −0.71 | ESI+ |
| 12 | PE(22:2(13Z,16Z)/P-18:1(11Z)) | PE2 | 13.59 | 781.5985 | 2.54 | 0.000 | −1.11 | ESI+ |
| 13 | PE(22:5(4Z,7Z,10Z,13Z,16Z)/P-18:1(11Z)) | PE3 | 15.64 | 775.5156 | 2.51 | 0.000 | −0.65 | ESI+ |
| 14 | PG(16:0/22:5(4Z,7Z,10Z,13Z,16Z)) | PG1 | 15.64 | 796.5254 | 2.51 | 0.000 | −0.64 | ESI+ |
| 15 | PG(16:0/18:3(6Z,9Z,12Z)) | PG2 | 10.83 | 744.4941 | 3.31 | 0.001 | 1.80 | ESI− |
| 16 | melibiitol | Mel | 4.10 | 344.1319 | 1.89 | 0.030 | 0.29 | ESI+ |
| 17 | physcion 8-gentiobioside | Phy | 16.35 | 608.1741 | 2.05 | 0.015 | 1.76 | ESI+ |
| 18 | L-tryptophan | Try | 12.69 | 204.0899 | 1.93 | 0.026 | 0.20 | ESI+ |
| 19 | hydroxylysine | Hyd | 6.03 | 162.1004 | 1.83 | 0.039 | 0.77 | ESI+ |
| 20 | tetradecanoic acid | Tet | 12.66 | 258.1831 | 1.83 | 0.038 | 0.09 | ESI+ |
| 21 | gibberellin A3 | Gib | 1.20 | 346.1416 | 1.77 | 0.048 | −0.80 | ESI+ |
| 22 | pisumic acid | Ps | 3.75 | 282.1467 | 2.45 | 0.001 | 1.02 | ESI+ |
| 23 | flavone | Fla | 12.66 | 222.0681 | 2.03 | 0.016 | 0.25 | ESI+ |
| 24 | homovanillic acid | Hom | 6.54 | 182.0579 | 2.36 | 0.002 | 0.35 | ESI+ |
| 25 | peonidin 3-ferulylglucoside-5-glucoside | Peo | 13.50 | 962.2692 | 2.98 | 0.006 | −0.27 | ESI− |
| 26 | isogermafurene | Iso | 3.32 | 216.1514 | 2.46 | 0.001 | 1.21 | ESI+ |
| 27 | barringtogenol C | Bar | 17.32 | 490.3658 | 1.99 | 0.020 | −0.60 | ESI+ |
| No | Compound                        | Abbreviation | RT (min) | Molecular Weight | VIP  | t test | Fold Change (CT/W) | Mode |
|----|---------------------------------|--------------|----------|------------------|------|--------|-------------------|------|
| 28 | indicine                        | Ind          | 3.75     | 299.1733         | 2.53 | 0.000  | 1.21              | ESI+ |
| 29 | anabasine                       | Ana          | 6.06     | 162.1157         | 2.36 | 0.002  | 0.67              | ESI+ |
| 30 | 3-acetyl-2, 7-naphthyridine     | 3-Ace        | 1.49     | 172.0637         | 2.34 | 0.002  | 1.52              | ESI+ |
| 31 | acetylintermedine               | Ace          | 1.12     | 341.1838         | 1.93 | 0.026  | –0.86             | ESI+ |

Acylamide compound

| No | Compound                        | Abbreviation | RT (min) | Molecular Weight | VIP  | t test | Fold Change (CT/W) | Mode |
|----|---------------------------------|--------------|----------|------------------|------|--------|-------------------|------|
| 32 | adrenoyl ethanolamide           | Adr          | 15.78    | 375.3137         | 2.64 | 0.000  | –0.85             | ESI+ |
| 33 | palmitic amide                  | Pal          | 15.22    | 255.2562         | 2.08 | 0.013  | –0.35             | ESI+ |

Pigment

| No | Compound                        | Abbreviation | RT (min) | Molecular Weight | VIP  | t test | Fold Change (CT/W) | Mode |
|----|---------------------------------|--------------|----------|------------------|------|--------|-------------------|------|
| 34 | xanthophyll                     | Xan          | 17.63    | 568.4280         | 2.03 | 0.016  | –0.47             | ESI+ |

Vitamin

| No | Compound                        | Abbreviation | RT (min) | Molecular Weight | VIP  | t test | Fold Change (CT/W) | Mode |
|----|---------------------------------|--------------|----------|------------------|------|--------|-------------------|------|
| 35 | vitamin D3                      | Vit          | 16.49    | 384.3392         | 2.45 | 0.001  | –0.73             | ESI+ |

S compound

| No | Compound                        | Abbreviation | RT (min) | Molecular Weight | VIP  | t test | Fold Change (CT/W) | Mode |
|----|---------------------------------|--------------|----------|------------------|------|--------|-------------------|------|
| 36 | allicin                         | All          | 8.15     | 162.0173         | 2.34 | 0.002  | 1.01              | ESI+ |

Other

| No | Compound                        | Abbreviation | RT (min) | Molecular Weight | VIP  | t test | Fold Change (CT/W) | Mode |
|----|---------------------------------|--------------|----------|------------------|------|--------|-------------------|------|
| 37 | phaseolotoxin                   | Pha          | 15.21    | 531.1989         | 1.96 | 0.023  | 1.62              | ESI+ |
| 38 | fraxin                          | Fra          | 15.23    | 370.0900         | 2.01 | 0.018  | 1.77              | ESI+ |
| 39 | dibutyl phthalate               | Dib          | 12.68    | 278.1518         | 1.91 | 0.028  | 0.17              | ESI+ |
| 40 | absinthin                       | Abs          | 7.40     | 496.2825         | 1.89 | 0.030  | 0.11              | ESI+ |
| 41 | cis, cis-3, 6-dodecadienoy-CoA  | Cis          | 13.58    | 945.2510         | 2.44 | 0.041  | –0.27             | ESI+ |

ESI+, electrospray ionization (ESI) operating in positive ionization mode; ESI–, operating in negative ionization mode; RT, retention time; VIP, variable importance in the projection statistics.
Four classes of phospholipids were detected in extracts from C. lanceolata leaves (Table 3), which included diglyceride (DG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG). Based on ESI+ detection, the concentrations of most of the phospholipid compounds significantly increased under the warming treatment. However, warming significantly decreased the concentrations of carbohydrates such as melibiitol and physcion 8-gentiobioside. Two kinds of amino acids (L-tryptophan and hydroxylysine) were identified to have significantly lower concentrations under the warming treatment than under the control treatment. The concentrations of organic acids, including tetradecanedioic acid and pisumic acid, drastically decreased under the warming treatment. Compared to those under the control treatment, the concentrations of phenolic compounds (homovanillic acid), terpene compounds (isogermafurene and barringtogenol C), and flavonoids (flavone) were significantly lower in the warming treatment. Except for acetylintermedine, most N containing molecules, including indicine, anabasine, and 3-acetyl-2,7-naphthyridine, drastically decreased in the warming treatment.

The concentrations of acylamide compounds significantly differed between treatments, whereby the warming treatment resulted in significant higher concentrations of adrenoyl ethanolamide and palmitic amide. Xanthophyll, an important pigment, was obviously higher in concentration under the
warming treatment than under the control treatment. Vitamin D3 also exhibited a similar response pattern, with a higher concentration under the warming treatment than under the control treatment (Table 3).

4. Discussion

4.1. Differential Nutrient Status in Response to Warming

In the present study, the N and P concentrations in leaves and roots of *C. lanceolata* declined under warming conditions (Table 2), although warming increased soil mineralization at the same experimental site [46,47]. Leaf N and P concentrations are often enhanced by the increase in soil N and P availability associated with a higher mineralization rate [8,48]. Similar decreases in the N concentration of leaves in response to warming were recorded by Suseela et al. [23]. Warming can influence the nutrient components of plants via different mechanisms: (1) Labile P can be quickly precipitated by Fe and Al minerals, especially in the sub-tropical regions with highly weathered soil, which transforms the active P forms into solid P forms that are not easily absorbed and utilized by plants [49]. (2) The promotion of biomass production might lead to a decrease in nutrient concentration, i.e., a dilution effect [50,51]. In the same experimental site, Xiong et al. [52] observed a positive effect of warming on annual fine root production; the warming treatment significantly increased the maximum net photosynthetic rate of *C. lanceolata* [53]. Thus, the decreases of N and P concentrations (especially in roots) were likely related to growth, and thus diluted nutrient concentrations in the leaves and roots under the warming condition.

Since the concentrations of C and N of *C. lanceolata* showed different responses to warming, the ratio of C:N increased, especially in the roots (Table 2), indicating that warming altered the chemical composition of *C. lanceolata*. Tharayil et al. [19] and Zhang et al. [54] also found that warming altered plant structural compositions and chemical components. Considering that the plant stoichiometric characteristic is an important regulatory factor that drives microbial community composition and activity [55], an increase in the C:N ratio of *C. lanceolata* under the warming condition may alter the soil nutrient cycling process and its ecological feedback.

4.2. Warming Effects on Reactive Oxygen Species and Antioxidant Enzymes

At the same site, Zhang et al. [53] found that warming treatment significantly increased the maximum net photosynthetic rate of *C. lanceolata*. Under such conditions, plants would be able to store a great abundance of photosynthetic products for growth. However, warming did not increase tree height dramatically (Table 2). Antioxidant enzymes, as electron donors, have the ability to change the oxidation dynamics and provide strong antioxidant effects for plants [28,56]. In this experiment, warming altered antioxidant enzyme activities but not significantly (Figure 2). Similar results were also observed by Zhao and Liu [30].

In our previous work [53], we sampled in a different season (in spring) and also measured the activities of antioxidant enzymes and the concentrations of reactive oxygen species. It is remarkable that the activities of antioxidant enzymes in summer (sampling time of the present study) were significantly lower than those in spring, regardless of treatments, whereas the concentrations of reactive oxygen species were significantly higher in summer than in spring. The different results demonstrated that seasonal changes significantly affect the antioxidant system [28]. This could be largely related to the significant changes in temperature and precipitation [34]. It has been reported that plants exposed to moisture stress increase antioxidant activities in response to oxidative stress [28]. Taking this one step further, the oxidative stress caused by lower rainfall and higher temperature in summer may exceed the antioxidant capacity of plants. Collectively, these findings suggest that different mechanisms may exist to control reactive oxygen species at such high concentrations in hot summers.
4.3. Variations of Warming-Induced Metabolite Response

Consistent with most studies, warming dramatically alters the metabolites in C. lanceolata [12,23,24]. Carbohydrates are the main source of energy for all living organisms. Moreover, some carbohydrates have a specific physiological activity [57,58]. However, we observed decreases in carbohydrates in C. lanceolata leaves under the warming treatment (Table 3). The same pattern was observed in the plant C concentrations (Table 2). Warming decreases soil moisture, as confirmed in many studies [15,59]. Our results suggested that moisture stress under warming may drive C deficiency and metabolic restriction (i.e., amino acids and carbohydrates) in plants [16].

According to Bartlett et al. [18], the investment in energy and C storage, and the resulting osmotic regulation, are key processes by which plants adapt to moisture stress [14]. However, as indicated by the low concentration of proline measured in the present study (Figure 3a), proline does not seem to be involved in cytosolic osmotic regulation. Our results concur with previous studies in wheat by Öncel et al. [60]. In addition, soluble proteins are also important macromolecular compounds that regulate osmotic pressure [61], their increase and accumulation can improve the water-retention capacity and defense of the cells [62]. Consistent with other studies [61,62], higher concentrations of soluble protein were observed under the warming treatments in the present study (Figure 3b), which indicates a higher allocation of N for the regulation of cell osmotic pressure to improve heat stress resistance in C. lanceolata.

Intracellular homeostasis is maintained not only through transport, but also by biosynthesis, utilization, and catabolism of metabolites [23]. In particular, phospholipids function as signaling compounds, maintaining the integrity of proteins and enzymes and scavenging hydroxyl radicals, thus allowing plants to tolerate abiotic stress [23,30]. The increase in phospholipid concentration observed in the present study was beneficial for the maintenance of the integrity of proteins when exposed to the warming, which resulted in reductions in the concentrations of amino acids derived from protein degradation (Table 3). These also led to a downregulation of metabolism at higher soil temperatures; this is related to a decrease in the concentration of amino acids [14].

A previous study suggested that warming enhanced the concentrations of organic acids of Quercus rubra (L.) such as fumaric acid [23]. However, our results showed that warming significantly decreased the concentrations of organic acids (tetradecanedioic acid and pisumic acid). Generally, organic acids are involved in anionic equilibrium. Dong et al. [63] found that the concentrations of organic acids in tomato seedlings decreased gradually as the ratio of ammonium N:nitrate N increased. In the present study, the ratio of ammonium N:nitrate N ratio in soil (1.71 and 0.76 for control and warming treatments, respectively; unpublished) did not explain the decrease in organic acids under warming treatments. In addition, organic acids are associated with the citric acid cycle. Plants can upregulate their citric acid cycle or shikimate pathway under moisture stress, which can lead to increases in tannin [64] and flavonoid [28,65] production. Flavonoids are a series of compounds with two benzene rings and a phenol hydroxyl connected by three central C atoms [58]. In response to the warming treatment (and compared to the control treatment), the green leaves of C. lanceolata had lower concentrations of flavonoids (Table 3).

Recently, the important role of phenolic compounds in the decomposition of litter via regulation of soil microbial activity was reviewed [13,61]. A study suggested that soil microbial respiration was significantly inhibited under the warming treatment because the relative abundance of phenolic compounds interferes with microbial metabolism [24]. Johnson and Hartley [12] demonstrated that warming caused a decline in the concentrations of phenolic compounds in Chloris gayana (Kunth). These results are in accordance with the results reported in the present study. Savoi et al. [66] have suggested that the flavonoid pathway responds to water deficiency at transcriptional and metabolite levels, thereby determining the change in phenolic concentrations. Considering that flavonoids and phenolic compounds provide photoprotection [67], the decreased flavonoids and phenolic compounds synthesis of C. lanceolata could contribute to low tolerance to warming and potentially increase the susceptibility of C. lanceolata to biotic stresses such as herbivory [68]. Forest pest control should be considered in
forests in response to warming. This phenomenon could potentially result from a greater biosynthesis of macromolecule proteins and reflects a trade-off with regard to plant defenses. In addition, there was a sharp decrease in some of the N-containing small molecules under warming conditions, such as indicine, anabasine, and 3-acetyl-2,7-naphthyridine (Table 3). This result indicates general reprogramming of the N metabolism of leaves in response to warming. *C. lanceolata* may change its energy utilization strategy and invest fewer resources in producing small molecule compounds (i.e., carbohydrates, amino acids, organic acids, phenolic compounds, and flavonoids), whereas more resources may be used to produce macromolecular compounds (i.e., soluble protein) for protecting the plant against heat stress. Thus, a lower concentration of aromatic-C as a result of climatic warming could have severe consequences for nutrient cycling and soil C sequestration in the future.

5. Conclusions

In the present study, warming led to changes in the nutrient levels of *C. lanceolata*. Leaf and root N and P concentrations did not increase in response to warming, probably owing to a dilution effect associated with biomass production. The ratio of C:N increased in response to warming, especially in the roots, which indicated that warming altered the chemical compositions of *C. lanceolata*. In addition, warming significantly altered the metabolite levels as well. Higher concentrations of soluble proteins and phospholipids in *C. lanceolata* under warming may prevent damage in the leaves, exemplified by no accumulation of \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), and MDA. Moisture stress under warming may drive C deficiency and metabolic restriction (i.e., amino acids and carbohydrates) in plants. Considering that most metabolites provide protection, forest pest control should be considered in future forest management. *C. lanceolata* changed its energy utilization strategy and invested fewer resources in producing small molecule compounds (i.e., carbohydrates, amino acids, organic acids, phenolic compounds, and flavonoids). Thus, a lower concentration of aromatic-C as a result of climatic warming could have severe consequences for nutrient cycling and soil C sequestration in the future. Warming in sub-tropical forests may have ultimate consequences for plant fitness and biogeochemical processes in this ecosystem.

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