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Relationship between leaf reddening, ROS and antioxidants in *Buxus microphylla* during overwintering

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Abstract To provide a theoretical basis for revealing the mechanism of winter leaf reddening in evergreen species, the relationships between winter leaf reddening, reactive oxygen species (ROS) and the antioxidant system of *Buxus microphylla* 'Wintergreen' were studied. The pigment changes, ROS production, lipid peroxidation and antioxidants activities of sun leaves during the reddening and regreening processes were investigated, using green shade leaves as controls. The carotenoids in the sun leaves increased linearly with reddening but decreased with the regreening. There was no significant difference in either the superoxide anions ($O_2^{-\cdot}$) or malondialdehyde (MDA) changes between the sun and shade leaves, and their $O_2^{-\cdot}$ contents were positively correlated with MDA. In contrast to the shade leaves, the sun leaves showed a trend in which the hydrogen peroxide (H_2O_2) changes were closely related to the reddening process and positively correlated with carotenoids content but not with the MDA content. A similar trend was observed for catalase (CAT) and ascorbate peroxidase (APX) activity between the sun and shade leaves, but superoxide dismutase (SOD) and peroxidase (POD) activity and the ascorbate (AsA) content differed between these two ecotypes. Furthermore, the sun leaves had higher CAT activity and AsA content than the corresponding shade leaves. These results suggested that H_2O_2 might play an important role in the winter reddening of sun leaves by promoting the accumulation of carotenoids. In addition, SOD, POD and AsA probably play a photoprotective role in winter-red sun leaves, while the changes in $O_2^{-\cdot}$, CAT and APX were independent of winter leaf reddening and were more likely responses to stress caused by low temperatures.

Keywords Antioxidants · *Buxus microphylla* · Carotenoids · Evergreen species · Reactive oxygen species (ROS) · Winter leaf reddening

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Introduction

The leaves of some evergreen species frequently change color to brown or reddish yellow in winter and are restored to green with springtime warming (Koiwa et al. 1986; Hughes 2011). Previous research on the winter leaf reddening mechanism mainly investigated the relationship between pigment changes in leaves and their photoprotective function (Hughes 2011). It is well documented that leaf reddening in winter evergreen species is mainly caused by the accumulation of anthocyanins (Kaku et al. 1992; Hughes and Smith 2007) or red carotenoids (Ida et al. 1995; Han et al. 2004). According to the photoprotection hypothesis, these red pigments accumulate in leaves to protect photosynthetic tissues from winter high light stress by acting as light attenuators to alleviate excess chlorophyll

excitation pressure in underlying cells (Hormaetxe et al. 2005; Carpenter et al. 2014; Hughes et al. 2014). These pigments also act as quenchers of reactive oxygen species (ROS) (Hormaetxe et al. 2004; Velissarios-Phaedon and Yiannis 2006). The former function has been studied directly in detail, but the latter remains a hypothesis because the direct relationship between pigments and ROS in the winter leaf coloration process has not been systematically studied.

Under normal growth conditions, the production of ROS, including hydrogen peroxide (H_2O_2), superoxide anions (O_2^-), hydroxyl radical ($OH^.$) and singlet oxygen ($^1O_2$), is low in cells (Mittler 2002). However, in winter, green leaves are often subjected to cold temperature stress or chilling-enhanced photo-oxidative stress (Wise 1995; Arora and Rowland 2011). This may disrupt the cellular homeostasis of cells and enhance the production of ROS (Wise 1995; Verhoeven et al. 2005; Ruelland et al. 2009). Excess ROS can cause oxidation of biomolecules and cell death. However, there are a series of antioxidant systems that tightly control ROS production (Mittler 2002). $^1O_2$ is a singular ROS that is produced constitutively in plant leaves under light conditions via chlorophylls that act as photosensitizers, and its production is enhanced under light stress conditions (Triantaphylidès and Havaux 2009). Carotenoids are considered to be the main $^1O_2$ quenchers in chloroplasts (Ramel et al. 2012; Fischer et al. 2013). $OH^.$, produced from O_2^- and H_2O_2, is one of the most highly reactive ROS (Gill and Tuteja 2010). Superoxide dismutase (SOD) can remove O_2^-, and hence decrease the risk of $OH^.$ formation, and H_2O_2 can be converted into water by other ROS-scavenging mechanisms (Pandhair and Sekhon 2006).

Research on the shrubs of two boxwood species have shown that winter leaf reddening in *Buxus sempervirens* and *B. microphylla* is closely related to the accumulation of carotenoids (Ida et al. 1995; Jiang et al. 2015). As an important antioxidant, carotenoids protect plants from ROS damage by scavenging free radicals and by quenching $^1O_2$ and the excited-state light-sensitive molecules (Domonkos et al. 2013). Therefore, in this study, the relationships between carotenoids, ROS and antioxidants in sun and shade leaves of *B. microphylla* 'Wintergreen' were investigated during winter reddening and regreening periods to provide an important theoretical basis for revealing the mechanism underlying winter reddening.

Materials and methods

Plant material

A cultivar of *B. microphylla* 'Wintergreen', introduced from Belgium more than 10 years ago, grown in the Beijing Botanical Garden Seedling Center, China ($40°01'N$, $116°19'E$), was selected for study. This shrub is spherical in nature with an average crown of 1.2×1.2 m, and a height of 1.3 m. Shrubs were planted in an east–west array in an open field. The morphology, growth and size were basically similar. According to our preliminary observations and the relevant literature, sun leaves exposed to light turn red in winter and become green again with springtime warming compared with shade leaves that remain green throughout winter (Hughes 2011; Peng et al. 2011).

The color change of *B. microphylla* sun leaves was divided into three stages during the winter, (1) the reddening process in which color changed from green to red as winter progressed, (2) red stable stage in mid-winter, (3) the regreening process in which color changed from red to green with springtime warming. Three sampling dates were selected in the first (reddening process, October to December 2013) and the third stage (regreening process, February to March 2014) respectively according to the leaf color change process in the 2013–2014 winter. Sun and shade leaves were randomly collected from the middle of the current year branches with south- and north-facing exposure.

Leaves designated as G-g (green leaves), G-m (intermediate state between green and red leaves) and G-r (red leaves) correspond to three different coloration stages of sun leaves changing from green to red as winter progresses. Similarly, leaves designated as R-r (red leaves), R-m (intermediate state between red and green leaves) and R-g (green leaves) correspond to the different colors of sun leaves changing from red to green with springtime warming. The coloration of all sun leaves sampled is presented in Fig. 1. All samples were immediately frozen in liquid nitrogen and stored at -80 °C until biochemical analysis. The field temperature during the test period was monitored using a HOBO U23-004 data logger (Onset Computer Corp., MA, USA) (Fig. 2).

Biochemical analysis

Chlorophyll and carotenoids contents

An extraction method was used following Zhang (1986), but with 80 % (v/v) acetone as the extraction solvent instead of the acetone–ethanol mixture. Leaves cut into filaments (0.15 g) were immersed in 10 ml 80 % acetone (v/v) and were incubated for 24 h at ambient temperature in the dark until the material was completely white. The absorbance of the extraction solution was measured at 663, 646 and 470 nm using a Biomate 3S UV–Visible Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) according to Lichtenthaler and Wellburn (1983). A
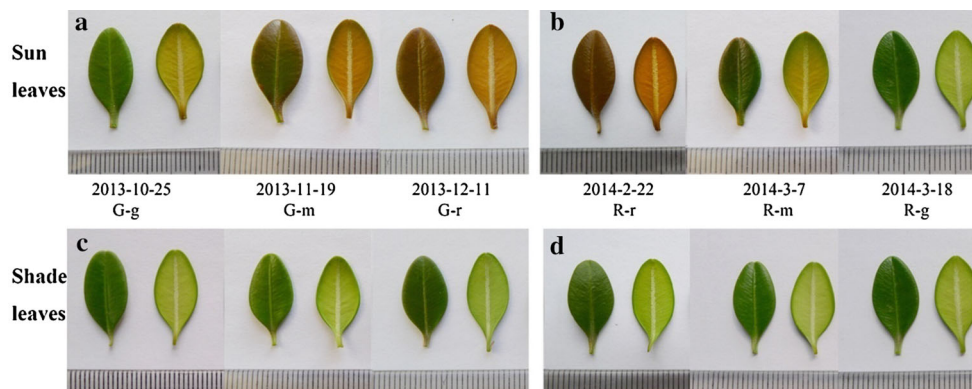
Fig. 1 Winter leaf color changes in *B. microphylla* 'Wintergreen'. **a**, **b** Sun leaves; **c**, **d** shade leaves in the corresponding period. *G-g*, G-m and *G-r* represent the *different colors* of the sun leaves changing from *green* to *red* during the reddening process as winter progresses; *R-r*, *R-m* and *R-g* represent the *different colors* changing from *red* to *green* during the regreening process with springtime warming
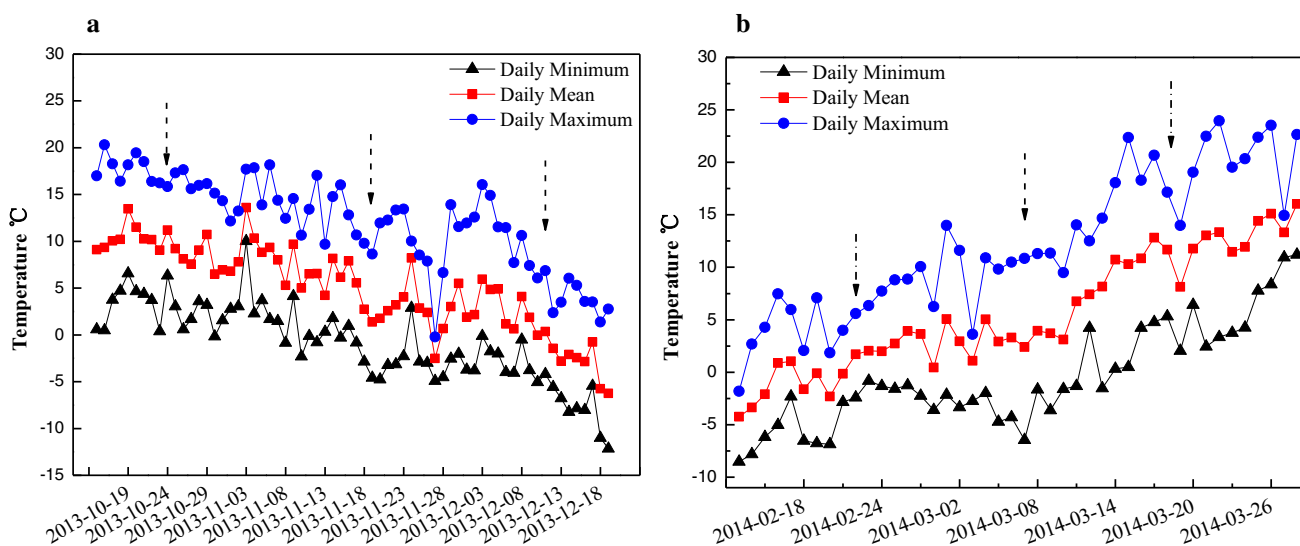

Fig. 2 Field temperature recorded from October 14 to December 18, 2013 (**a**) and February 12 to March 26, 2014 (**b**). The specific sampling dates (for analysis of pigments, ROS and antioxidant systems) are shown with the *dotted arrows* pointing downwards

similar method was also used in the total carotenoids determination of red needles in Qilian juniper in Wen and Chen (2014). The contents of chlorophyll *a*, *b* and total carotenoids were calculated using the following formulas,

Chlorophyll *a* content (C_a) = $\dfrac{(12.21\,A_{663} - 2.81\,A_{646})\,V}{1000\,W}$

Chlorophyll *b* content (C_b) = $\dfrac{(20.13\,A_{646} - 5.03\,A_{663})\,V}{1000\,W}$

Total chlorophyll content = $C_a + C_b$

Total carotenoids content = $\dfrac{A_{470}\,V}{229\,W} - \dfrac{3.27\,C_a - 104\,C_b}{229}$

where V = volume of 80 % (v/v) acetone (ml) and W = weight of sample (g). The chlorophyll and carotenoids contents = [mg g^{-1} fresh weight (FW)].

Determination of ROS ($O_2^{-\cdot}$ and H_2O_2)

The $O_2^{-\cdot}$ content was measured according to the procedure described by Wang and Luo (1990). Leaves (0.3 g) were ground in a pre-chilled mortar with 5 ml of 50 mM sodium phosphate buffer (PBS, pH 7.8) in an ice bath. The homogenates were centrifuged at $4000 \times g$ for 15 min at 4 °C. One milliliter of the reaction mixture, which consisted of 0.5 ml supernatant, 50 mM PBS (pH 7.8) and 10 mM hydroxylamine hydrochloride, was incubated at 25 °C for 1 h. Then, 1 ml of 17 mM p-amino-benzene sulfonic acid and 1 ml 7 mM α-naphthylamine were added, and mixed well. After 20 min of incubation at 25 °C, the absorbance was recorded at 530 nm. Standard curves were obtained using different concentrations of NaNO$_2$ for each independent experiment. The $O_2^{-\cdot}$ content (nmol g^{-1} FW)

was calculated according to a chemical equation as follows: $NH_2OH + 2O_2^{\cdot -} + H^+ \rightarrow NO_2^- + H_2O_2 + H_2O$.

The H_2O_2 level was determined according to the method of Sergiev et al. (1997). Frozen leaf tissue (0.3 g) was ground in a pre-chilled mortar with 4 ml 0.1 % (w/v) TCA in an ice bath. The extraction solution was centrifuged at $4000 \times g$ for 15 min at 4 °C. The absorbance of the reaction mixture consisted of 0.5 ml supernatant, 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) and 1 ml 1 M KI, was measured at 390 nm. The H_2O_2 content was determined using a standard curve.

Malondialdehyde (MDA) assay

The MDA content was determined according to Li et al. (2000). Leaves (0.3 g) were homogenized in 4 ml 5 % (w/v) trichloroacetic acid (TCA) solution in an ice bath. The homogenate was centrifuged at $10,000 \times g$ for 20 min. The supernatant was collected and make up to a final volume of 4 ml for the next set of measurements. The reaction mixture, which consisted of 1 ml sample and 1 ml 0.67 % (w/v) thiobarbituric acid (TBA, dissolved in 20 % TCA), was incubated in boiling water for 30 min, and the reaction was stopped by placing the reaction tube in an ice bath. Then, the mixture was centrifuged again at $10,000 \times g$ for 5 min, and the absorbance of the supernatant was read at 450, 532 and 600 nm.

Extraction and assay of antioxidant enzymes

Antioxidant enzymes were extracted according to Prochazkova et al. (2001) and Wang et al. (2009). Using a pre-cooled mortar and pestle in an ice bath, a frozen leaf sample (0.3 g) was ground in 5 ml of 100 mM PBS (pH 7.5), containing 0.5 mM ethylene diamine tetraacetic acid (EDTA) and 2 % polyvinylpyrrolidone (PVP-40), with the addition of 2 mM ascorbate (AsA) in the case of the ascorbate peroxidase (APX) assay. The extract was centrifuged at $12,000 \times g$ for 20 min at 4 °C and the supernatant was collected as enzyme extracts for the following assays.

SOD activity was assayed at 560 nm using the nitroblue tetrazolium (NBT) method according to Dhindsa et al. (1981). The reaction mixture was composed of 1.7 ml 50 mM PBS (pH 7.8), 0.3 ml 130 mM L-methionine, 0.3 ml 750 μM nitroblue tetrazolium, 0.3 ml 100 μM EDTA-Na$_2$ and 0.1 ml enzyme extract (same volume of PBS as the control). One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition in the reduction of NBT.

Catalase (CAT) activity was determined by monitoring the decrease in absorbance at 240 nm according to Prochazkova et al. (2001). The 2.7 ml mixture contained 100 mM PBS (pH 7.0) and 0.2 ml enzyme extract. Then, 0.3 ml 10 mM H_2O_2 was added to initiate the reaction. A decrease in absorbance of 0.01 per minute at 240 nm was defined as one unit of enzyme activity.

APX activity was determined by monitoring the rate of drop in absorbance (per min) at 290 nm as a result of ascorbate oxidation according to Nakano and Asada (1981) and Wang et al. (2009). The 2.7 ml reaction mixture contained 50 mM PBS (pH 7.4), 0.5 mM AsA, 0.1 mM EDTA, and 50 μl enzyme extract. Then, 0.3 ml of 10 mM H_2O_2 was added to initiate the reaction.

Peroxidase (POD) activity was determined according to guaiacol oxidation caused by H_2O_2 at 470 nm (Prochazkova et al. 2001). The 3 ml reaction mixture contained 100 mM PBS (pH 7.0), 20 mM guaiacol and 12 mM H_2O_2. Then, 0.1 ml enzyme extract (same volume of PBS as control) was added and scanned at 470 nm immediately. An increase in absorbance of 0.1 per minute at 470 nm was defined as one unit of enzyme activity.

AsA content

The AsA content was determined according to the method of Kampfenkel et al. (1995). Frozen leaf material (0.3 g) was ground to a fine powder in a mortar (placed on ice) that was pre-chilled using liquid N_2. Then, 4 ml of 6 % (w/v) TCA was added. The mixture was continually homogenized in an ice bath until it was completely thawed, after which it was centrifuged at $4000 \times g$ for 15 min at 4 °C. The supernatant was transferred to a reaction vessel (on ice) and adjusted to a volume of 4 ml using 6 % (w/v) TCA for AsA analysis. The assay mixture contained 0.2 ml of sample extract, 0.6 ml of 0.2 M PBS (pH 7.4), 0.2 ml of double-distilled H_2O, 1.0 ml of 10 % TCA, 0.8 ml of 4.2 % H_3PO_4, 0.8 ml of 0.4 % of 2,2′-dipyridyl dissolved in 70 % (v/v) ethanol and 0.4 ml of 3 % $FeCl_3$. The mixture was immediately and vigorously mixed after the addition of $FeCl_3$. Then, incubate at 42 °C in a water bath for 40 min, and the absorbance was read at 525 nm. The AsA content (μmol g^{-1} FW) was determined using a standard curve.

Statistical analysis

All statistical analyses were performed using Microsoft Excel 2007 software (Microsoft Corp, Richmond, CA, USA) and SPSS software (version 18.0 SPSS Inc., Chicago, IL). Multiple comparisons were carried out using one-way ANOVA followed by Duncan's multiple range test when significant differences were detected. Mean comparisons between sun and shade leaves in each period were subjected to an independent sample t test. Correlations between ROS, carotenoids and MDA were evaluated

using Pearson's correlation coefficient. Three replications were performed for all experiments.

Results

Changes in pigment contents

The chlorophyll content decreased (Fig. 3a) and the carotenoids content increased (Fig. 3b) linearly in the sun leaves as the color changed from green to red during the reddening process. In contrast, these pigments exhibited the opposite pattern during the regreening process. The chlorophyll content increased but there was no significant difference between R-r and R-m (Fig. 3c) while carotenoids declined linearly (Fig. 3d) as the color changed from red to green. In addition, the carotenoids content in the winter-red sun leaves was higher than that in the green shade leaves during the corresponding period, but the chlorophyll content was lower. Unlike the sun leaves, the chlorophyll and carotenoids contents in shade leaves did not show a linear change over the winter.
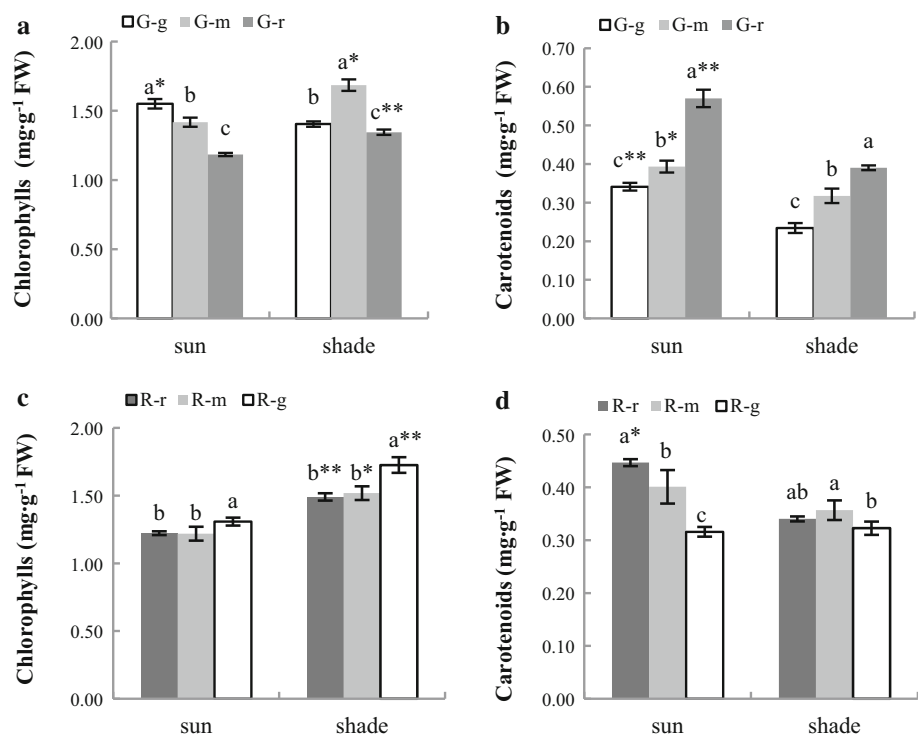
Changes in ROS and MDA

MDA accumulation has been used as an important biomarker of oxidative stress-induced damage caused by ROS. The $O_2^{-\cdot}$, H_2O_2 and MDA contents (Fig. 4a–c) in the sun leaves increased during reddening process as the winter

progressed and reached the highest values at the G-r stage, which were significantly higher than that at the G-g stage. Correlation analysis showed that the $O_2^{-\cdot}$ content in the sun leaves was significantly positively correlated with the carotenoids and MDA, and H_2O_2 was positively correlated with the carotenoids but not with MDA during the reddening process (Table 1). In addition, no significant difference was found in either the $O_2^{-\cdot}$ or MDA content between the sun and shade leaves (Fig. 4a, c). This result indicated that the changes in the $O_2^{-\cdot}$ content did not occur specifically as a result of the reddening of the sun leaves and that the two types of ecological leaves were subjected to the same oxidative damage. However, the changes in the H_2O_2 content were different; the sun leaves had consistently higher H_2O_2 levels than the corresponding shade leaves (Fig. 4b).

The $O_2^{-\cdot}$ and MDA contents in the sun leaves showed a downward trend with the lowest values measured in the R-g leaves (Fig. 4d, f), while the H_2O_2 remained relatively constant as the color changed from red to green. Correlation analysis performed on the sun leaf data indicated that $O_2^{-\cdot}$ was positively related to the carotenoids and MDA, in contrast to the H_2O_2 content, during the regreening process (Table 1). In addition, the $O_2^{-\cdot}$ and MDA contents in the sun and shade leaves were similar at the R-m and R-r stages and decreased to the lowest values at the R-g stage (Fig. 4d, f). In contrast to the sun leaves, the H_2O_2 content in the shade leaves remained unchanged initially but then increased at the R-g stage (Fig. 4e), but this value was

Fig. 3 Changes in the pigment contents of *B. microphylla* 'Wintergreen' leaves. **a**, **b** The reddening process; **c**, **d** the regreening process. *G-g*, *G-m* and *G-r* represent the *different colors* of sun leaves changing from *green* to *red* during the reddening process as winter progresses; *R-r*, *R-m* and *R-g* represent the *different colors* of sun leaves changing from *red* to *green* during the regreening process with springtime warming. Each value is the mean ± SE. *Different letters* denote significant differences at *P* < 0.05. * and ** indicate significant differences at *P* < 0.05 and *P* < 0.01, respectively, between the sun and shade leaves
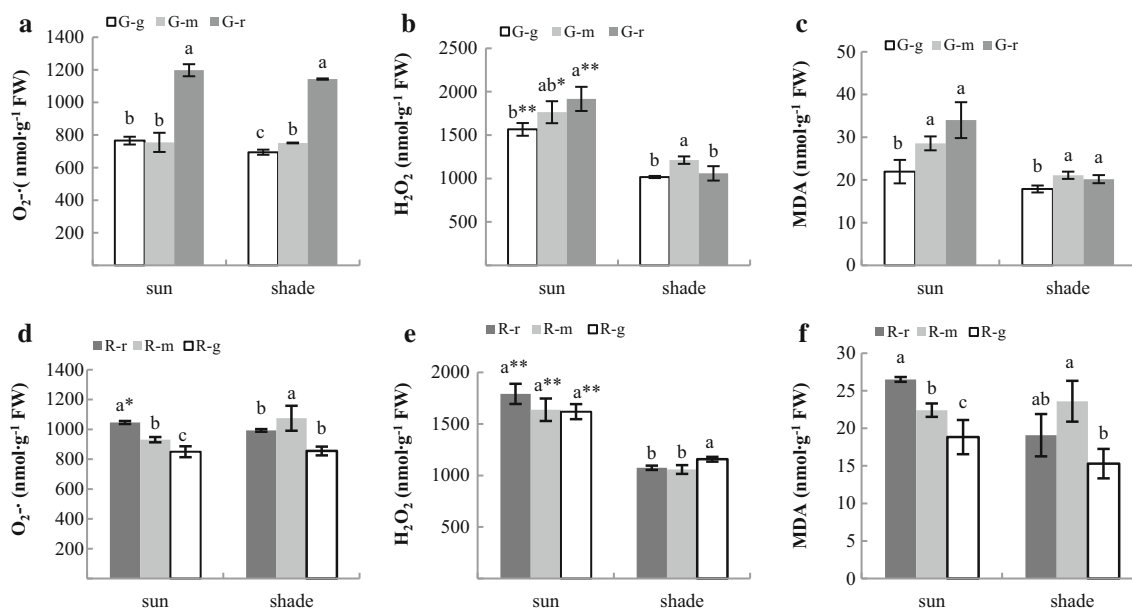
Fig. 4 Changes in the $O_2-\cdot$, H_2O_2 and MDA contents of *B. microphylla* 'Wintergreen' leaves. **a-c** The reddening process; **d–f** the regreening process. *G-g*, *G-m* and *G-r* represent the *different colors* of sun leaves changing from *green* to *red* during the reddening process as winter progresses; *R-r*, *R-m* and *R-g* represent the *different colors* of sun leaves changing from *red* to *green* during the regreening process with springtime warming. Each value is the mean ± SE. *Different letters* denote significant differences at $P < 0.05$. * and ** indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively, between the sun and shade leaves

Table 1 Pearson's rank correlation coefficients between ROS, carotenoids and MDA of *B. microphylla* 'Wintergreen' sun leaves during the leaf color changing period in winter

Color change period	Indices	$O_2^{-\cdot}$	H_2O_2
Reddening process	Carotenoids	**0.947****	**0.711***
	MDA	**0.773***	0.648
Regreening process	Carotenoids	**0.881****	0.602
	MDA	**0.958****	0.526

Bold values indicate a significant correlation between two indices

* and ** signify significant correlation at $P < 0.01$ and $P < 0.001$, respectively

much lower than that of the corresponding sun leaves (Fig. 4e).

Changes in the antioxidant system

Antioxidant system changes in the leaves of *B. microphylla* 'Wintergreen' during the reddening process are shown in Fig. 5a–e. Changes in the SOD, CAT and POD activities in the sun leaves exhibited similar trends, i.e., an increase at the G-m stage and a decrease at the G-r stage. Compared with the G-g stage, leaves at the G-r stage had lower SOD activity (Fig. 5a, b) but higher POD activity (Fig. 5d). Both the APX activity and the AsA content increased as winter progressed but the former reached a maximum value at the G-r stage (Fig. 5c) while the latter had the highest value at

the G-r and G-m stages (Fig. 5e).The above results demonstrate that the antioxidant systems were mobilized in cooperation and responded synchronously in sun leaves, with the same changes in the trends of their activity. In addition, the change in CAT activity was similar between these two ecotypes, but it was higher in sun leaves compared with the corresponding shade leaves (Fig. 5b), and no difference was found in APX activity between the sun and shade leaves (Fig. 5c). However, the SOD and POD activities and AsA content of the sun leaves were different from those of the shade leaves, in which the SOD activity continued to increase (Fig. 5a), the POD activity decreased before increasing (Fig. 5d) and the AsA content remained constant before increasing (Fig. 5e). Furthermore, the sun leaves had a higher AsA content than the shade leaves during each step of the color changing process (Fig. 5e).

The antioxidant system changes in the leaves of *B. microphylla* during the regreening process are presented in Fig. 5f–j. In the sun leaves, both the SOD and APX activities decreased at the R-m stage and then increased at the R-g stage (Fig. 5a, h). In contrast, the CAT activity increased at the R-m stage and decreased at the R-g stage (Fig. 5g). The POD activity increased linearly (Fig. 5i), but the AsA content remained steady (Fig. 5j) with spring time warming. Compared with R-g, the winter-red sun leaves (R-r) had higher CAT and APX activities (Fig. 5g, h) and lower POD activity (Fig. 5i) but the same SOD activity and AsA content (Fig. 5f, j). In addition, there was no
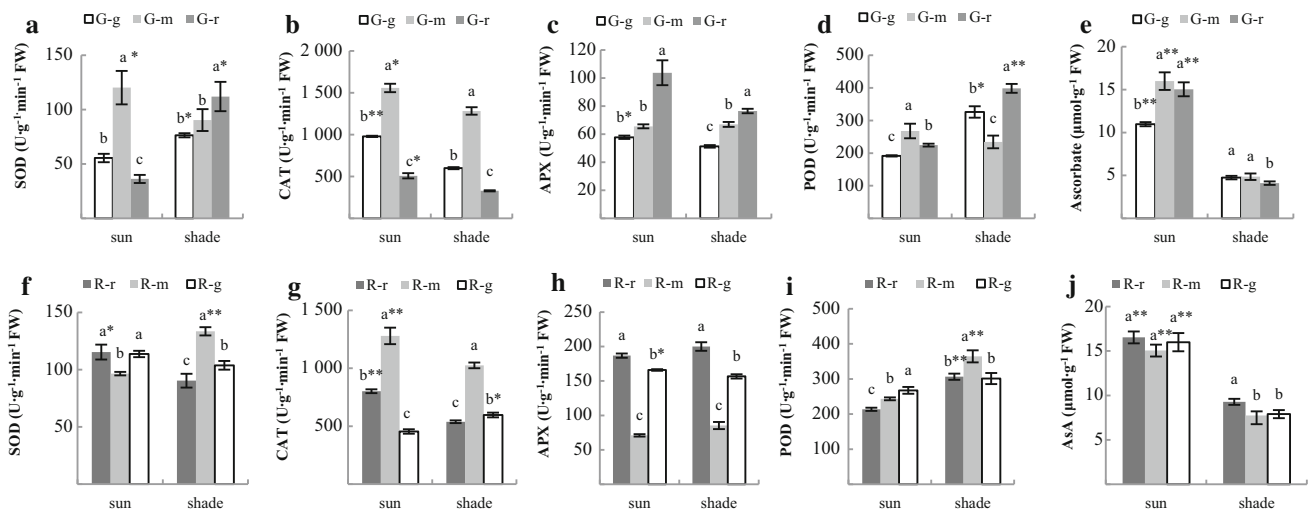
Fig. 5 Changes in the antioxidant system of *B. microphylla* 'Wintergreen' leaves. **a–e** The reddening process; **f–j** the regreening process. *G-g*, *G-m* and *G-r* represent the *different colors* of sun leaves changing from *green* to *red* during the reddening process as winter progresses; *R-r*, *R-m* and *R-g* represent the *different colors* of sun leaves changing from *red* to *green* during the regreening process with springtime warming. Each value is the mean ± SE. *Different letters* denote significant differences at $P < 0.05$. * and ** indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively, between the sun and shade leaves

difference in the change in the CAT and APX activities (Fig. 5g, h) between the sun and shade leaves, although the CAT activity was higher than that of the corresponding sun leaves. This result was similar to what occurred during the reddening process. However, the SOD and POD activities and the AsA content differed between the sun and shade leaves. In the shade leaves, the SOD and POD activities decreased after the first increase (Fig. 5f, i) and the AsA content decreased (Fig. 5j) with springtime warming. Furthermore, the sun leaves had a higher AsA content than the shade leaves at each step during the color changing process (Fig. 5j), similar to the trend in the reddening process.

Discussion

According to Koiwa et al. (1986), the winter reddening process of *B. sempervirens* leaves was accompanied by simultaneous chloroplast disintegration into chromoplasts which were restored to functional chloroplasts in regreening leaves. Consistent with their results, our study also showed similar pigment changes in *B. microphylla* 'Wintergreen' leaves during the reversible color transformation process (Fig. 3). However, during the regreening process, no difference was found in the chlorophyll content of the sun leaves at the R-r and R-m stages, though their colors were significantly different. This might be attributed to the carotenoids having already degraded without the immediate de novo synthesis of chlorophyll at the R-m stage (Ruelland et al. 2009). Therefore, the total carotenoids

content can better reflect the degree of discoloration of sun leaves compared with chlorophyll. We should point out that the Lichtenthaler and Wellburn (1983) equation for total carotenoids was usually used for the "usual" yellow carotenoids present in green leaves and it could be less precise for the quantification of red carotenoids.

Excess ROS generated under low temperatures can cause oxidative damage to plants, and this damage would be exacerbated in the presence of light (Wise 1995). In this study, there was no difference in the $O_2^{-\cdot}$ content between the sun and shade leaves in the same period, and their change trends were also similar during overwintering (Fig. 4a, d). This indicated that $O_2^{-\cdot}$ may not be involved in sun leaf winter reddening, although the $O_2^{-\cdot}$ content was positively related to carotenoids (Table 1). MDA is the ultimate product of membrane lipid peroxidation and its content is often related to the extent of oxidative damage caused by ROS. Our present findings clearly revealed that the sun and shade leaves showed similar changes in the MDA (Fig. 4c, f) content in winter, and their $O_2^{-\cdot}$ contents were positively correlated with MDA (Table 1). These findings led us to believe that sun leaves undergo winter reddening, and that were exposed to high light did not suffer from additional damage compared with the green shade leaves, and $O_2^{-\cdot}$ may have caused cell membrane lipid peroxidation.

The H_2O_2 content in the sun leaves was consistently higher than that of the corresponding shade leaves (Fig. 4b, e), suggesting that the winter photo-oxidative stress promoted the accumulation of H_2O_2 in the sun leaves. It is known that as antioxidants, carotenoids can protect

photosynthetic organisms from oxidative damage by quenching $^1O_2$ (Ramel et al. 2012; Fischer et al. 2013). According to Bouvier et al. (1998), down-regulation of CAT induced the synthesis of carotenoids in excised green pericarp discs, indicating that H_2O_2 might play an important role in mediating carotenoids synthesis by acting as a novel class of secondary messengers. Under photo-oxidative stress, H_2O_2 is sufficient for $^1O_2$-mediated oxidative damage protection though a reduction in the degree of carotenoid breakdown by the activation of de novo carotenoid synthesis (Chang et al. 2013; Iigusa et al. 2005). In our study, the reddening process was accompanied by an increase in H_2O_2 (Fig. 4b), and the level of H_2O_2 was correlated with carotenoids but not with the MDA content (Table 1). Therefore, it is conceivable that H_2O_2 may play an important role in sun leaf winter reddening by promoting carotenoids accumulation for photoprotection.

Although plants actively produce H_2O_2 as a signaling molecule, its presence above threshold levels is also toxic. Thus, plants also have scavenging mechanisms, including enzymatic and nonenzymatic antioxidants, to ensure that H_2O_2 level are controlled in cells (Shabnam and Pardha-Saradhi 2016). Located in the peroxisome, CAT directly removes H_2O_2 and is indispensable for H_2O_2 detoxification under stressed conditions (Garg and Manchanda 2009). However, APX has a higher affinity for H_2O_2 than CAT (Mittler 2002). APX is involved in scavenging H_2O_2 in water–water and AsA-GSH cycles and utilizes AsA as the specific electron donor (Gill and Tuteja 2010; Sofo et al. 2015). In our research, the APX activities of the sun and shade leaves of *B. microphylla* increased synchronously during the cold period in winter (Fig. 5c, h), whereas the CAT activities declined (Fig. 5b, g). The reduction in CAT was specific to low temperature treatments and might trigger the induction of APX by increasing the H_2O_2 level (Distelbarth et al. 2013). This indicated that APX may be more effective in maintaining the cold hardiness of both sun and shade leaves compared with CAT, which was consistent with some studies (e.g., Cansev et al. 2011). Therefore, CAT and APX were probably generated in response to low temperature stress and did not play a role in sun leaf winter reddening. The observation of higher CAT activity in sun leaves compared with shade leaves was consistent with their high accumulation of H_2O_2.

The significant differences between the sun and shade leaves with respect to the changes in SOD (Fig. 5a, f) and POD (Fig. 5d, i) activities and the AsA content (Fig. 5e, j) throughout the overwintering period suggested that these three antioxidants were not only affected by low temperature but also by high light. It is known that SOD can convert $O_2^{-\cdot}$ into H_2O_2 (Breusegem et al. 2001). In the sun leaves, the SOD activity decreased at the G-r stage (Fig. 5a), though the $O_2^{-\cdot}$ (Fig. 4a) and H_2O_2 (Fig. 4b)

contents were much higher. This may have occurred because excess H_2O_2 inhibited the SOD activity during that period (Casano et al. 1997). However, POD and AsA may enable the reactivation of SOD by reducing H_2O_2 production and through the continuous scavenging of $O_2^{-\cdot}$ (Zipor and Oren-Shamir 2013), and together these antioxidants played a role in photoprotection. POD is widely involved in plant growth and development (Passardi et al. 2005). Our research revealed that the increase in POD activity corresponded to the regreening process of the sun leaves, but the role this enzyme plays in the regreening process requires further investigation. High light can enhance the AsA content (Yang et al. 2008). Studies have also shown that AsA can maintain excess energy dissipation as a cofactor of violaxanthin de-epoxidase (Eskling et al. 1997). Therefore, a higher AsA content (Fig. 5e, j) in the sun compared with the shade leaves was not only due to the higher level of H_2O_2 (Fig. 4b, e), but was also related to the higher light energy absorption of the sun leaves.

In conclusion, H_2O_2 which was positively correlated with carotenoids but not with the MDA content during the entire overwintering period may play an important role in sun leaf winter reddening by promoting the accumulation of carotenoids. By contrast, the level of $O_2^{-\cdot}$ in the sun leaves was similar to that in the shade leaves, which suggests that it is independent of sun leaf winter reddening, but mainly causes plant cell membrane lipid peroxidation under low temperature stress. SOD, POD and AsA have a high sensitivity to light oxidative stress. They participate in regulating H_2O_2 production and play a photoprotective role in the winter-red sun leaves of *B. microphylla* 'Wintergreen' by cooperating with carotenoids. However, CAT and APX are independent of sun leaf winter reddening and their changes are probably common responses to low temperature.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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