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Mechanisms on inhibition of photosynthesis in *Kandelia obovata* due to extreme cold events under climate change

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Abstract

Introduction: Mangroves that grow at the latitudinal extremes of their distribution are susceptible to extreme cold events. Successive enhancement of low temperature stress (seLTS) is a typical characteristic of extreme cold events. Low temperature stress can inhibit mangrove photosynthesis, which often inhibits the growth and development of mangroves. However, the possible reasons for impairment to photosynthesis of mangroves due to extreme cold events remain unclear.

Methods: *Kandelia obovata* seedlings in a growth chamber were exposed to 5°C/−2°C (day/night) for 36 h (−2°C for 16 h) with 12 h light per day at 600 μmol m^{−2} s^{−1} photosynthetic photon flux density (PPFD) (a low temperature stress, aLTS), then the plants were transferred to the control condition (15°C/10°C (day/night) and allowed to recover for 5 days (R1). The other seedlings were subjected to low temperature treatment with a day/night temperature of 5°C/−1°C in a growth chamber for 24 h. Then these plants were transferred to 5°C/−2°C (day/night) under the same light and climate conditions for 36 h (two low temperature stresses, tLTS). Following the successive enhancement of low temperature treatment, these plants were returned to 15°C/10°C (day/night) for another 5-day recovery period (R2).

Results: Results showed that aLTS treatment significantly reduced leaf net photosynthetic rate (P_n) and stomatal conductance (G_s), while increased intercellular CO₂ concentration (C_i). Photosynthetic activity of leaves quickly recovered after the plants were returned to control temperature for 5 days (R1). However, decreases in leaf P_n were more obvious under tLTS treatment than those under aLTS treatment. This reduced P_n might be attributed to stomatal and non-stomatal limitations. Moreover, non-stomatal limitation played a major role in reducing P_n during tLTS treatment, as proven by reduced ribulose 1,5-biphosphate carboxylase (RuBPCase) activity. This limitation also enhanced lipid peroxidation in chloroplasts, decreased sucrose-metabolizing enzymes and ratios of both chlorophyll a/chlorophyll b (Chl a/b) and chlorophyll/carotenoids (Chl/Car), and increased protease senescence. Damages of tLTS treatment to photosynthesis were insufficiently alleviated even after the plants were returned to control temperature for 5 days (R2).

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Conclusions: Successive enhancement of low temperature depressed successful recovery of leaf photosynthesis of *K. obovata* seedlings mainly by impairing pigment synthesis, antioxidant metabolism, and sucrose translocation, as well as accelerating senescence of endopeptidase. Furthermore, our results suggest that decreases in sucrose content in leaves might also account for increases in reactive oxygen species (ROS) in chloroplasts.

Keywords: *Kandelia obovata*, Low temperature, Gas exchange, Sucrose synthesis, Peptidase

Introduction

Low temperature is a major environmental constraint for plants as a result of the injury provoked on leaf photosynthesis. Chloroplasts are the site of photosynthesis in cells and are a rich source of reactive oxygen species (ROS). ROS production is dramatically enhanced in response to adverse environmental conditions, often resulting in an excessive demand of the cellular antioxidant machinery (Fahnenstich et al. 2008). In general, to limit the action of ROS, plant cells evolve anti-oxidative systems including superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), and peroxidase (POD, EC 1.11.1.7). Inhibition of antioxidant enzyme activities by chilling stress leads to production of ROS, which confers serious oxidative damages to *Zingiber officinale* (Li et al. 2014). Moreover, a significant rise in H₂O₂ content in the leaves of cucumber, as well as higher activities of APX and SOD in the leaves, was found under low temperature stress (Lee et al. 2004). When tomato leaves are exposed to low temperature stress, sucrose contents are determined by photosynthetic rate, and sucrose phosphate synthase (SPS) activity is determined by sugar concentration (Artuso et al. 2000). That is, variations in photosynthesis are related to stomatal closure, leaf chlorosis, chloroplast malfunction, photo-inhibition or photo-oxidation, and sucrose metabolism. However, it is unclear whether physiological metabolic processes that depress the photosynthesis of mangroves have unique characteristics, in comparison with terrestrial plants.

The latitudinal distribution of mangrove species is limited mainly by temperature-related drivers (Quisthoudt et al. 2012). Mangroves are mainly tropical tree species that grow in the intertidal zone of tropical and subtropical coasts. According to the tropical origin, climate warming may enable mangroves to extend their distribution to higher latitudes (Gilman et al. 2008; Naidoo 2016). However, mangroves that lie along the latitudinal extremes of their distribution are still vulnerable to the extreme cold events (Ellis et al. 2006; Gilman et al. 2008; Saintilan et al. 2014). In general, successive enhancement of low temperature is a typical characteristic of extreme cold events. Several researches reported that low temperature, especially freezing stress, seriously influenced mangrove development and survival (Ellis et al. 2006; Stuart et al.

2006; Liu et al. 2014). For example, a severe winter in Florida, occurred between the 1995 and 1996 growing seasons (minimum air temperature of -8°C in January 1996), killed 12% mangrove trees, and the severe freeze season occurred once approximately 8 years (Stevens et al. 2006; Ross et al. 2009). Studies have shown that frost may restrict nutrient resorption during leaf senescence. *K. obovata* resorbed 61% N and 42% P during normal leaf senescence (Wang et al. 2011). Peng et al. (2013) found that three *CBF/DREB1* (C-repeat binding factor/dehydration responsive element-binding factor 1) genes play different roles in *Avicennia marina*, and the *AmCBF2* might be involved in the signaling pathway of cold stress response. But little attention has been paid to physiological mechanisms on these damages to mangroves by seLTS.

K. obovata Sheue et al. (2003) is a common mangrove species along southeastern coastlines of China. This species has high tolerance against low temperature stress. However, low temperature still decreases the total number of leaves that emerge from *K. obovata* yearly (An-luddin et al. 2009). In 2010, a blizzard severely damaged or killed many mangrove trees in southern China. It was found that seLTS severely damaged the development of *K. obovata* by disturbing photosynthetic capacity in the Yueqing bay, Zhejiang Province, China (Zheng et al. 2013). Unfortunately, the possible reasons for impairment to photosynthesis of mangroves by seLTS remain unclear. In the present study, we investigated the effects of seLTS and subsequent recovery on photosynthetic characteristics, photosynthetic pigment contents, chloroplast anti-oxidation system, sucrose synthesis, and proteolysis in the leaves of *K. obovata* seedlings to assess harmful effects on photosynthesis and find reasons for reduction in photosynthesis. These efforts will be useful in restoring and protecting mangrove forests especially in high latitude area.

Methods

Plant materials and culture

Propagules of *K. obovata* were collected from Fuding (the northern limit of natural mangrove distribution in China, $27^{\circ}17'N$), Fujian Province, China. Undamaged propagules with 12 ± 0.5 cm length were randomly planted in plastic pots (30 cm in height and 20 cm in diameter) filled with 3.0 kg of intertidal soil and

maintained in a temperature-controlled growth chamber under 25°C/20°C day/night cycles. The everyday light and climate conditions in this chamber were 12 h of light at 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD) and 70% relative humidity. The pots were immersed in nutrient solution (Haines and Dunn 1976), which was replenished once a week.

Experimental treatments

The experiment was conducted in Wenzhou (28°1'N), Zhejiang Province, China. Wenzhou is the northern limit of mangroves introduced in China. In Wenzhou, extreme low temperatures sporadically occur, causing substantial frost damage to mangroves. In January, 2011, low temperature in mangrove communities steadily decreased, and the minimal air temperature dropped from -1.1°C (about 10 h in 24 h) to -3.2°C (about 4 h in 72 h). The lowest temperature occurred during flood tide and the minimal surface seawater temperature during this period was about 5.5°C. The seedlings of *K. obovata* were therefore protected by warmer seawater. But seedlings were exposed to freezing temperature (-2.0°C) for about 16 h in 72 h. That is, there was a rare cold event in 2011, resulting in badly damaged mangroves (Zheng et al. 2016). To simulate this cold event, an experiment was performed as follows: The seedlings were maintained at 15°C/10°C day/night (near minimum monthly mean temperature in Wenzhou) with 12 h light per day at 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for 15 days. Then, they were divided into three batches after the tenth pair of leaves expanded. One batch was in a chamber at a day/night temperature of 15°C/10°C (control plants). Another batch in a growth chamber was exposed to -2°C from 8:00 pm to 8:00 am in the next morning in the dark and then the temperature was shifted to 5°C from -2°C for 12 h with light at 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD. Afterwards, the seedlings continuously grew at -2°C for 4 h in the dark and at 5°C for 8 h with light at 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD. This treatment was designated as a low temperature stress (aLTS). After aLTS treatment, the plants were transferred to the control condition and allowed to recover for 5 days (R1). The last batch was subjected to low temperature treatment with a night temperature of -1°C in a growth chamber from 8:00 pm to 8:00 am in the next morning in the dark, then the day temperature was 5°C with light at 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD by setting the control from 8:00 am to 8:00 pm. Then, these plants were allowed to continue processing according to the methods of aLTS treatment (designated as two low temperature stresses (tLTS)). Following the successive enhancement of low temperature treatment, these plants were returned to 15°C/10°C (day/night)

for another 5-day recovery period (R2). In total, three treatments were established. Physiological parameters were determined by using the second pair of fully expanded leaves. The experiment was replicated in five separate plants for each treatment.

Gas exchange assay

Net photosynthetic rate (P_n), stomatal conductance (G_s), and intercellular CO_2 concentration (C_i) were measured on the youngest fully expanded leaves using a Li-6400 portable photosynthesis system (Li-Cor, Lincoln, NE, USA). A leaf chamber equipped with a red/blue LED light source was used. All measurements were taken at a constant flow rate of 500 mL min^{-1} and CO_2 concentration of 390 $\mu\text{mol mol}^{-1}$ under a photosynthetically active radiation of 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Leaf chlorophyll content assay

The youngest fully expanded leaf sample (0.1 g) was sliced and incubated in 50 mL of pigment extraction solution containing acetone and anhydrous ethanol (1:1, v/v) in the dark for 24 h at 25°C. Chlorophyll contents were determined as described by Arnon (1949).

Ribulose 1,5-biphosphate carboxylase activity assay

RuBPCase activity was determined according to the method of Sui et al. (2005) by spectrophotometry. In brief, the reaction mixture was comprised of 5 mM NADH, 50 mM ATP, 50 mM phosphocreatine, 0.2 mM NaHCO_3 , 100 mM Tris-HCl buffer (pH 7.8), 12 mM MgCl_2 , 0.4 mM EDTA- Na_2 , 160 U mL^{-1} 3-phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, creatine phosphokinase, and 0.1 mL enzyme extract solution. The changes of A_{340} in 1 min of the assay mixture were measured with a UV-VIS recording spectrophotometer (UV-160A, Shimadzu, Japan).

Chloroplast preparation

Chloroplasts were isolated using the method described by Seigneurin-Berny et al. (2008). Fresh leaves were homogenized in ice-cold isolation buffer (0.4 mM sorbitol, 20 mM Tricine-KOH, 10 mM EDTA, 10 mM NaHCO_3 , and 0.15% bovine serum albumin (BSA)). The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at 1465 $\times g$ for 90 s (including deceleration). The pellets were resuspended in 2 mL of buffer (0.4 mM sorbitol, 20 mM Hepes-KOH, 2.5 mM EDTA, 5 mM MgCl_2 , 10 mM NaHCO_3 , and 0.15% BSA), placed into a tube containing 8 mL of resuspension medium with 40–80% (v/v) Percoll and then centrifuged at 13300 $\times g$ for 10 min. The chloroplasts were

collected at the 40%/80% interface. All procedures were performed at 0–4°C.

Assays of malondialdehyde content, hydrogen peroxide release, superoxide anion release rate, and antioxidant enzyme activity in leaf chloroplasts

Malondialdehyde (MDA) content was measured in terms of the total content of thiobarbituric acid (TBA) reactive substances as described by Hodges et al. (1999). Hydrogen peroxide (H_2O_2) content was assayed using the method by Moloi and van der Westhuizen (2006). Release rate of O_2^- was measured as previously described by Sui et al. (2007).

Superoxide dismutase (SOD) activity was assayed following the method from Giannopolitis and Ries (1977), while peroxidase (POD) activity was measured as described by Lee and Lin (1995). Ascorbate peroxidase (APX) activity and soluble protein (SP) content were determined according to Nakano and Asada (1981) and Bradford (1976), respectively.

Total soluble sugar, sucrose, and free amino acid assays

Approximately 0.1 g of dried leaf sample powder was extracted with 8 mL of 80% (*v/v*) ethanol. The extract was incubated at 80°C for 30 min and then centrifuged at 10000×*g* for 10 min. The supernatant solution was collected. The extraction procedure was repeated thrice to ensure the complete extraction of sugars and amino acid. The total soluble sugar content was measured as previously described by Zhang et al. (2011). The sucrose content was determined by a similar method from Hendrix (1993) with minor modifications. Ethanol extract (0.4 mL) was mixed with 0.2 mL of 2 M NaOH, heated in a boiling water bath for 5 min and then allowed to cool. The reactants were mixed with 2.8 mL of 30% HCl and 0.8 mL of 0.1% resorcin (0.1 g of resorcin in 100 mL of 95% aqueous ethanol). The mixture was incubated at 80°C for 10 min. The reaction mixture was then measured at 480 nm absorbance.

The content of free amino acids was measured by a similar method from Moore and Stein (1954) with minor modifications. Ninhydrin reagent (3 mL, pH 5.4), distilled water (1 mL), 0.1% ascorbic acid, and ethanol extract (1 mL) were mixed. The mixture was heated in a boiling water bath for 15 min and then allowed to cool in tap water. The content of free amino acids was spectrophotometrically determined at 570 nm.

Sucrose phosphate synthase and sucrose synthase assays

Enzyme extraction and assay were according to Hubbard et al. (1989). Approximately 0.5 g of fresh leaves were ground and homogenized in a prechilled mortar and pestle with 5 mL of extraction buffer [50 mM Mops–NaOH (pH 7.5), 5 mM MgCl_2 , 1 mM

EDTA, 2.5 mM dithiothreitol (DTT), 0.05% (*v/v*) Triton X-100, and 0.5 mg mL^{-1} BSA]. The homogenate was centrifuged at 10,000×*g* for 30 min, and the supernatant was immediately used to analyze activities of sucrose synthase (SS) and sucrose phosphate synthase (SPS). All extraction procedures were carried out at 0–4°C.

The assay mixture contained 50 mM Mops–NaOH buffer (pH 7.5), 15 mM MgCl_2 , 5 mM fructose-6-phosphate, 15 mM glucose-6-phosphate, 5 mM UDP–glucose, and 0.1 mL of enzyme extract. The mixture was incubated at 25°C for 30 min, and the reaction was terminated by adding 0.1 mL of 2 M NaOH. The tubes were placed in boiling water bath for 10 min and then allowed to cool. The mixture was added with 1 mL of 0.14% (*w/v*) anthrone in 13.8 M H_2SO_4 and then incubated in 40°C water bath for 20 min. The tubes were allowed to cool, and sucrose content was spectrophotometrically determined at 620 nm absorbance. SS assay was the same as above, except that the reaction mixture contained 10 mM fructose instead of fructose-6-phosphate and was devoid of glucose-6-phosphate.

Endopeptidase and carboxypeptidase assays

Enzymes were extracted according to the procedure described by Mehta and Mattoo (1996). Fresh leaves (1 g) were homogenized in 5 mL of refrigerated grinding medium (50 mM Tris–HCl buffer, 4 mM DTT, 1 mM EDTA, and 1% PVP). The extract was centrifuged at 15000×*g* for 30 min, and the supernatant was collected to estimate activities of endopeptidase and carboxypeptidase.

Carboxypeptidase activity was assayed as described by Wang et al. (2003). The reaction mixture (0.4 mL of enzyme supernatant, 0.4 mL of 100 mM acetate buffer, and 0.2 mL of 0.05 M bovine hemoglobin) was incubated at 38°C for 1 h. Then, the reaction was terminated by adding 1 mL of 10% TCA. After centrifugation at 4000×*g* for 10 min, the suspension was used for ninhydrin reaction.

The reaction mixture for endopeptidase assay was similar to that for carboxypeptidase assay except that 0.3% (*w/v*) hippuryl-L-phenylalanine was used instead of 0.05 M bovine hemoglobin. Activities of endopeptidase and carboxypeptidase were expressed in $\mu\text{mol } \alpha\text{-NH}_2 \text{ g}^{-1} \text{ FW h}^{-1}$.

Statistical analysis

Data of each parameter were normal and reported as mean + standard deviation (SD) of five replicates. The one-way ANOVA was used to test any significant differences among the treatments. Pearson's correlation was used to analyze relationships among physiological indices.

Significant level was at 0.05. All statistical analyses were performed by using the SPSS software (SPSS Ver. 19.0, Chicago, IL, USA).

Results

Leaf gas exchange

A drastic impact was found under both low temperature stresses, reaching null and negative value in P_n (Fig. 1a). Compared with the control, aLTS treatment significantly decreased P_n and G_s but significantly increased C_i in the seedling leaves of *K. obovata* ($p < 0.05$, Fig. 1a–c). By contrast, no significant differences in P_n , G_s , and C_i were observed within 5 days of recovery after aLTS treatment between the stressed and control plants (Fig. 1a–c). tLTS significantly reduced P_n and G_s but significantly increased C_i ($p < 0.05$). P_n , G_s , and C_i were not recovered (i.e., the values returned to the level of the control, the same as below) even after the subsequent returned to 15°C/10°C (day/night) for 5 days.

Pigment contents

Treatment aLTS significantly decreased ratios of chlorophyll a/chlorophyll b (Chl a/b) and chlorophyll/carotenoids (Chl/Car) compared with those of the control (Fig. 2a, b). Both ratios became completely recovered within 5 days of recovery after the aLTS treatment. Both ratios significantly decreased to levels lower than those of the control plants under tLTS treatment (Fig. 2a, b), and both ratios slightly increased after 5 days of recovery. However, significant differences in each of the two ratios were observed between tLTS treatment and the control.

RuBPCase activity

Both aLTS and tLTS treatments significantly decreased RuBPCase activity compared with the control, particularly under tLTS treatment (Fig. 3). RuBPCase activity recovered within only 5 days of recovery after aLTS treatment.

Hydrogen peroxide and malondialdehyde contents, O_2^- release rate, and anti-oxidative enzyme activities in chloroplasts

Contents of MDA and H_2O_2 and release of O_2^- in chloroplasts of the *K. obovata* leaves were significantly increased under aLTS and tLTS treatments (Fig. 4a, b and f). Only the contents of MDA and H_2O_2 and the release rate of O_2^- in the chloroplasts recovered within 5 days of recovery after aLTS treatment. Significant differences in the contents of MDA and H_2O_2 and release rate of O_2^- were still observed in the chloroplasts between tLTS treatment and the control within 5 days of recovery after tLTS treatment.

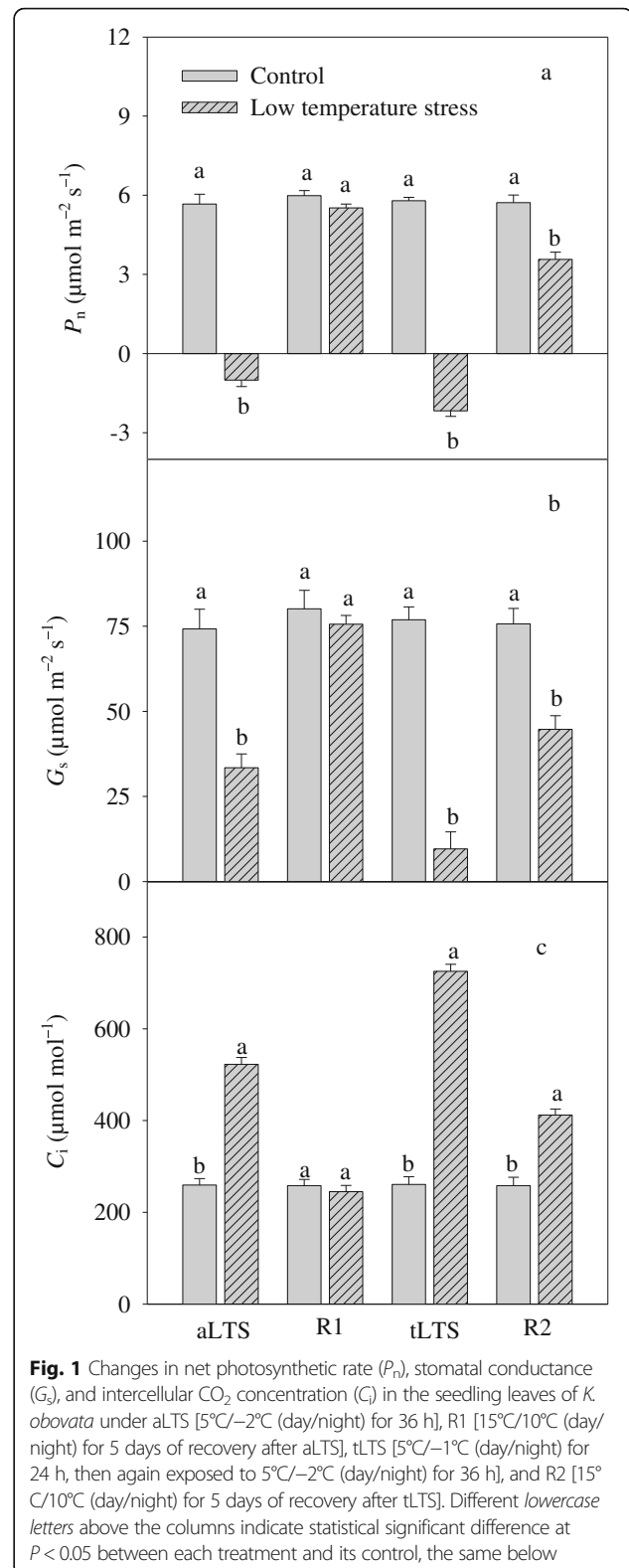


Fig. 1 Changes in net photosynthetic rate (P_n), stomatal conductance (G_s), and intercellular CO_2 concentration (C_i) in the seedling leaves of *K. obovata* under aLTS [5°C/–2°C (day/night) for 36 h], R1 [15°C/10°C (day/night) for 5 days of recovery after aLTS], tLTS [5°C/–1°C (day/night) for 24 h, then again exposed to 5°C/–2°C (day/night) for 36 h], and R2 [15°C/10°C (day/night) for 5 days of recovery after tLTS]. Different lowercase letters above the columns indicate statistical significant difference at $P < 0.05$ between each treatment and its control, the same below

Compared with the control, treatment aLTS significantly increased the activities of SOD and POD in the chloroplasts (Fig. 4c, d). Both activities recovered within

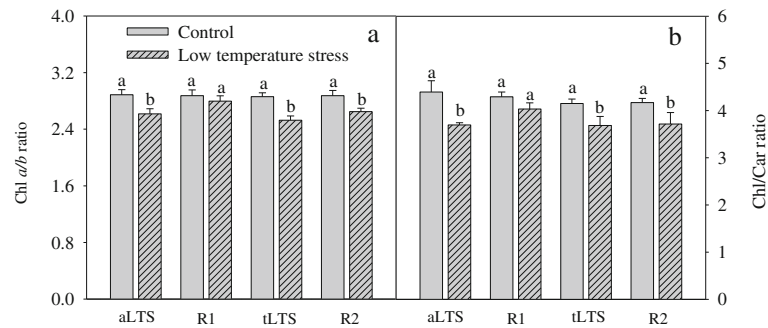


Fig. 2 Changes in ratios of chlorophyll a/b (Chl a/b) and chlorophyll/carotenoid (Chl/Car) in the seedling leaves of *K. obovata* under aLTS [5°C/−2°C (day/night) for 36 h], R1 [15°C/10°C (day/night) for 5 days of recovery after aLTS], tLTS [5°C/−1°C (day/night) for 24 h, then again exposed to 5°C/−2°C (day/night) for 36 h], and R2 [15°C/10°C (day/night) for 5 days of recovery after tLTS]

5 days recovery treatment. However, treatment tLTS significantly decreased these activities, compared with the control plants. Subsequent recovery only slightly improved the activities of SOD and POD in chloroplasts; thus, significant differences were still observed between the treatments. Compared with the control, aLTS and tLTS treatments significantly decreased activity of APX (Fig. 4e). Activity of APX was recovered within only 5 days of recovery after aLTS treatment.

TSS and sucrose contents and sucrose synthase and sucrose phosphate synthase activities in leaves

Compared with the control, treatment aLTS significantly increased contents of TSS and sucrose and activity of SS in leaves, while significantly decreased activity of SPS in leaves (Fig. 5a, d). However, contents of TSS and sucrose

and activities of SS and SPS in leaves recovered after 5 days recovery treatment. Compared with the control, treatment tLTS significantly increased TSS content but reduced sucrose content and activities of SS and SPS (Fig. 5b, c). Significant differences in contents of TSS and sucrose and activities of SS and SPS were still observed between the stressed and control plants even after 5 days recovery treatment (Fig. 5a, b).

Soluble protein and free amino acid contents and endopeptidase and carboxypeptidase activities

Compared with the control, aLTS and R1 increased contents of SP and free amino acid (FAA) and activity of carboxypeptidase in leaves but decreased activity of endopeptidase (Fig. 6a–d). Significant differences in the contents of SP and FAA and activities of endopeptidase and carboxypeptidase were only observed between aLTS treatment and the control (Fig. 6a–d). These differences were more significant than those between tLTS treatment and the control. Additionally, there were still significant differences in the contents of SP and FAA and activities of endopeptidase and carboxypeptidase between low temperature stress and control plants after 5 days recovery treatment (Fig. 6a–d).

Correlation coefficient among physiological indices

P_n was significantly positively correlated with G_s , ratios of Chl a/b and Chl/Car, activities of APX and SOD in chloroplasts, and activities of RuBPCase, SS, SPS, and endopeptidase in leaves ($p < 0.01$, Table 1). By contrast, P_n was significantly negatively correlated ($p < 0.01$) with C_i , contents of MDA and H_2O_2 and release rate of O_2^- in the chloroplasts, contents of TSS, SP, and FAA, and activity of carboxypeptidase in the leaves.

Discussion

Exposure of *K. obovata* seedlings to low temperature reduces P_n and G_s (Kao et al. 2004). After a rare cold event

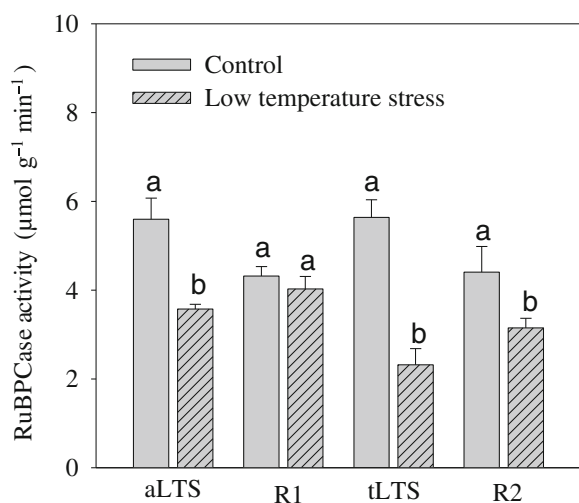
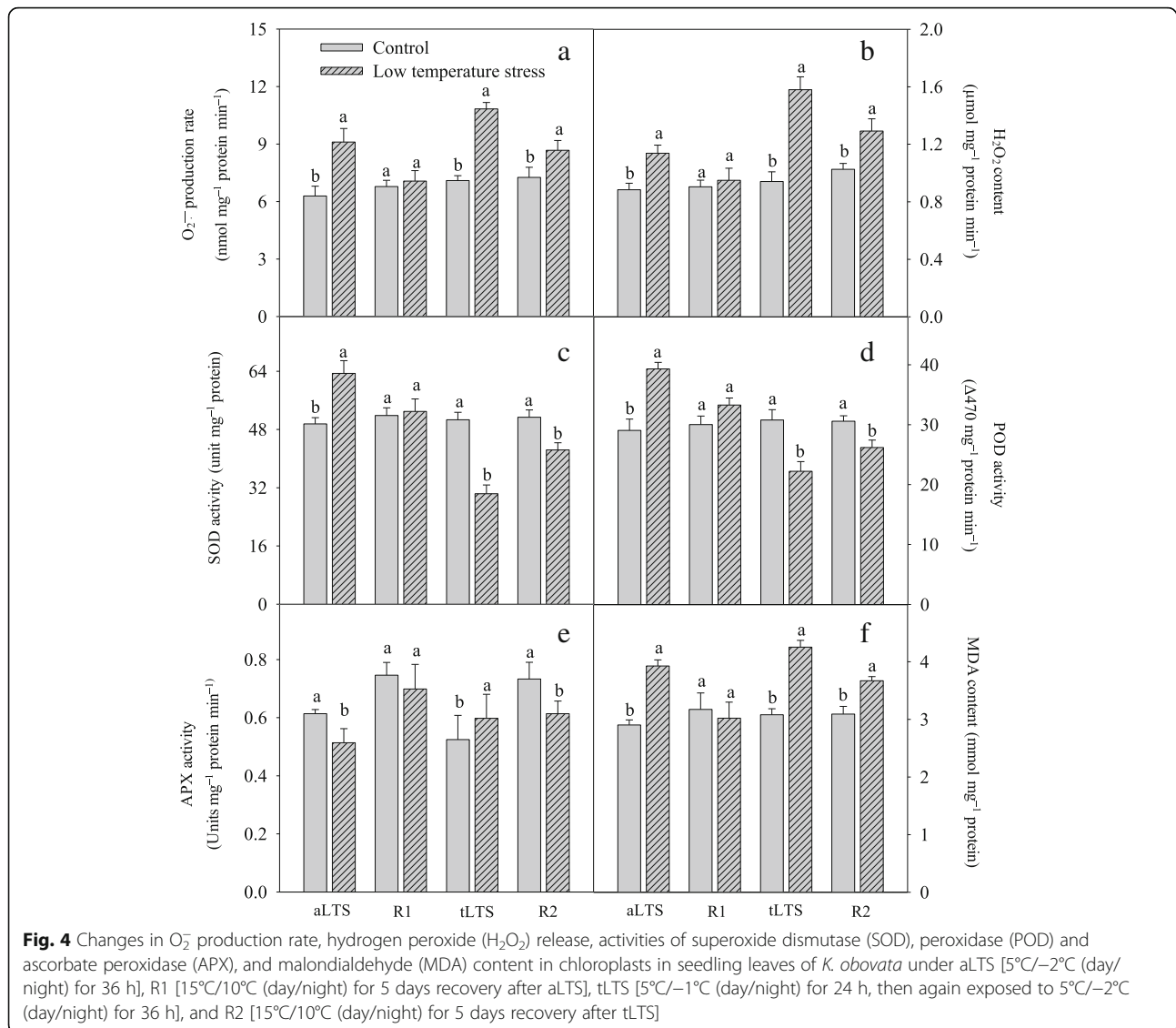


Fig. 3 Changes in ribulose 1,5-biphosphate acrboxylase (RuBPCase) activity in the seedling leaves of *K. obovata* under aLTS [5°C/−2°C (day/night) for 36 h], R1 [15°C/10°C (day/night) for 5 days of recovery after aLTS], tLTS [5°C/−1°C (day/night) for 24 h, then again exposed to 5°C/−2°C (day/night) for 36 h], and R2 [15°C/10°C (day/night) for 5 days of recovery after tLTS]



in winter of 2010, the recovery of photosynthetic capacity in *K. obovata* seedlings in the northern limit of introduced mangroves in China is difficult during a short period (Zheng et al. 2016). In the present study, P_n rapidly decreased after low temperature stress treatments, particularly tLTS treatment. The seedlings could not recover photosynthesis efficiency even for 5 days recovery treatment under optimum conditions after tLTS treatment (Fig. 6). This indicated that seLTS inhibited recovery of P_n in *K. obovata* seedlings. In general, P_n is affected by stomatal and non-stomatal factors. Stomatal or non-stomatal limitation on P_n depends on changes in C_i (Farquhar and Sharkey 1982). In addition, it is reported that non-stomatal limitations of P_n to C_i and biochemical assay were related to the decline in the activity of RuBPCase (Ziska et al. 1990). The seedlings of *K. obovata* were subjected to aLTS and tLTS treatments and

were returned to the initial growth conditions to assess their response during recovery. Treatment aLTS significantly decreased P_n and G_s in leaves with increasing C_i . And RuBPCase activity was significantly decreased under aLTS treatment. This suggested that reduction in P_n under aLTS treatment could be principally ascribed to non-stomatal limitation. Recovery for 5 days at $10/15^\circ C$ (day/night) was sufficient for complete recovery of photosynthesis from aLTS treatment, while the photosynthetic capacity was not recovered under tLTS treatment.

P_n positively correlated with ratios of Chl a/b and Chl/Car in leaves (Table 1). This suggested that decreases in ratios of Chl a/b and Chl/Car in leaves limit photosynthesis under low temperature stress. In the present study, decreases in ratio of Chl a/b in leaves under low temperature stress and subsequent recovery resulted

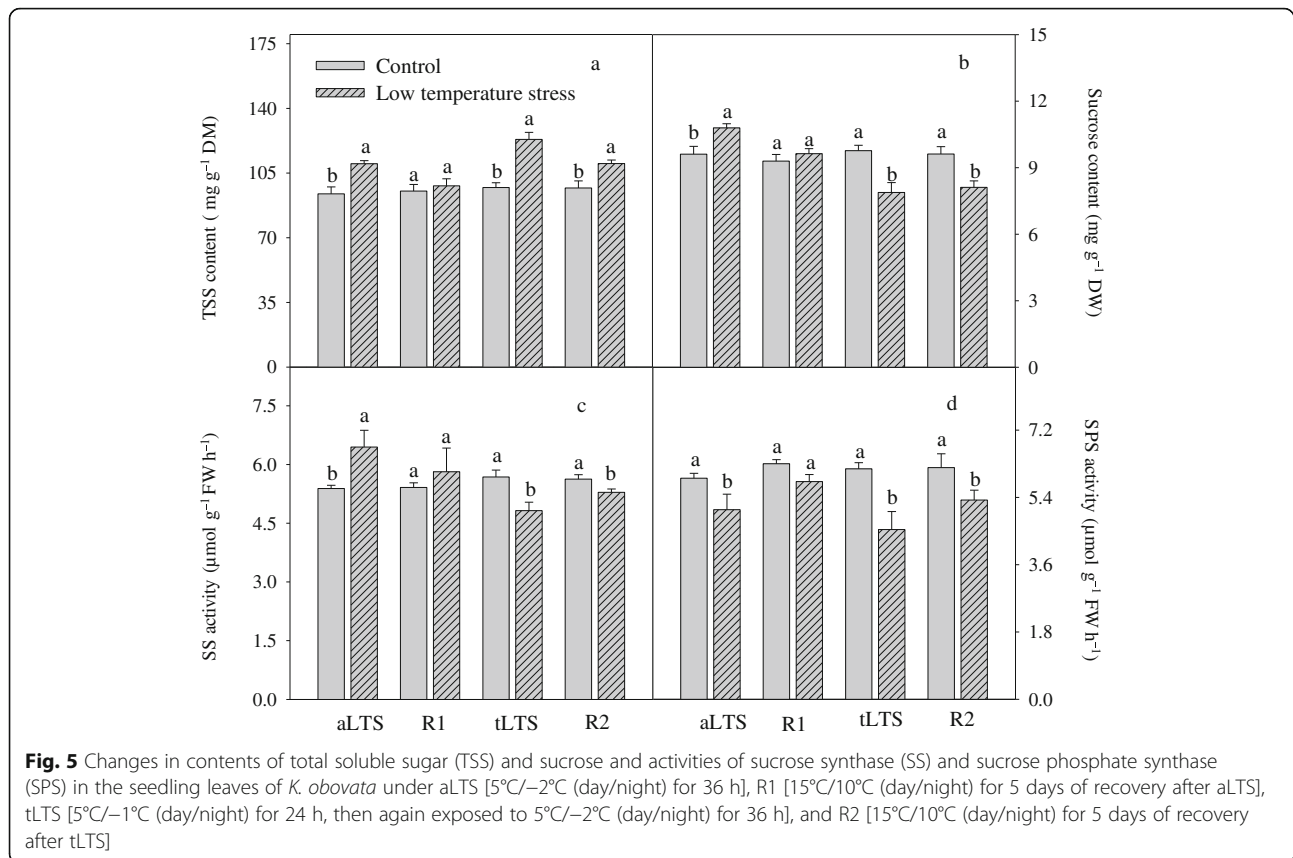


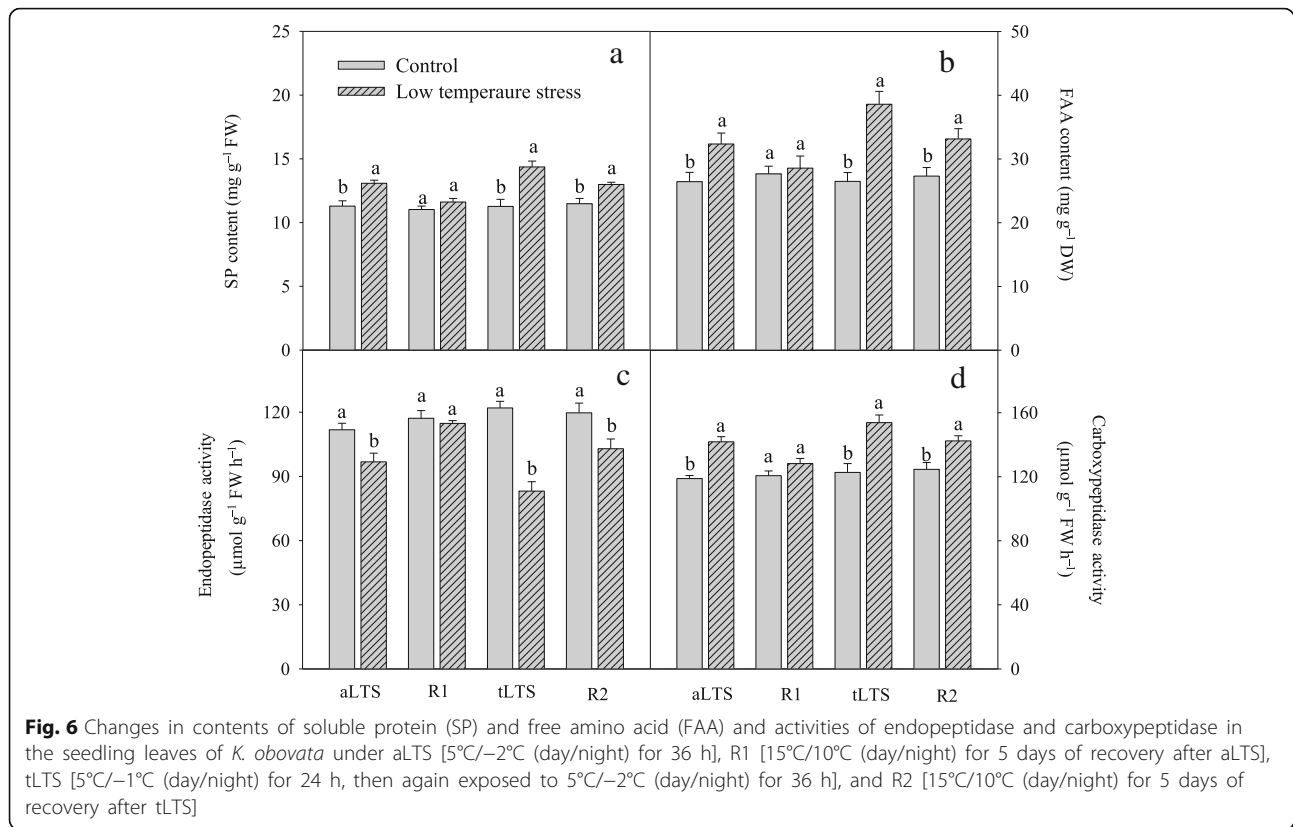
Table 1 Correlation coefficients between net photosynthetic rate (P_n) and physiological indices in *K. obovata*

Parameters	
Stomatal conductance (G_s)	0.963**
Intercellular CO_2 concentration (C_i)	-0.962**
Chlorophyll a/chlorophyll b (Chl a/b) ratio	0.677**
Chlorophyll/carotenoid (Chl/Car) ratio	0.779**
Ribulose 1,5-biphosphate acboxylase (RuBPCase)	0.564**
Ascorbate peroxidase (APX)	0.575**
Superoxide dismutase (SOD)	0.544**
Peroxidase (POD)	0.327
Malondialdehyde (MDA)	-0.918**
Superoxide anion (O_2^-)	-0.935**
Hydrogen peroxide (H_2O_2)	-0.913**
Total soluble sugar (TSS)	-0.931**
Sucrose	0.398
Sucrose synthase (SS)	0.265
Sucrose phosphate synthase (SPS)	0.914**
Soluble protein (SP)	-0.947**
Free amino acid (FAA)	-0.926**
Endopeptidase	0.921**
Carboxypeptidase	-0.940**

**Significant correlation at $P < 0.01$

from more increases in Chl b than in Chl a. One explanation is that, when Chl a preferential assembling reaction centers are limited, the reaction centers no longer incorporate the majority of Chl a. Thus chlorophyllide a oxygenase synthesizes Chl b in significant amounts (Wu et al. 2007). In addition, low ratios of Chl/Car in leaves under low temperature stress and subsequent recovery could be attributed to the faster decreases in Chl than in Car. These indicated that aberration of Chl synthase function decreases Chl a synthesis. Moreover, ratios of Chl a/b and Chl/Car in leaves under tLTS treatment were lower than those of aLTS treatment, indicating that seLTS more severely affected pigment components, in accordance with the findings from Zheng et al. (2013).

Reduction in P_n under low temperature stress is associated with increase in ROS in the chloroplasts of cucumber, and low temperature increases activities of SOD and APX in chloroplasts (Hu et al. 2008). In the present study, although activities of APX and POD increased, decreases in SOD activity would still probably lead to the accumulation of H_2O_2 and O_2^- in chloroplasts for the first low temperature injury. This is in accordance with the findings of Peng et al. (2015). And levels of H_2O_2 , O_2^- , and MDA in chloroplasts recovered after the first recovery period of 48 h under control conditions (Fig. 3a, b, and d). However, treatment tLTS aggravated



damage extent and then depressed activities of SOD, POD, and APX and caused massive accumulation of H_2O_2 , O_2^- , and MDA in chloroplasts. This indicated that seLTS induced oxidative bursts because of ROS overproduction in chloroplasts, thereby reduced utilization of the absorbed light energy for CO_2 assimilation. Correlation coefficients between P_n and activities of APX, SOD, and POD, and levels of H_2O_2 , O_2^- , and MDA in chloroplasts agreed with this proposition. After the plants were subjected to two freezing injuries, levels of H_2O_2 , O_2^- , and MDA and activities of SOD, POD, and APX did not recover even when they were transferred to control conditions for 5 days.

Sucrose metabolism is crucial to carbohydrate translocation and accumulation, and low temperature stress might impair sucrose translocation (Artuso et al. 2000). Photosynthesis rate also determines sucrose content in sugarcane leaves exposed to low temperature stress (Du and Nose 2002). Therefore, positive relationship between P_n and sucrose content indicated that decreases in P_n restrained sucrose synthesis under low temperature. Sucrose synthesis and/or degradation referred to SPS and SS. Genetically, SPS catalyzes a step in sucrose synthesis. In addition, protein SS is preferentially expressed during cold stress, suggesting that the enzyme might be involved in plant responses to low temperature stress (Crespi et al. 1991). SS participates in sucrose cleavage

or sucrose synthesis. In the present study, SS activity in leaves increased after aLTS treatment, but recovered after treatment R1. Meanwhile, treatment aLTS significantly increased contents of TSS and sucrose but decreased activity of SPS. These suggested that SS activity in leaves during aLTS treatment participated in sucrose synthesis and that sucrose accumulation induced by low temperature injury principally depended on increases in SS activity. Treatment tLTS significantly reduced content of sucrose and activities of SS and SPS but significantly increased content of TSS. These parameters did not recover even after 5 days recovery treatment, suggesting that sucrose metabolism in *K. obovata* leaves was deteriorated after seLTS treatment. It was also found that sucrose acted as a direct ROS scavenger (Van den Ende and Valluru 2009). Therefore, decreases in sucrose content in leaves might also account for increases in ROS in chloroplasts.

Decreases in SP and increases in free amino acids might be related with activities of endopeptidase and carboxypeptidase (Cohen and Demaggio 1986). Endopeptidase is a protease that catalyzes the cleavage of peptide bonds within a polypeptide or protein, and carboxypeptidase is another protease enzyme that hydrolyzes (cleaves) a peptide bond at the C-terminal end of a protein or peptide, which has a stronger preference for free amino acids. In the present study, aLTS and tLTS

treatments improved the accumulation of SP and FAA and activity of carboxypeptidase but decreased activity of endopeptidase, indicating that carboxypeptidase was critical to cleave peptide bonds for FAA. In addition, the principal reason for protein degradation under low temperature stress is the accumulation of ROS that promotes oxidation of proteins and lipids and inhibits activity of endopeptidase (Prasad 1996). Protein degradation in *K. obovata* was completely recovered after R1, but proteolysis was insufficiently recovered after R2. Furthermore, FAA content positively correlated with carboxypeptidase activity but negatively correlated with endopeptidase activity, indicating that low temperature stress induced senescence of endopeptidase but improved efficiency of carboxypeptidase to synthesize FAA.

Conclusions

In conclusion, tLTS treatment decreased P_n in the leaves of *K. obovata* seedlings because of stomatal closure and non-stomatal factors. Increase in C_i might be partly explained by declined ratios of Chl a/b and Chl/Car, enhanced peroxidation in chloroplasts, weakened activities of sucrose-metabolizing enzymes, and accelerated senescence of endopeptidase. Therefore, photosynthetic capacity could not completely recover after tLTS treatment.

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Authors' contributions

WL, YY, CZ, and JT designed the study and collected and analyzed the data. CZ, JC, JQ, and XP performed the experiment work and analyzed the data. CZ and JT drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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