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Ecophysiological responses of *Cunninghamia lanceolata* to nongrowing-season warming, nitrogen deposition, and their combination

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Abstract

Warming winter and atmospheric nitrogen (N) deposition are expected to have effects on net primary production (NPP) of Chinese fir (*Cunninghamia lanceolata*) plantation and implications for plantation carbon sequestration. The effects of nongrowing-season warming on plant morphological and physiological traits were investigated in a greenhouse experiment with two-year-old *C. lanceolata* seedlings. Elevated temperature (ET) during the nongrowing season significantly increased the net photosynthetic characteristics. The strongest effects occurred during warming period from 1 December 2014 to 1 February 2015 (W1). Moreover, the carbohydrate concentration was elevated due to the warming during W1, but it declined during four months of the warming (from 1 December 2014 to 1 April 2015, W2). The seedlings kept under N deposition (CN) showed a positive effect in all the above-mentioned parameters except $\delta^{13}C$. Significant interactions between ET and N deposition were observed in most parameters tested. At the end of the experiment (W2), the seedlings exposed to a combined ET and N deposition treatment exhibited the highest carbon contents. Our results showed that N deposition might ameliorate the negative effects of the winter warming on the carbon content.

Additional key words: chlorophyll fluorescence; fructose; gas exchange; malondialdehyde; reactive oxygen species; starch.

Introduction

Global climate models predict a strong potential for warmer winters (Easterling 1997). Substantial evidence suggests that warming has large effects on plant C-assimilation rates (Saxe *et al.* 2001) and growth (Hudson and Henry 2009). However, most studies are conducted during the growing season. Thus, little is known about the effect of nongrowing-season warming. Nitrogen (N) deposition, which is caused by human activities, such as fossil fuel combustion and forest disturbance, also plays an important role in the forest ecosystem (Matson *et al.* 2002, Gruber and Galloway 2008). N deposition may reach 35.6–38.4 kg(N) ha^{-1} per year in high-N deposition regions of China (Zhou and Yan 2001), which exceeds the N demand of forests. An increase in atmospheric N input usually stimulates plant growth (Nakaji *et al.* 2001). However, it has been reported that the high N deposition reduced photosynthesis (Tripodi and Sievering 2010) and an uptake of nutrients (Elser *et al.* 2009). Although many studies have examined the effects of individual environmental factors on forests, few studies have examined whether N deposition influences plant ecophysiology under nongrowing-season warming. Winter warming has been demonstrated to increase plant biomass, which may increase plant N demand (Andresen and Michelsen 2005). Yet, we do not know whether the increased N demand might be compensated for by the N deposition.

Chinese fir [*Cunninghamia lanceolata* (Lamb.) Hook.], a typical subtropical coniferous tree species, is one of

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Abbreviations: AT – ambient temperature; C – control (ambient temperature and 0 g N deposition); Chl – chlorophyll; C_i – intercellular CO_2 concentration; CN – control + nitrogen deposition (+1 g N); DM – dry mass; ET – elevated temperature; ETN – nitrogen deposition (+1 g N) + elevated temperature (+2°C); FM – fresh mass; F_v/F_m – maximal quantum yield of PSII photochemistry; g_s – stomatal conductance; MDA – malondialdehyde; P_N – net photosynthetic rate; R_D – dark respiration; ROS – reactive oxygen species; W1 – two months of warming treatment; W2 – four months of warming treatment.
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the most important timber species in southern China due to its fast growth, high yield, and excellent wood quality (Wu 1984). *C. lanceolata* plantations are also very important for studying the carbon budget of terrestrial ecosystems, since the C uptake of *C. lanceolata* plantation was $3.28 \times 10^3 – 6.26 \times 10^3$ kg ha^{-1} per year (Yao *et al*. 2015), which therefore has a fundamental role in mitigating the build-up of atmospheric CO_2. Since higher winter temperatures and N deposition have recently been observed simultaneously in the *C. lanceolata* ecosystems, plant responses to temperature and N deposition can be expected, which might have implications for a plantation carbon sequestration. Nevertheless, few studies have investigated the ecological consequences of elevated temperatures (ET) in the winter and N deposition (EN) in these ecosystems. A key to understanding of the role of plantation in carbon sequestration is determining how plants would respond to future climate. In order to understand how ET modifies traits in woody plants, we investigated the impact of ET (+2°C in the air) during the nongrowing season (1 December 2014 to 1 April 2015) on carbohydrate metabolism in *C. lanceolata* seedlings. We hypothesized that a large set of parallel changes in physiological responses could occur, when plants were exposed to the winter warming and N deposition, and these changes could eventually affect plant growth. The specific objectives were as follows: (*1*) to monitor the physiological changes of *C. lanceolata* under nongrowing-season warming, N deposition, and their combination, and (*2*) to test whether the effects of experimental warming varied with the N contents.

Materials and methods

Plant material and experimental design: Two-year-old *Cunninghamia lanceolata* (Lamb.) Hook. seedlings were collected from the Hongya National Forest Farm (29°38'N, 102°58'E), which is one of the major natural distribution regions of *C. lanceolata* in southern China (Dong *et al*. 2015). Healthy seedlings ($n = 80$) with a similar crown size and uniform height (~ 40 cm) were chosen and planted in 10-L plastic pots (one seedling per pot). A soil was collected from a depth of 20–40 cm at the plot of a 30-year-old *C. lanceolata* forest plantation. Four climate-controlled chambers (south facing) were employed for warm treatments about 2°C above ambient air temperature (Naudts *et al.* 2011, 2013). The interior surface area was 2 × 2 m, the height at the north side was 2 m, and the south side of 1.5 m. Two small electronic fans were installed at the height of 1.5 m on east and west sides in order to keep the CO_2 concentrations in the chambers the same as outside. The top of the chambers consisted of a colourless polycarbonate plate (4 mm thick), whereas the sides were made of polyethylene film (200 μm thick), both UV transparent. Fig. 1S (*supplement available online*) shows the elevated air temperature and the ambient air temperature from December 2014 to March 2015.

The experimental layout was completely randomized with eight factorial combinations of two temperature regimes [ambient (AT) and elevated temperature (ET)], two N concentrations [0 g per pot (C) and 1 g per pot (EN)] (Ti *et al.* 2010, Xie *et al.* 2015), and two different durations of the treatment [two months (W1) and four months of warming (W2)] were employed. N deposition was supplied by adding aqueous solution of NH_4NO_3 five times as small simulated rain events (2 mm), with control (C) and ET pots receiving equal quantities of deionized water. The 2-month warming (W1), started on 1 December 2014 and harvested on 1 February 2015. The 4-month warming (W2), started on 1 December 2014 and plants were harvested on 1 April 2015. On 1 December 2014, 40 seedlings under ET and ETN treatments were kept in a semi-controlled greenhouse where four climate-controlled chambers were employed to elevate temperature, while 40 plants (C and CN) were kept under AT. All treatments are summarized in the following table, each treatment had 10 replicates.

Experiment duration	Abbreviation	Treatment
W1: 2-month warming (1 December 2014 – 1 February 2015)	C	control
	ET	elevated temperature (+2°C)
	CN	control + nitrogen deposition (+1 g N)
	ETN	nitrogen deposition (+1 g N) + elevated temperature (+2°C)
W2: 4-month warming (1 December 2014 – 1 April 2015)	C	control
	ET	elevated temperature (+2°C)
	CN	control + nitrogen deposition (+1 g N)
	ETN	nitrogen deposition (+1 g N) + elevated temperature (+2°C)

The experiments were carried out in a semi-controlled greenhouse (Chen *et al*. 2015) at the Chengdu Institute of Biology (CIB), the Chinese Academy of Sciences (CAS). The seedlings were watered in the evening every two days.

Gas-exchange determination and chlorophyll (Chl) fluorescence measurements: Before harvest in both W1 and W2, eight seedlings were selected randomly from each treatment for gas-exchange measurements. The net photosynthetic rate (P_N), dark respiration (R_D), stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) were measured using the *LI-COR 6400* portable photosynthesis measuring system (*LI-COR*, Lincoln, NE, USA). Gas-exchange measurements were taken between 08:00 and 11:30 h, and CO_2 gas cylinders (*LI-COR*, Lincoln, NE, USA) were used to provide a constant and stable CO_2 concentration. Prior to measurement, samples were illuminated with saturating irradiance (1,000 μmol m^{-2} s^{-1} PPFD) provided by the *LI-COR* LED light source for 10 min in order to achieve full photosynthetic induction. A standard *LI-COR* leaf chamber (2 × 3 cm^2) was used. P_N, g_s, and C_i were measured under the following conditions: leaf temperature of 25°C, leaf air vapour pressure deficit of 1.5 ± 0.5 kPa, relative humidity of 50%, PPFD of 1,500 μmol m^{-2} s^{-1}, and CO_2 concentration of 400 ± 5 μmol mol^{-1}. Because the measured leaves did not fill the chamber, the actual leaf areas were used to normalize the data. The leaves in the chamber were bordered and photographed (600 dpi), and leaf areas were calculated using a scanner (*Cannon Scanner 5600F*, Chengdu, China) and imaging software (*Image J*, *National Institutes of Health*, MD, USA). Measurements of R_D were obtained through gas-exchange measurements after covering the leaf chamber for 15 min.

The Chl fluorescence measurements were performed on the intact leaves with a *PAM-2100* portable fluorometer (*Walz*, Effeltrich, Germany) following the procedures of Schreiber *et al*. (1986). A leaf was dark-adapted for at least 30 min prior to the measurements in order to reoxidize completely PSII electron transporters. The minimal fluorescence (F_0) was determined with a weak light pulse [< 1 μmol(photon) m^{-2} s^{-1}] at a low frequency (1 Hz). The maximal fluorescence yield of a dark-adapted leaf (F_m) was measured during an 800-ms exposure to irradiation of approximately 2,600 μmol(photon) m^{-2} s^{-1}. Maximal PSII quantum yield (F_v/F_m) was estimated from the variable to maximum fluorescence ratio [$F_v/F_m = (F_m - F_0)/F_m$].

Pigment determinations: The Chl content was determined using a spectrophotometer (*UV-330*, *Unicam*, Cambridge, UK). Fresh leaves were extracted in 80% (v/v) chilled acetone and quantified according to Lichtenthaler (1987) at wavelengths of 470, 646, and 663 nm. Chl *a* and Chl *b* were calculated from equations derived by Porra *et al*. (1989).

Carbon (C) and nitrogen (N) determinations: Dried samples were ground to fine powder and passed through a mesh (pore diameter *ca*. 275 μm). The concentrations of nitrogen (N) and carbon (C) were determined by the semimicro Kjeldahl method (Mitchell 1998) and the rapid dichromate oxidation technique (Nelson and Sommers 1982), respectively. The total C to N ratio (C/N) was calculated as an estimate of the long-term nitrogen-use efficiency (Livingston *et al*. 1999).

Nonstructural carbohydrate determination: Total soluble sugars, fructose, and sucrose were extracted from dried leaves, stems, and roots in 80% (v/v) ethanol. The total soluble sugars were detected colorimetrically (*Multiskan GO 1510, Thermo Fisher Scientific*, Finland) at 625 nm following the anthrone-sulphuric acid method (Yemm and Willis 1954). Fructose and sucrose were detected colorimetrically (*Multiskan GO 1510, Thermo Fisher Scientific*, Finland) at 480 nm following the modified resorcinol method (Murata 1968). Then, the starch content was determined from the pellet of plant material that remained after the removal of ethanol (Zhao *et al.* 2011). The solutions were filtered through *Whatman GF/C* filters and diluted in 10-ml volumetric flasks. The concentrations of starch as glucose equivalents were determined colorimetrically (*Multiskan GO 1510, Thermo Fisher Scientific*, Finland). The absorption of an enzyme blank was subtracted from each sample's absorbance prior to the calculation of the sugar content.

Nitrate reductase (NR) activity determination: Five replications of 0.2 g fresh leaves were randomly selected from each treatment for leaf nitrate reductase (NR) activity determination. The NR (EC 1.6.6.1) activity was measured following the method of Li *et al.* (2014), and the absorbance of the supernatants was determined at 540 nm using a spectrophotometer (*UV-330*, *Unicam*, Cambridge, UK). The NR activity was expressed as the amount of generated nitrite in [μmol(NO_2) g^{-1}(FM) h^{-1}].

The malondialdehyde (MDA) content was measured by the method as described by Li *et al*. (2011). Fresh leaves (0.3 g) from each sample were homogenized in 4 ml of 10% trichloroacetic acid (TCA) and centrifuged at 12,000 × *g* for 10 min, after which 1 ml of the supernatant was mixed with 1 ml of 0.6% thiobarbituric acid, heated at 95°C for 30 min, and then quickly cooled down on ice, after being measured at 532 nm using a spectrometer (*UV-330*, *Unicam*, Cambridge, UK) and corrected for nonspecific turbidity by subtracting the absorbance at 600 and 450 nm. The concentration of MDA in extracts was calculated from the difference in absorbance at 532, 600, and 450 nm, where MDA is calculated in [μmol ml^{-1}] = $6.45(A_{532} - A_{600}) - 0.56A_{450}$. Then MDA content was expressed as [μmol g^{-1}(FM)].

^{15}N and ^{13}C analysis: Leaf samples from harvested plants were used for the carbon isotope analysis, and the carbon and nitrogen isotopes were measured as following: dried leaves were ground, and $^{13}C/^{12}C$ ratios were determined with an isotope ratio mass spectrometer (*Thermo Fisher Scientific*, *Inc.*, USA). The carbon isotope composition was expressed as $\delta^{13}C$ values. The overall precision of the

δ-values was better than 0.1‰, as determined by repetitive samples. The $\delta^{13}C$ values are reported relative to the international standard, Pee Dee Belemnite (PDB). The entire analysis was performed in the Stable Isotope Laboratory for Ecological and Environmental Research (SILEER), CAS. In addition, in the ^{15}N-trace experiment, labelled $^{15}NH_4NO_3$ and $NH_4{}^{15}NO_3$ solutions were injected into the soil at 5-cm depth around the rhizosphere of the plants (20 mg per plant) in all treatments. Half of all the plants were randomly chosen for the ^{15}N-trace experiment in W1, and the remainder of the seedlings were injected with ^{15}N solution in W2. Seventy-two hours after the application of the ^{15}N solution, plants were harvested, dried in an oven at 70°C for 48 h and ground for the ^{15}N isotope composition analysis. The $^{15}N/^{14}N$ ratios were determined with an isotope ratio mass spectrometer (*Thermo Fisher Scientific*, *Inc.*, USA). The atomic standard of ^{15}N (N_2: 0.3663 atom %) was used as the reference for the calculation of excess ^{15}N. The standard deviation of the repeated measurements of a laboratory standard was 0.1‰ for $\delta^{15}N$.

Transmission electron microscopy (TEM) observations: Small intact leaf sections (1–3 mm in length) were cut by a sharp scissor in order to obtain a front part of current-year leaves in each treatment. The selected leaf sections were fixed in 3% glutaraldehyde (v/v) in 0.2 M PBS (sodium phosphate buffer, pH 7.2) for 6–8 h, and post-fixed in 1% osmium tetraoxide for 1 h, and washed in 0.2 M PBS (pH 7.2) for 1–2 h. Dehydration was carried out in a graded ethanol series (50, 60, 70, 80, 90, 95, and 100%) followed by acetone, after which the samples were infiltrated and embedded in Epon-araldite resin. Ultrathin sections (80 nm) were sliced (*Ultracut E ultramicrotome, Reichert-Jung*, Austria), stained with uranyl acetate and lead citrate, and mounted on copper grids for viewing in the *H-600IV* TEM (*Hitachi*, Tokyo, Japan) at an accelerating voltage of 60.0 kV.

Statistical analysis: Prior analysis of variance (*ANOVA*), data were checked for normality and the homogeneity of variances and log transformed in order to correct deviation from these assumptions when needed. One-way and three-way *ANOVAs* were performed using the *Statistical Package for the Social Sciences* (*SPSS*, Chicago, IL, USA) *version 19.0*. Three-way *ANOVA* was used to test the overall effects of temperature, nitrogen, and warming time. One-way *ANOVA* was used to evaluate the differences between treatments. The independent sample *t*-test was employed to test for differences between the two different warming durations (W1 and W2). *Post hoc* comparisons were performed using the *Duncan*'s multiple tests at a significance level of $P<0.05$.

Results

Gas exchange and pigments: Compared with the control treatment, the P_N, R_D, and g_s in both W1 and W2 increased significantly by the ET, CN, and ETN treatments (Table 1); the R_D/P_N ratio under the ET treatment was higher in both

Table 1. Net photosynthetic rate (P_N), dark respiration (R_D), R_D/P_N ratio, stomatal conductance (g_s), and intercellular CO_2 concentration (C_i) of *Cunninghamia lanceolata* as affected by nitrogen and elevated temperature under 2-month (W1) and 4-month (W2) warming treatment. Each value is the mean ± SE ($n = 5$). F_{ET} – elevated temperature effect; F_N – nitrogen effect; $F_{ET\times N}$ – elevated temperature × nitrogen effect. *Different lowercase letters* indicate significant differences between each treatment in W1 or W2 according to *Duncan*'s tests ($P<0.05$). The asterisks demonstrate statistically significant differences according to independent samples *t*-test between two different warming durations (W1 and W2) within each treatment ($^{*}0.01<P<0.05$; $^{**}0.001<P\leq0.01$). C – control; ET – elevated temperature; CN – control + nitrogen; ETN – elevated temperature + nitrogen.

Treatments	P_N [μmol(CO_2) m^{-2} s^{-1}]	R_D [μmol(CO_2) m^{-2} s^{-1}]	R_D/P_N	g_s [mol(H_2O) m^{-2} s^{-1}]	C_i [μmol(CO_2) mol^{-1}]
W1					
C	2.43 ± 0.01^{d}	0.65 ± 0.11^{d}	0.27 ± 0.05^{c}	0.10 ± 0.01^{c}	157.98 ± 2.09^{b}
ET	5.17 ± 0.03^{a}	2.38 ± 0.03^{a}	0.46 ± 0.01^{a}	0.15 ± 0.01^{a}	166.06 ± 5.29^{b}
CN	3.50 ± 0.05^{c}	1.09 ± 0.04^{c}	0.31 ± 0.01^{bc}	0.13 ± 0.00^{b}	153.61 ± 2.59^{b}
ETN	4.32 ± 0.02^{b}	1.50 ± 0.03^{b}	0.35 ± 0.01^{b}	0.14 ± 0.01^{b}	185.10 ± 3.31^{a}
$P > F_{ET}$	0.000	0.000	0.001	0.000	0.001
$P > F_N$	0.003	0.004	0.184	0.067	0.071
$P > F_{ET\times N}$	0.000	0.000	0.006	0.001	0.011
W2					
C	3.16 ± 0.02^{d}	$1.28 \pm 0.05^{d*}$	$0.40 \pm 0.02^{bc**}$	0.12 ± 0.00^{c}	164.51 ± 3.81^{ab}
ET	5.10 ± 0.06^{b}	$2.71 \pm 0.07^{a*}$	$0.53 \pm 0.01^{a**}$	0.15 ± 0.01^{ab}	172.75 ± 2.86^{ab}
CN	4.04 ± 0.03^{c}	$1.44 \pm 0.06^{c*}$	$0.37 \pm 0.01^{c**}$	0.13 ± 0.00^{b}	160.13 ± 4.73^{b}
ETN	5.73 ± 0.05^{a}	$2.37 \pm 0.04^{b*}$	$0.41 \pm 0.01^{b**}$	0.15 ± 0.00^{a}	178.75 ± 3.26^{a}
$P > F_{ET}$	0.000	0.000	0.000	0.000	0.007
$P > F_N$	0.000	0.081	0.000	0.008	0.833
$P > F_{ET\times N}$	0.014	0.000	0.003	0.172	0.202

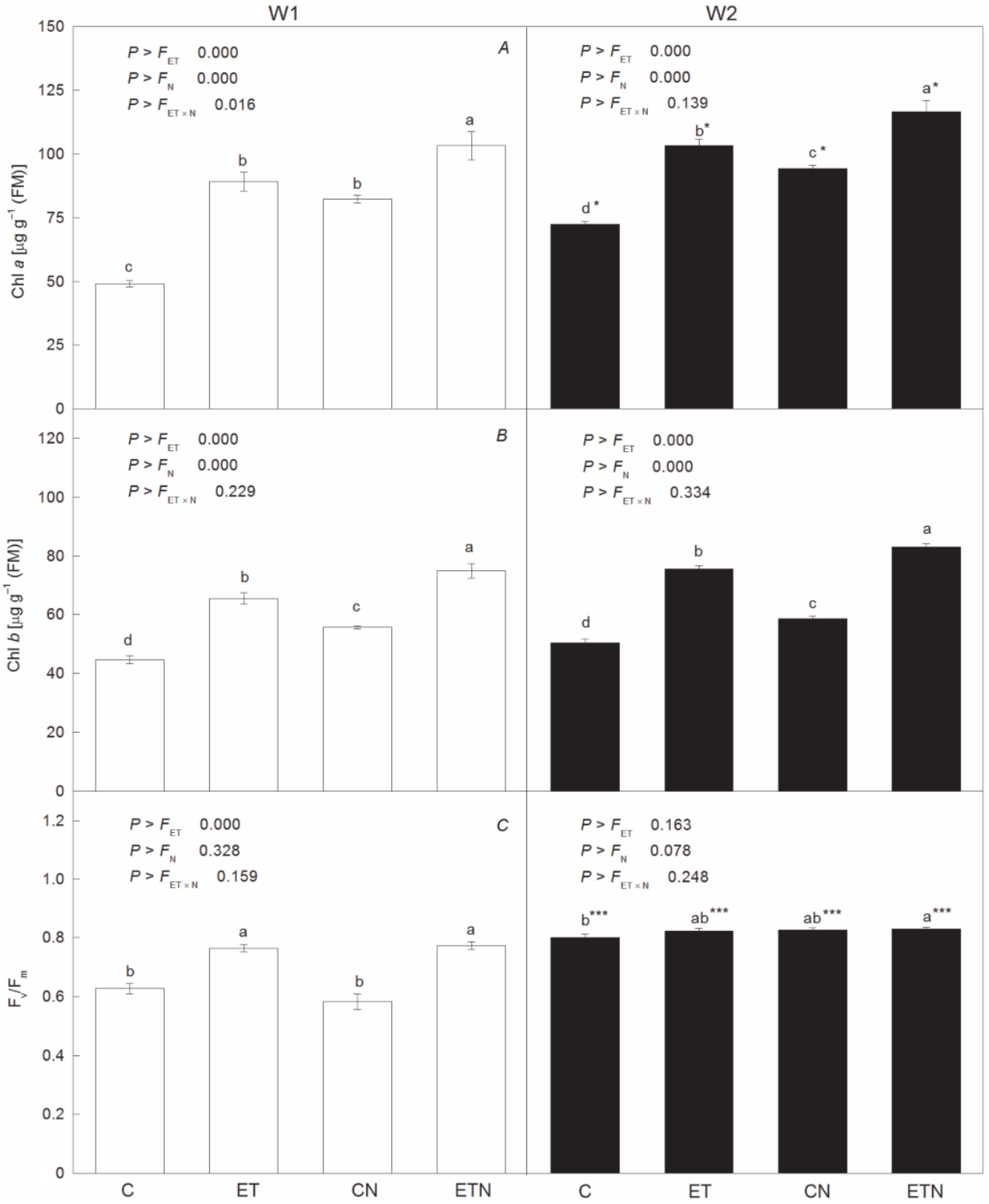

Fig. 1. Chllorophyll (Chl) *a*, Chl *b*, and F_v/F_m (maximal quantum yield of PSII photochemistry) of *Cunninghamia lanceolata* as affected by nitrogen and elevated temperature under 2-month (W1) and 4-month (W2) warming treatment. F_{ET} – elevated temperature effect; F_N – nitrogen effect; $F_{ET\times N}$ – elevated temperature × nitrogen effect. *Different lowercase letters* indicate significant differences between each treatment in W1 or W2 according to *Duncan*'s tests ($P<0.05$). The asterisks demonstrate statistically significant differences according to independent samples *t*-test between two different warming durations (W1 and W2) within each treatment ($^{*}0.01<P<0.05$; $^{***}P\leq0.001$). C – control; ET – elevated temperature; CN – control + nitrogen; ETN – elevated temperature + nitrogen. Mean ± SE, $n = 5$.

W1 and W2 than that under the C, CN, and ETN treatments (Table 1). In addition, there were no significant differences in P_N, g_s, and C_i between W1 and W2.

Compared with the C treatment, the Chl *a* and Chl *b* contents significantly increased in both W1 and W2 by the ET, CN, and ETN treatments (Fig. 1*A*,*B*). The ET and ETN treatments significantly increased the F_v/F_m in both W1 and W2, except F_v/F_m under the ET treatment in W2. In addition, there were no significant differences in F_v/F_m between the C and CN treatments in either W1 or W2 (Fig. 1*C*).

Nitrate reductase (NR) activity: Compared with the C treatment, the ET treatment significantly decreased the NR activities in both W1 and W2, and the CN treatment increased the NR activity (Fig. 2). In addition, there were significant differences in NR activities between the CN and ETN treatments in both W1 and W2. However, there were no significant differences in NR activities between the ET and ETN treatments in both W1 and W2, except the NR activity in W2 (Fig. 2).

MDA content: Compared with the C treatment, the MDA content significantly decreased by the ET, CN, and ETN treatments in both W1 and W2, except CN treatment in W1. In addition, the MDA content significantly decreased in W2 compared with that in W1 (Fig. 3).

Carbon (C) and nitrogen (N) content: Compared with the C treatment, the ET treatment significantly increased the C contents in the roots and the C/N ratio in the roots and stems and significantly decreased the C contents in the leaves and the N contents in the roots and stems in W1 (Table 2). Furthermore, compared with the C treatment, the ET treatment decreased the C contents in the roots and stems and the C/N ratio in the stems and leaves in W2. Moreover, compared with the ET treatment, the ETN treatment significantly increased the C contents in roots, stems, and leaves in W2 (Table 2).

Nonstructural carbohydrates: Compared with the C treatment, the fructose concentration significantly decreased in the leaves and increased in the roots in W1 under the ET treatment, whereas the ET treatment significantly increased the fructose concentration in the leaves and significantly decreased that in roots when compared with the C treatment in W2 (Fig. 4*A*). Furthermore, the fructose concentrations were significantly higher in the leaves and significantly lower in the roots under both the CN and ETN treatments than under the C treatment in W2 (Fig. 4*A*).

Compared with the C treatment, the ET, CN, and ETN treatments all significantly increased the starch concentrations in each organ in W1, except for the stems under the CN treatment (Fig. 4*B*). Furthermore, when comparing the two warming periods, W1 and W2, the starch concentrations in W2 were significantly lower than those in W1 (Fig. 4*B*). Compared with the C treatment, the ET treatment significantly increased the sucrose concentrations in each organ in W1, but the sucrose concentrations were significantly lower in both the stems and roots under the ET treatment than those under the C treatment in W2. Moreover, when compared with the ET treatment, the sucrose concentrations significantly increased in the leaves and stems under the ETN treatment in W2 (Fig. 4*C*).

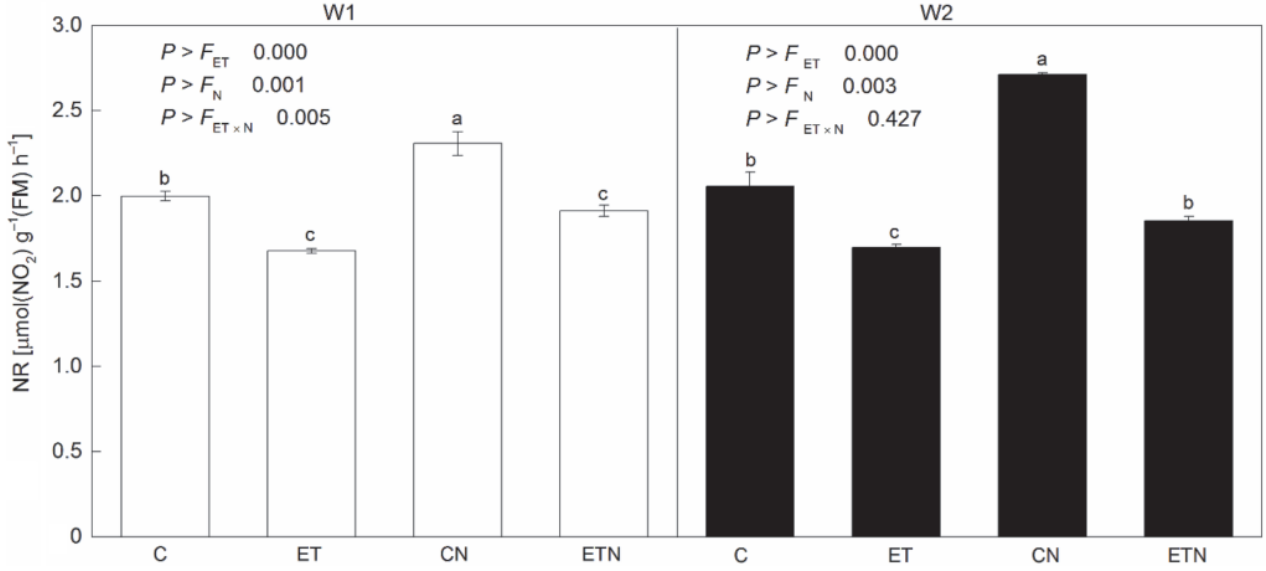

Fig. 2. The nitrate reductase (NR) activity in leaves of *Cunninghamia lanceolata* as affected by nitrogen and elevated temperature under 2-month (W1) and 4-month (W2) warming treatment. F_{ET} – elevated temperature effect; F_N – nitrogen effect; $F_{ET\times N}$ – elevated temperature × nitrogen effect. *Different lowercase letters* indicate significant differences between each treatment in W1 or W2 according to *Duncan*'s tests (P<0.05). C – control; ET – elevated temperature; C – control + nitrogen; ETN – elevated temperature + nitrogen. Mean ± SE, n = 5.

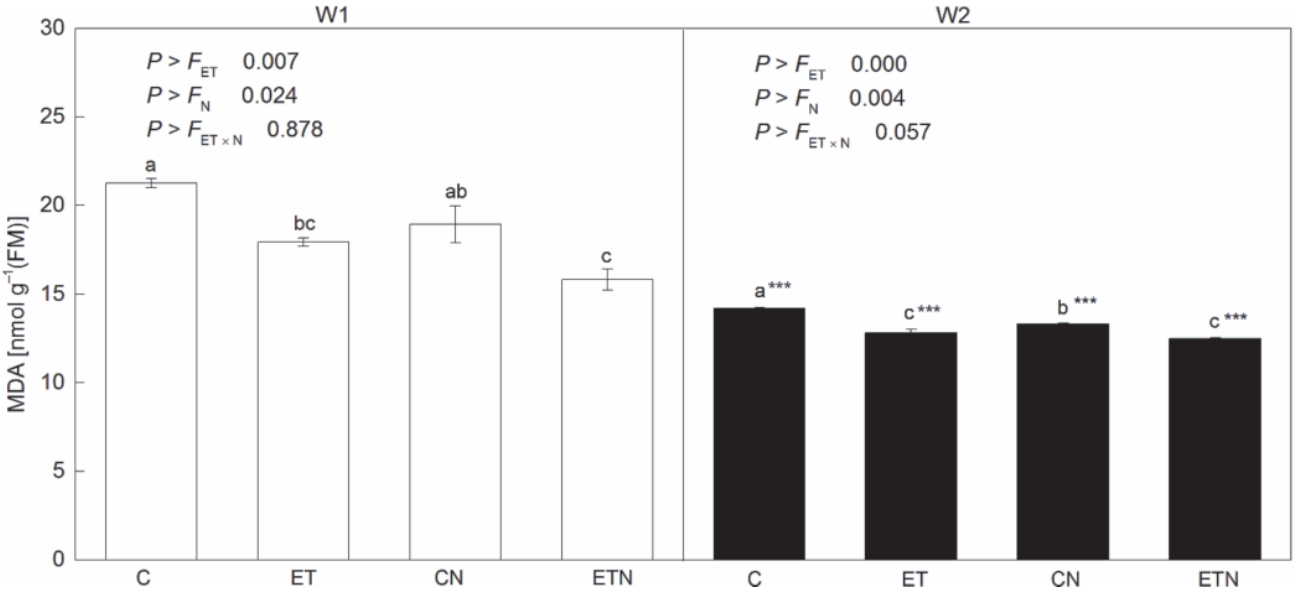

Fig. 3. The malondialdehyde (MDA) content in leaves of *Cunninghamia lanceolata* as affected by nitrogen and elevated temperature under 2-month (W1) and 4-month (W2) warming treatment. F_{ET} – elevated temperature effect; F_N – nitrogen effect; $F_{ET \times N}$ – elevated temperature × nitrogen effect. *Different lowercase letters* indicate significant differences between each treatment in W1 or W2 according to *Duncan*'s tests ($P<0.05$). The asterisks demonstrate statistically significant differences according to independent samples *t*-test between two different warming durations (W1 and W2) within each treatment ($^{***}P \leq 0.001$). C – control; ET – elevated temperature; CN – control + nitrogen; ETN – elevated temperature + nitrogen. Mean ± SE, $n = 5$.

Compared with the C treatment, the total soluble sugar concentrations significantly increased in the leaves but significantly decreased in the roots and presented no obvious differences in the stems under the ET, CN, and ETN treatments in W2. The total soluble sugar concentrations were significantly lower in all organs in W2 compared with those in W1 (Fig. 4*D*).

$\delta^{13}C$ and $\delta^{15}N$: There were no significant differences in the foliar $\delta^{13}C$ between the C, ET, CN, and ETN treatments in W1, whereas in W2, when compared with the C treatment, the ET and ETN treatments significantly decreased the foliar $\delta^{13}C$, while the CN treatment exhibited no obvious differences (Fig. 5*A*). Furthermore, the ET, CN, and ETN treatments significantly increased the foliar $\delta^{15}N$ when compared with the C treatment in W1. However, in W2, compared with the C treatment, the CN and ETN treatments significantly decreased foliar $\delta^{15}N$ (Fig. 5*B*).

Ultrastructure: The ultrastructural alterations of the leaf mesophyll cells were investigated during the experiment. Under the C and CN treatments in W1 (Fig. 6*A*,*C*,*E*,*G*), the ultrastructure of mesophyll cells exhibited the great modifications, with the chloroplasts filled by starch granules and with grana and mitochondria being almost absent. Moreover, under the C treatment in W1 (Fig. 6*A*), there were visible mainly starch granules and almost no other organelles. However, under the ET and ETN treatments (Fig. 6*B*,*D*,*F*,*H*), the ultrastructure of the mesophyll cells showed lesser alterations of the smooth cell membranes and cell walls and numerous organelles in the cytoplasm. The chloroplasts exhibited a typical structure and well-arranged thylakoids in the granum regions and clear mitochondria under the ET and ETN treatments.

Discussion

Nongrowing-season warming significantly enhanced the photosynthetic parameters (Table 1). Moreover, our data provided insight into responses to winter warming varying with time. Generally, the response to nongrowing-season warming differed between W1 and W2. Notably, carbon contents were significantly higher in all organs under the ETN treatments compared to ET treatments in W2 (Table 2). This finding suggests that N deposition mitigated the potentially negative effects on the carbon balance if the foliage was exposed to nongrowing-season warming.

In our study, the N deposition resulted in higher gas exchange and elevated photosynthetic pigment contents (Table 1, Fig. 1*A*,*B*). The observed accumulation of photosynthetic pigments indicated that the nutrient supply stimulated the synthesis of these pigments (Li *et al.* 2014). These findings suggest that the increase in the tissue carbohydrates could be attributed to the increased gas exchange and photosynthetic pigment contents. The seedlings grown under the N deposition conditions did not change $\delta^{13}C$ when compared with the control treatment

Table 2. Carbon (C) and nitrogen (N) content and C/N ratio of different organs in *Cunninghamia lanceolata* as effected by nitrogen and elevated temperature under 2-month (W1) and 4-month (W2) warming treatment. Each value is the mean ± SE (n = 5). F_{ET} – elevated temperature effect; F_N – nitrogen effect; $F_{ET\times N}$ – elevated temperature × nitrogen effect. *Different lowercase letters* indicate significant differences between each treatment in W1 or W2 according to *Duncan*'s tests (P<0.05). The asterisks demonstrate statistically significant differences according to independent samples t-test between two different warming durations (W1 and W2) within each treatment ($^{**}0.001<P\leq0.01$). C– control; ET – elevated temperature; CN – control + nitrogen; ETN – elevated temperature + nitrogen.

Treatment	C content [mg g^{-1}(DM)]			N content [mg g^{-1}(DM)]			C/N ratio		
	Root	Stem	Leaf	Root	Stem	Leaf	Root	Stem	Leaf
W1									
C	437.14 ± 0.80^{d}	455.70 ± 0.50^{b}	472.90 ± 0.68^{a}	9.03 ± 0.07^{c}	8.00 ± 0.52^{b}	14.63 ± 0.74^{ab}	48.40 ± 0.30^{b}	57.47 ± 3.90^{b}	32.50 ± 1.56^{ab}
ET	459.09 ± 0.87^{a}	455.62 ± 0.49^{b}	458.73 ± 0.23^{c}	8.21 ± 0.06^{d}	6.70 ± 0.44^{c}	13.41 ± 0.04^{b}	55.90 ± 0.46^{a}	68.60 ± 4.85^{a}	34.21 ± 0.12^{a}
CN	456.19 ± 0.71^{b}	453.53 ± 1.41^{b}	465.74 ± 0.36^{b}	14.99 ± 0.09^{a}	9.27 ± 0.23^{a}	14.95 ± 0.09^{a}	30.43 ± 0.14^{d}	49.01 ± 1.30^{b}	31.16 ± 0.18^{b}
ETN	442.24 ± 0.93^{c}	458.47 ± 0.02^{a}	456.72 ± 1.50^{c}	11.15 ± 0.16^{b}	7.99 ± 0.13^{b}	14.36 ± 0.10^{ab}	39.67 ± 0.52^{c}	57.41 ± 0.92^{b}	31.80 ± 0.26^{ab}
$P > F_{ET}$	0.001	0.015	0.000	0.000	0.008	0.045	0.000	0.016	0.176
$P > F_N$	0.221	0.678	0.001	0.000	0.008	0.131	0.000	0.016	0.047
$P > F_{ET\times N}$	0.000	0.013	0.017	0.000	0.979	0.427	0.055	0.682	0.516
W2									
C	449.30 ± 0.94^{a}	446.68 ± 1.20^{c**}	453.09 ± 0.45^{c}	8.95 ± 0.12^{c}	6.33 ± 0.08^{c}	10.79 ± 0.08^{d}	50.23 ± 0.58^{a}	70.63 ± 0.97^{a}	42.01 ± 0.36^{a}
ET	428.26 ± 0.80^{b}	440.82 ± 0.23^{d**}	453.88 ± 0.79^{c}	8.50 ± 0.43^{c}	6.61 ± 0.02^{b}	13.29 ± 0.10^{c}	50.64 ± 2.72^{a}	66.73 ± 0.19^{b}	34.16 ± 0.21^{b}
CN	446.94 ± 1.34^{a}	452.20 ± 0.67^{b**}	458.26 ± 0.46^{b}	17.88 ± 0.04^{a}	11.73 ± 0.05^{a}	20.06 ± 0.08^{b}	25.00 ± 0.04^{b}	38.54 ± 0.23^{c}	22.85 ± 0.11^{c}
ETN	444.91 ± 8.22^{a}	456.76 ± 0.27^{a**}	468.37 ± 0.59^{a}	6.38 ± 0.22^{b}	11.63 ± 0.03^{a}	22.07 ± 0.02^{a}	27.16 ± 0.28^{b}	39.27 ± 0.10^{c}	21.22 ± 0.03^{d}
$P > F_{ET}$	0.025	0.387	0.000	0.005	0.117	0.000	0.386	0.014	0.000
$P > F_N$	0.128	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
$P > F_{ET\times N}$	0.054	0.000	0.000	0.069	0.005	0.013	0.548	0.002	0.000

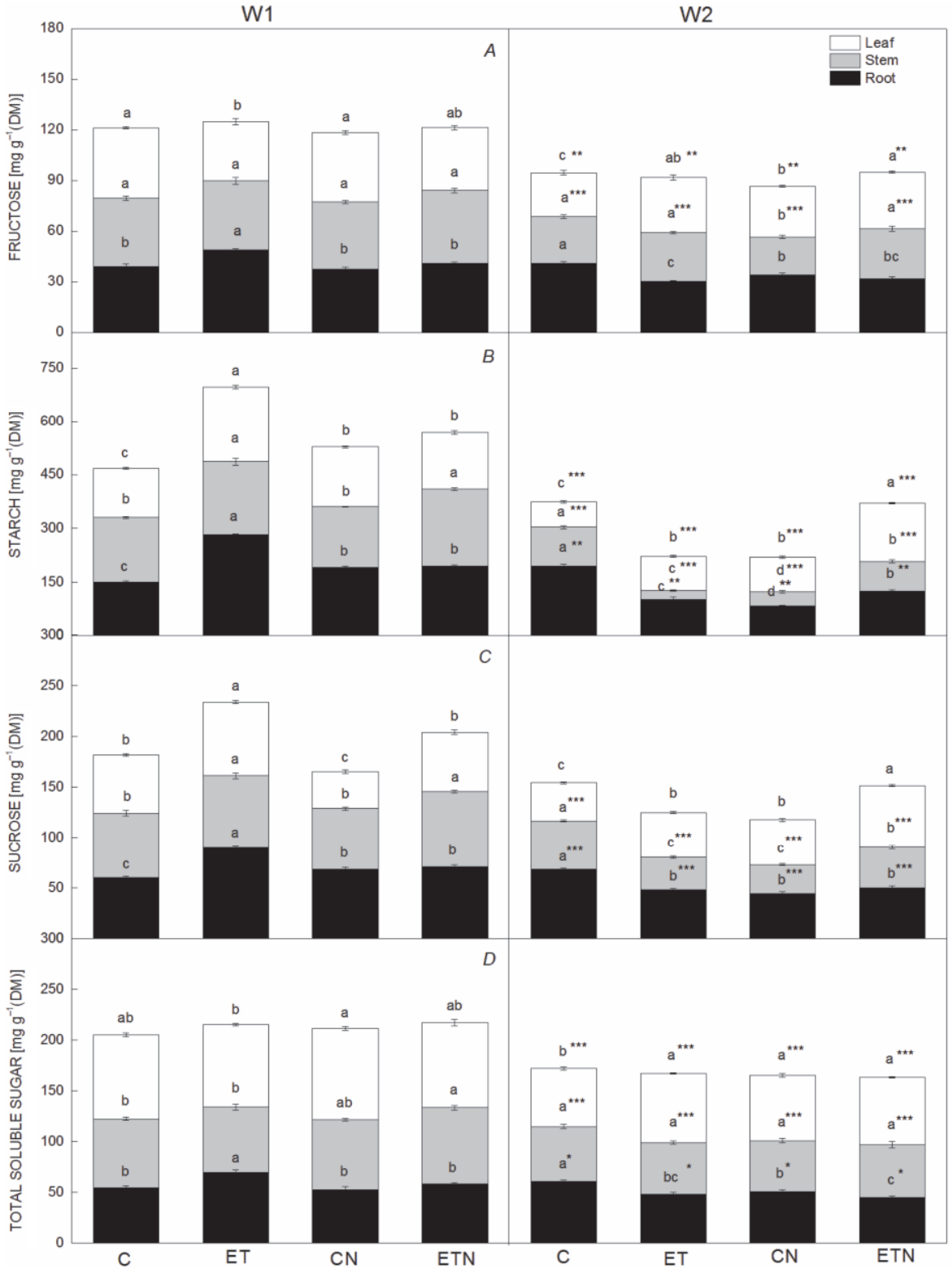

Fig. 4. Fructose, starch, sucrose, and total soluble sugar contents in different organs of *Cunninghamia lanceolata* as effected by nitrogen and elevated temperature under 2-month (W1) and 4-month (W2) warming treatment. *Different lowercase letters* indicate significant differences between each treatment in W1 or W2 according to *Duncan*'s tests (P<0.05). The asterisks demonstrate statistically significant differences according to independent samples *t*-test between two different warming durations (W1 and W2) within each treatment ($^{*}0.01<P<0.05$; $^{**}0.001<P\leq0.01$; $^{***}P\leq0.001$). C – control; ET – elevated temperature; CN – control + nitrogen; ETN – elevated temperature + nitrogen. Mean ± SE, n = 5.

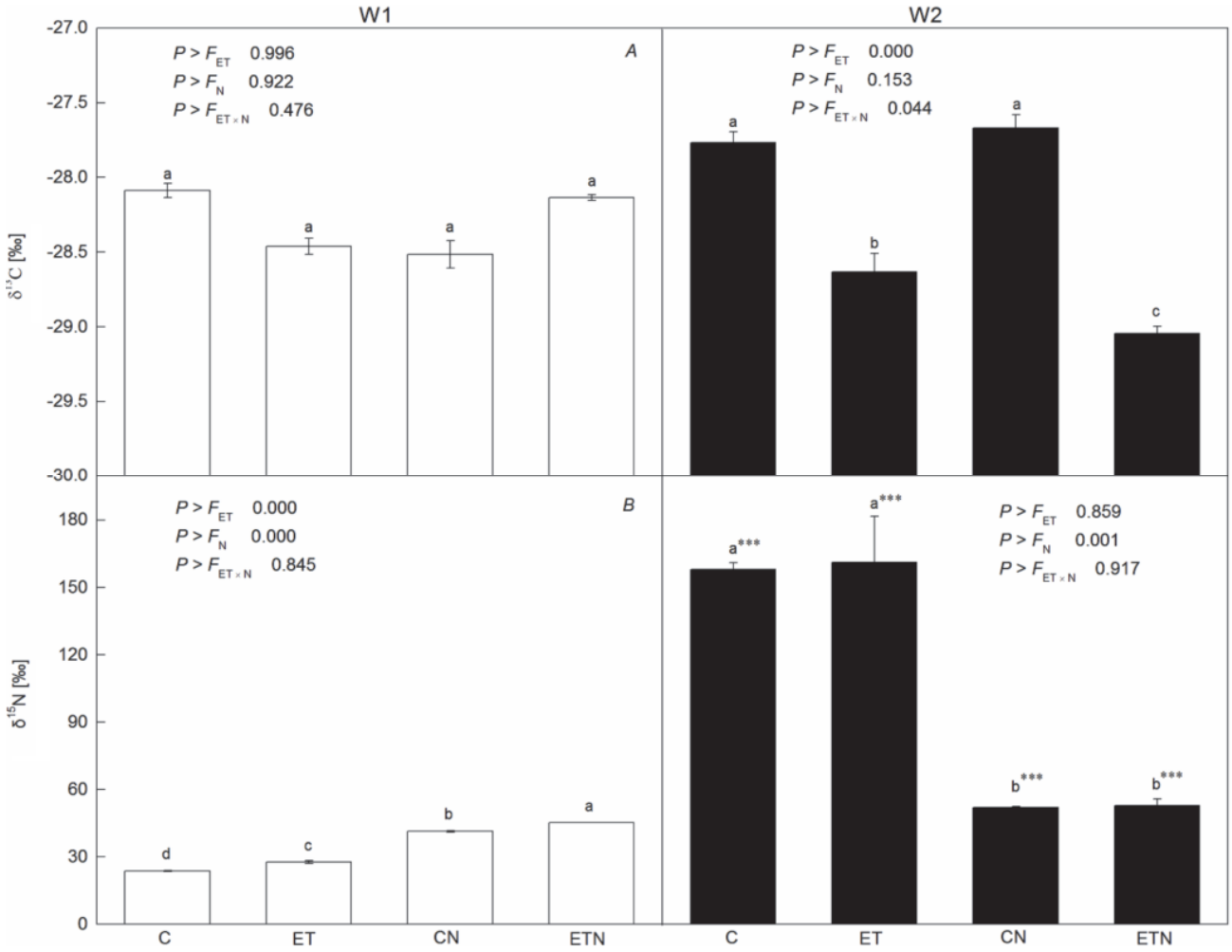

Fig. 5. Carbon isotope composition ($\delta^{13}C$) and nitrogen isotope composition ($\delta^{15}N$) in leaves of *Cunninghamia lanceolata* as affected by nitrogen and elevated temperature under 2-month (W1) and 4-month (W2) warming treatment. F_{ET} – elevated temperature effect; F_N – nitrogen effect; $F_{ET \times N}$ – elevated temperature × nitrogen effect. *Different lowercase letters* indicate significant differences between each treatment in W1 or W2 according to *Duncan*'s tests ($P<0.05$). The asterisks demonstrate statistically significant differences according to independent samples *t*-test between two different warming durations (W1 and W2) within each treatment ($^{***}P \leq 0.001$). C – control; ET – elevated temperature; CN – control + nitrogen; ETN – elevated temperature + nitrogen. Mean ± SE, $n = 5$.

(Fig. 5*A*). This result suggests that the N deposition induced the same changes in g_s and P_N, with no changes in C_i (Table 1), and thus no changes in carbon isotope discrimination. In contrast, the $\delta^{15}N$ and NR activities were significantly affected by the N deposition, as proposed previously (Li *et al.* 2012, 2014). NR is an important enzyme that affects N assimilation and absorption, which was used as an integrative indicator of direct uptake of N by species from the atmosphere and N transported from the soil. An increase in NR under the N deposition conditions may result in an increase in Chl biosynthesis and photosynthesis (Reich 1994, Li *et al.* 2014, Shi *et al.* 2015). As the NR activity was higher under the N deposition than after the other treatments (Fig. 2), it is possible that the better performance of the plants exposed to higher N was mainly associated with a greater capacity for N assimilation and absorption indicated by the higher NR activity. The high NR activity in plants exposed to the high N deposition might contribute to the fact that *C. lanceolata* is a fast-growing species and tends to be high N-requiring species (Wu 1984). Fast-growing plants remain physiologically active over the winter and could take advantage of the increased N deposition.

MDA is an important product of lipid peroxidation and the MDA content reflects the degree of the peroxidation of membrane lipids (Masia 2003). Reactive oxygen species (ROS) can damage the membrane lipids (Bowler *et al.* 1992). In our study, the MDA content was significantly higher under control treatments than that under ET and ETN treatments in W1 and W2 (Fig. 3). The increase in the MDA content detected under the C treatment (without warming) proved the occurrence of cold stress to membranes (Fazeli *et al.* 2007). We also observed that the mesophyll cell ultrastructure of *C. lanceolata* was damaged

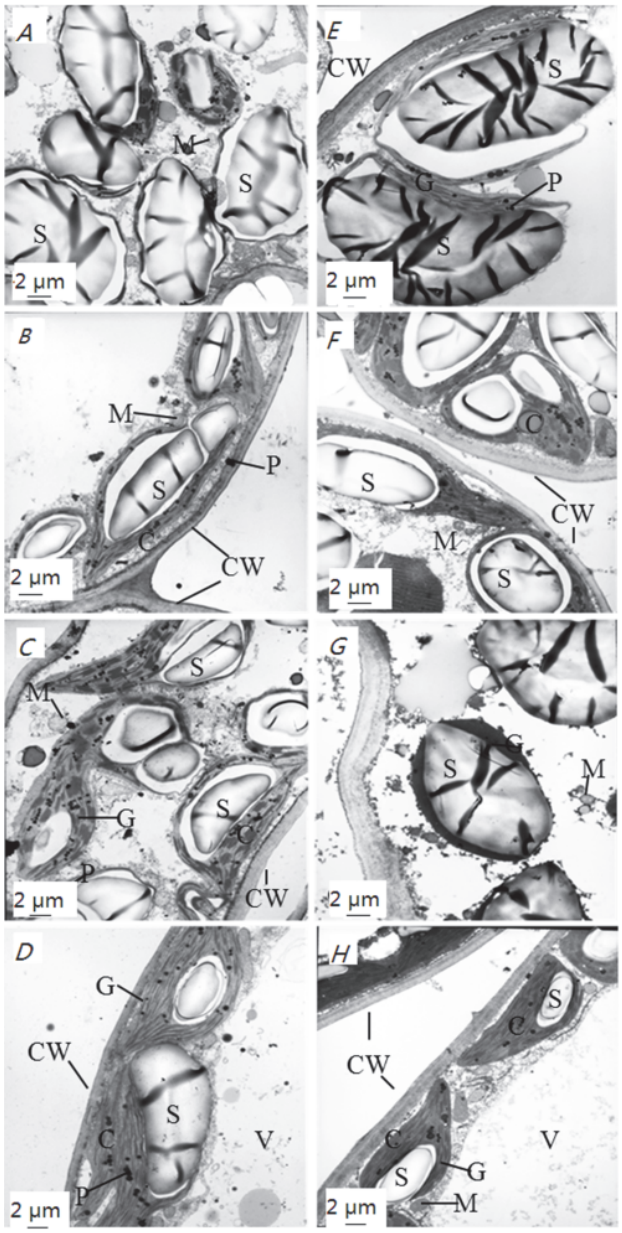

Fig. 6. Mesophyll cell observations of *Cunninghamia lanceolata* as affected by nitrogen and elevated temperature under 2-month (W1) and 4-month (W2) warming treatment by transmission electron microscopy (TEM). The left four graphs (*A–D*) were in W1: control (C), elevated temperature (ET), control + nitrogen (CN) and elevated temperature + nitrogen (ETN), respectively. The right four graphs (*E–H*) were in W2: control (C), elevated temperature (ET), control + nitrogen (CN) and elevated temperature + nitrogen (ETN), respectively. The bars shown are 2 μm. C – chloroplast; CW – cell wall; G – granum; P – plastoglobulus; S – starch granule; M – mitochondrion; V – vacuole.

at sites of the nuclear envelope, mitochondrial membranes, thylakoids, and stomata under the C treatment without warming (Fig. 6*A*,*C*,*E*,*G*); such a damage might occur due to an increased production of ROS as a result of the cold stress (Stefanowska *et al.* 2002, Zhang *et al.* 2005). Cold damage to the mitochondria might be caused by ROS accumulation in different cell compartments (Vollenweider *et al.* 2006, Gunthardt-Goerg and Vollenweider 2007).

Winter warming clearly stimulated the P_N and higher Chl contents (Chl *a*, Chl *b*) (Table 1, Fig. 1*A*,*B*). This response was similar to the results obtained in the previous studies (Aiken and Smucker 1996, Danby and Hik 2007, Shi *et al.* 2015). These results suggest that seedlings grown under nongrowing-season warming possessed the higher capacity for photosynthesis related to Chl biosynthesis. However, nongrowing-season warming reduced the N contents in the roots and stems in W1 (Table 2). The lower N contents in the seedlings can result from the growth-induced dilution effects or from the elevated N uptake. In addition, the higher C/N ratio in the roots and stems observed in W1 indicated relatively larger N-limitation. The increase in the C/N ratio caused by warming is a widely observed phenomenon in other tree species (Olszyk *et al.* 2003). However, the seedlings exposed to nongrowing-season warming had significantly higher starch contents than those under the C treatment in W1 (Fig. 4*B*). Increased carbohydrate uptake could be one explanation for the accumulations of soluble sugar and starch (Gandin *et al.* 2011). In contrast, in W2, the seedlings grown under warming treatment (ET) exhibited significantly lower starch and sucrose contents in all organs except the leaves (Fig. 4*B*) compared with the C treatment. It indicated that winter warming altered the plant carbohydrate metabolism, likely due to a higher respiration rate under winter warming (Bokhorst *et al.* 2010). Hence, the consumption of soluble sugars due to the increasing respiration rates might accelerate climate inadaptation. Many studies have indicated that warming effects are expected to vary with a season due to differences in low and high temperatures (Bokhorst *et al.* 2010) and soil moisture (Sardans *et al.* 2006, Ogaya and Penuelas 2007). In our study, the effect of warming varied with time. The effect of winter warming on the carbohydrate contents was more positive in W1 than that in W2 (Fig. 4*A*). One possibility is that in W1, plants exposed to winter warming are less susceptible to low temperature-induced negative effects (Shi *et al*. 2015).

Under the combination of the N-deposition treatments and winter warming, P_N and Chl content significantly increased (Table 1, Fig. 1*A*,*B*). In addition, under the ETN treatment, carbon, sucrose, and starch contents were significantly higher than those under the ET treatment in W2 (Table 2, Fig. 4*B*,*C*). These results demonstrated a possible role of N deposition in alleviating the damage in plant carbon storage during the nongrowing season. This phenomenon may be attributed to the N deposition compensating for reductions in carbohydrates (Bingham and Rees 2008) induced by warming. Thus, we concluded that the supplemental nutrient was favourable for the growth of the plants under winter warming. The carbohydrate contents differed in different organs with higher amounts occurring in the leaves and lower contents in the

roots in W2 (Fig. 4). The reasons for this variation might be in the fact that the mobile carbohydrates produced by leaf photosynthesis were transported to the roots for growth, and any surplus exceeding growth needs was transformed into starch reserves in the stems and roots (Korner 2003).

In conclusion, our study documented that winter warming modified the physiological traits of *C. lanceolata*, but the magnitude of the responses varied with the duration of the experiment. Under the present conditions, the response to warming was greater after W1 period than that after W2. Because winter warming decreased the carbon reserves during W2, it is likely that winter warming had negative effects on *C. lanceolata via* its strong influence on balance in carbohydrate reserve of the plants. Further, the N deposition stimulated positively a large set of parallel changes in physiological responses to warming of the *C. lanceolata* seedlings. Our results also showed that the N deposition might ameliorate the negative effects of winter warming on carbohydrate contents. Such detailed information is useful for assessing the responses of forest plantations to climate change.

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