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Anthocyanins function as a light attenuator to compensate for insufficient photoprotection mediated by nonphotochemical quenching in young leaves of *Acmena acuminatissima* in winter

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Abstract

Anthocyanins and nonphotochemical quenching (NPQ) are two important tools that provide photoprotection in plant leaves. In order to understand how plants use these tools for acclimation to changing seasonal conditions, we investigated pigments, antioxidative capacity, and photosynthesis in leaves of an evergreen tree (*Acmena acuminatissima*) in two contrasting seasons. Young leaves of *A. acuminatissima* appeared in distinct colors, being light green in summer and red in winter due to the presence of anthocyanins. In the winter young leaves, anthocyanins contributed less than 2% to the antioxidant pool. In the summer, young leaves had higher NPQ than that of mature leaves, but in the winter, they did not derive any NPQ-related advantage over mature leaves. These results suggest that the accumulation of anthocyanins in young leaves in the winter may compensate for the insufficient photoprotection afforded by NPQ and that anthocyanins function as a light attenuator to protect the photochemical apparatus against excess light.

Additional key words: chlorophyll fluorescence; evergreen tree; gas exchange; photoprotection; pigment; season.

Introduction

Lower temperatures suppress electron transport, CO_2 assimilation and photorespiration and reduce the fluidity of thylakoid membranes in plants. Photoinhibition or photodamage may occur in subtropical plants after exposure to cold atmospheric currents combined with strong sunlight, due to the absorbed light exceeding the photon utilization capacity in the photosynthetic apparatus. Therefore, plants have developed several photoprotective strategies to maintain the balance of energy flow or ameliorate the effects of excess light energy. Photoprotection of the photochemical apparatus involves mitigating the impact of excessive excitation energy and removal of oxygen free radicals formed in the chloroplast (Niyogi 1999). Nonphotochemical quenching (NPQ) is a xanthophyll cycle-dependent photoprotection tool that harmlessly dissipates excess energy captured by the photochemical apparatus as heat from the PSII antenna (Müller *et al.* 2001). The development of NPQ in plants under suboptimal temperatures is influenced by irradiance and stress duration (Kościelniak and Biesaga-Kościelniak 2006; Mai *et al.* 2010). On exposure to low temperatures for short periods (several hours), NPQ is increased to cope

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Abbreviations: C_i – intercellular CO_2 concentration; Car – carotenoid; Chl – chlorophyll; DPPH – 1,1-diphenyl-2-picrylhydrazyl; E – transpiration rate; ETR – electron transport rate; F_v/F_m – maximum photochemical efficiency of PSII; g_s – stomatal conductance; ML – mature leaves; NPQ – nonphotochemical quenching; P_N – net light-saturated photosynthetic rate; Φ_{PSII} – effective photochemical efficiency of PSII.
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with the excess light energy reaching the photochemical apparatus in leaves of rice (Hirotsu *et al.* 2004), cucumber (Hu *et al.* 2008), coffee (Guo and Cao 2004), and grapevine (Hendrickson *et al.* 2003). On exposure of leaves to low temperatures for an extended period (several days), NPQ no longer provides an advantage to dispose of excess light in *Hevea brasiliensis* (Mai *et al.* 2009). This suggests that the response of NPQ to low temperatures is complex, and not always sufficient to protect plants from the impact of harmful excess light.

As a class of water-soluble nonphotosynthetic pigments, anthocyanins also have photoprotective function in foliar and fruit peels (Liakopoulos *et al.* 2006; Steyn *et al.* 2009). Unlike the mechanism of NPQ, however, anthocyanin-mediated photoprotection occurs outside of the photochemical apparatus in plant leaves at a given development stage, particularly in stress conditions when additional photoprotection is required, *e.g.*, pathogen attack (Hipskind *et al.* 1996), nutrient deficiency (Kumar and Sharma 1999, Lea *et al.* 2007), high irradiance (Albert *et al.* 2009), and high irradiance combined with low temperature (Pietrini *et al.* 2002). Anthocyanins can serve as ideal photoprotective agents because of their spectral absorption characteristics and antioxidative properties. They modify the quantity and spectral quality of the light by absorbing the yellow, green, and blue components of visible light (Feild *et al.* 2001, Karageorgou and Manetas 2006, Hughes and Smith 2007), thereby intercepting excess light, which is otherwise absorbed by chlorophylls. The attenuation of blue light by anthocyanins may be particularly important, since blue light is especially effective in photoinactivation of PSII (Hakala *et al.* 2005, Onishi *et al.* 2005, He *et al.* 2015). Additionally, anthocyanins have a strong ability to scavenge oxygen free radicals (Zeng *et al.* 2010). Therefore, anthocyanins may function as both light attenuators and antioxidants in plant vegetative tissues depending on whether their location is in the vacuoles of the upper or lower leaf epidermis, or in the cytosol of mesophyll cells (Gould *et al.* 2000, Neill and Gould 2003). Although different views exist on photoprotection mechanism of anthocyanins, an increasing number of studies support the idea that anthocyanins afford photoprotection in leaves through functioning as a light screen that reduces the amount of light reaching chloroplasts (Neill and Gould 2003, Hughes *et al.* 2005, Tucić *et al.* 2009, Zhang *et al.* 2010, Zhang *et al.* 2016).

Acmena acuminatissima (Blume) Merr. et Perry is an evergreen tree that belongs to the Myrtaceae family. In China, it is one of the dominant canopy trees distributed in lower-latitude subtropical forests at late successional stages (Peng and Wang 1994). The present study was prompted by the observation that young leaves of *A. acuminatissima* turn red by late autumn and remain so up to early spring, but remain green during the rest of the year. The redness of the young leaves is particularly striking in winter. This annual anthocyanin pigment pattern in young leaves of *A. acuminatissima* gives the opportunity to investigate the physiological basis linked to the redness. For this purpose, changes in gas exchange, antioxidative activity, and chlorophyll fluorescence parameters were determined in *A. acuminatissima* in two contrasting seasons, summer and winter.

Material and methods

Plant material: *Acmena acuminatissima* saplings, collected from a typical subtropical evergreen forest of the Dinghu Mountain National Natural Reserve (112°30′39″–112°33′41″E, 23°09′21″–23°11′30″N), were grown in the biological garden of South China Normal University, Guangzhou, China, for ten years. The monsoon dominates the local climate, resulting in a distinct dry season from October to March. The annual mean temperature and rainfall are 21.4°C and 1,900 mm, respectively. Experiments were conducted in August of 2015 (summer) and January of 2016 (winter). The mean temperature in August 2015 and January 2016 was 30°C and 12.6°C, respectively; the maximum PPFDs of natural daylight were 1,800 and 1,100 μmol m^{-2} s^{-1}, respectively. The first, second, and third young leaves and fully expanded mature leaves were selected for study.

Pigment estimation: Three leaf discs of 10 mm diameter were punched from leaves and ground with liquid nitrogen. The leaf powder was submerged in 4 mL of 80% acetone at 4°C in the dark overnight for chlorophyll (Chl) extraction. Chl and carotenoid (Car) concentrations in the extract were spectrophotometrically assessed (*UV-2450*, *Shimadzu*, Kyoto, Japan) and calculated according to Wellburn (1994). Anthocyanin pigments were extracted from four leaf discs (10-mm diameter) in 4 mL of methanol:HCl (99:1, v/v) solution at 4°C in the dark for 24 h. Chls were removed from the extract by adding 4 mL of chloroform and 1.5 mL of purified water. After blending, Chls were dissolved in the lower chloroform phase and anthocyanins were dissolved in the upper water-methanol phase. The absorbance of the upper water-methanol phase was measured at 530 nm against methanol:HCl (99:1, v/v) as a blank. Anthocyanin concentration was calculated from a calibration curve constructed with 5–200 μM cyanidin-3-O-glucoside (dissolved in 99:1 methanol:HCl, v/v).

Total phenols were extracted from two 10-mm-diameter leaf discs in 1.5 mL of 95% methanol for 24 h at 4°C. Folin–Ciocalteu reagent was used to determine total phenol content in the methanol extracts according to a previously reported procedure (Ainsworth and Gillespie 2007). One mL of 10% Folin–Ciocalteu reagent and 2 mL of 0.7 M Na_2CO_3 were added to 0.5 mL of 20-fold diluted sample. After standing for 5 min, the absorbance of the

mixture was measured at 760 nm (*UV-2450*, *Shimadzu*, Kyoto, Japan) against the same mixture, with deionized water instead of the sample, as a blank. Gallic acid (50–250 μM) was used to construct a calibration curve for calculating concentrations of phenols.

Total flavonoids were extracted in the same way as total phenols, determined using a colorimetric method described by Heimler *et al*. (2005) with a slight modification. Aliquots of 0.2 mL of 5% $NaNO_2$, 0.3 mL of 10% $AlCl_3$ (freshly prepared), and 1 mL of 1 M NaOH solution were in turn added to 0.5 mL of 10-fold diluted sample. The final volume was adjusted to 3.5 mL with deionized water. After standing for 5 min, the absorbance of the mixture was recorded at 510 nm using a *UV-2450* spectrophotometer (*Shimadzu*, Kyoto, Japan). The amount of total flavonoids was expressed as catechin equivalents [μM cm^{-2}(leaf area)] through a standard curve. The standard curve range was in a range of 25–1,000 μM.

DPPH-scavenging capacity: Free radical-scavenging capacity of methanol extracts (two 10-mm diameter leaf discs extracted in 1.5 mL of 95% methanol) was evaluated by a 1,1-diphenyl-2- picrylhydrazyl (DPPH) test according to the previously reported procedure (Liu *et al*. 2007, Saha *et al*. 2008), where 3 mL of freshly prepared 100 μM DPPH (dissolved in 95% methanol) was added to 10 μL of the sample. The mixture was allowed to stand for 5 min at ambient temperature, and the decrease in absorbance at 517 nm (*UV-2450*, *Shimadzu*, Kyoto, Japan) was measured against a blank of 95% methanol. The DPPH radical-scavenging capacity of each antioxidant sample was expressed as μmol(DPPH) per unit area through a calibration curve established by a DPPH solution series (20–100 μM). Anthocyanin contribution to DPPH radical-scavenging capacity was calculated by using the scavenging coefficient of cyanidin-3-O-glucoside [4 μM(DPPH) μM^{-1}].

Rubisco: Total proteins were extracted from six 10-mm-diameter leaf discs in a mortar and pestle with liquid nitrogen and a little inert quartz, homogenized in 1.2 mL of 60 mM Tris-HCl (pH 7.8) buffer, containing 0.1% (w/v) NaCl, 5% (w/v) PVP, and 2% (v/v) glycerol. After centrifugation at 12,000 × *g* and 4°C for 10 min, total protein content in the supernatant was determined by the Bradford (1976) method with bovine serum albumin as the standard. To prepare for SDS-PAGE analysis, 0.1 mL of supernatant was added to an equal volume of protein loading buffer, incubated at 100°C for 5 min. Rubisco content was determined by SDS-PAGE as previously described by Zhang *et al*. (2016). A 10-μL sample was loaded onto an SDS gel (4% stacking, 12.5% resolving). The large and small subunits of Rubisco were recognized according to their molecular mass and relative abundance. Relative volume of Rubisco protein bands and bovine serum albumin series were detected by using a *TotalLab Quant* software (*TotalLab*, UK). Rubisco concentration was calculated through a calibration curve (band volume *vs*. concentration) constructed with bovine serum albumin (10–400 μg mL^{-1}).

Gas-exchange: An infrared gas analyser *Li-6400* (*LI-COR, Inc*., USA) was used to measure leaf gas exchange. Photosynthetic light-response curves were measured on young and mature leaves of four to five plants using nine descending irradiance steps [1,200; 800, 500, 200, 100, 50, 20, 10, and 0 μmol(photon) m^{-2} s^{-1}]. These irradiances were provided by red and blue LED lights integrated into the *LI-6400* leaf measurement chamber. During the measurements, reference CO_2 concentration in the leaf chamber was maintained at *ca*. 400 μmol mol^{-1}. The waiting times were 90–150 s for each irradiance. The mean relative air humidity during summer and winter measurements were 58 and 46%, respectively, and the corresponding mean temperatures were 31°C and 19°C, respectively. To ensure that plants were in a better photosynthetic state, the measurements were performed in the morning (08:30–12:00) and leaves were fully adapted for ~10 min to a saturating light intensity [1,000 μmol (photon) m^{-2} s^{-1}] provided by an LED light source. The net photosynthetic rate (P_N), intercellular CO_2 concentration (C_i), stomatal conductance (g_s), and transpiration rate (E) were automatically recorded and stored by computer systems of the device.

Chl fluorescence was determined by using a Chl fluorescence imaging system (*Technologica*, UK). Young and mature leaves were detached from five plants in the morning and kept for 30 min in the dark. The minimum fluorescence (F_0) and the maximum fluorescence (F_m) were then measured on the dark-adapted leaves by using a 6,000 μmol(photon) m^{-2} s^{-1} saturating pulse. Chl fluorescence light curves were measured by using blue actinic light (470 nm) at ten ascending PPFD steps (15, 30, 60, 100, 200, 400, 600, 800; 1,000; and 1,200 μmol m^{-2} s^{-1}). The leaves were adapted for 90 s at each irradiance, and then the corresponding fluorescence (F) and maximum fluorescence (F_m') were recorded. The maximum photochemical efficiency (F_v/F_m) of PSII was calculated as: $F_v/F_m = (F_m - F_0)/F_m$ (Oxborough and Baker 1997). The effective photochemical efficiency (Φ_{PSII}) was calculated as: $\Phi_{PSII} = (F_m' - F)/F_m'$ (Genty *et al*. 1989). Electron transport rate (ETR) was calculated as ETR = Φ_{PSII} × PPFD × 0.85 × 0.5, where the coefficient 0.85 was the leaf absorptance and the coefficient 0.5 indicated that the absorbed PPFD was equally allocated between PSI and PSII (Krall and Edwards 1992). Nonphotochemical quenching (NPQ) was calculated as NPQ = $(F_m/F_m') - 1$ (Bilger and Björkman 1990).

Statistical analysis: *IBM SPSS Statistics 19.0* (*IBM*, NY, USA) was used to perform one-way analysis of variance (*ANOVA*), and the *Tukey*'s test was used for post hoc analysis at the 0.05 level. *Sigmaplot 12.5* (*Systat Software*

Inc., USA) was used to conduct linear regression analysis and plot the data. All data are shown as means ± standard error (SE) from measurements made on four to five different plants.

Results

Anthocyanins and antioxidative capacity: Young leaves of *A. acuminatissima* displayed distinct colors in the summer and winter. Young leaves in the summer appeared light green, but winter young leaves were red (Fig. 1*A*). Beside coloration, no significant differences in leaf anatomy structure was found between summer and winter young leaves (Fig. 1*B*). The cross-section showed that the redness of winter young leaves mainly occurred in the uppermost two layers and the lowest layer of mesophyll cells (Fig. 1*B*). Winter young leaves accumulated dramatically higher anthocyanin concentrations than the summer young leaves (Fig. 2*A*). Both summer and winter young leaves exhibited greater flavonoid and phenol concentrations and DPPH-scavenging capacity than that of mature leaves (Fig. 2*B–D*). Flavonoid concentrations in winter leaves were slightly higher than in summer leaves, though there was no statistical difference (Fig. 2*B*). Differences between phenol concentrations in summer and winter leaves were also statistically insignificant (Fig. 2*C*). DPPH-scavenging capacity in developmentally earlier young leaves (first leaves) was comparable between the two seasons (Fig. 2*D*). However, the third young and mature leaves tended to have higher DPPH-scavenging capacity than the corresponding summer leaves.

Although anthocyanins belong to flavonoids, anthocyanins only comprised a very small proportion of the flavonoid pool. The biggest proportion of anthocyanins in flavonoids in *A. acuminatissima* leaves was less than 4% (Fig. 3*A*). Although anthocyanins are competent to scavenge oxygen free radicals, the largest contribution of anthocyanins to DPPH-scavenging capacity in *A. acuminatissima* leaves was less than 2% (Fig. 3*B*). Along the direction of leaf development, there was a notable positive correlation between DPPH-scavenging capacity and anthocyanin concentrations in first, second, and third young leaves and mature leaves (ML) in winter, but not in the summer (Fig. 3*C*). By contrast, DPPH-scavenging capacity was significantly and positively correlated with both flavonoids and phenols in the young and mature leaves in either summer or winter (Fig. 3*D*,*E*).

Photosynthetic pigments, proteins and gas exchange: Contents of Chl *a*, Chl *b*, total Chl, and Car were markedly lower in young leaves than mature leaves in both summer and winter (Fig. 4*A–E*). In addition, no statistically significant differences were found in Chl *a*, Chl *b*, Chl (*a*+*b*), and Car in young leaves between the two seasons. Due to Chl *b* concentrations in the winter young leaves being slightly above those of summer leaves (Fig. 4*C*), Chl *a*/*b* was significantly lower in winter than that in summer young leaves (Fig. 4*D*). Similarly, the concentration of Car in the summer young leaves was a slightly higher than that in winter leaves (Fig. 4*E*), resulting in a greater ratio of Car/Chl in the summer young leaves than that the winter leaves (Fig. 4*F*). Along the direction of leaf development, Car/Chl showed a slowly descending trend in the summer leaves, but remained unchanged in the winter leaves. In both summer and winter, young leaves had lower Rubisco content than that of the mature leaves, regardless of whether Rubisco was expressed as mass per unit of total soluble protein or mass per unit of leaf area (Fig. 5). Furthermore, young leaves in summer exhibited higher amounts of Rubisco than in winter. Under summer conditions, increasing Rubisco accumulated in the young leaves as they were growing towards maturity. Under winter conditions, however, accumulation of Rubisco was inhibited in young leaves.

Because of reduced Chl and Rubisco concentrations, photosynthetic rates were lower in the young leaves than that in mature leaves (Fig. 6). Light-saturated P_N obviously declined in mature leaves in winter, whereas it was comparable in young leaves between the two seasons (Fig. 6*A*). The low temperatures in winter caused a significant reduction in dark respiration of both young and mature leaves. C_i was slightly lower in winter than summer young leaves (Fig. 6*B*), because the net release of CO_2 declined in the winter. Young leaves demonstrated lower g_s and E than mature leaves in either summer or winter (Fig. 6*C*,*D*). Compared to values in summer, g_s and E were sharply reduced in winter leaves.

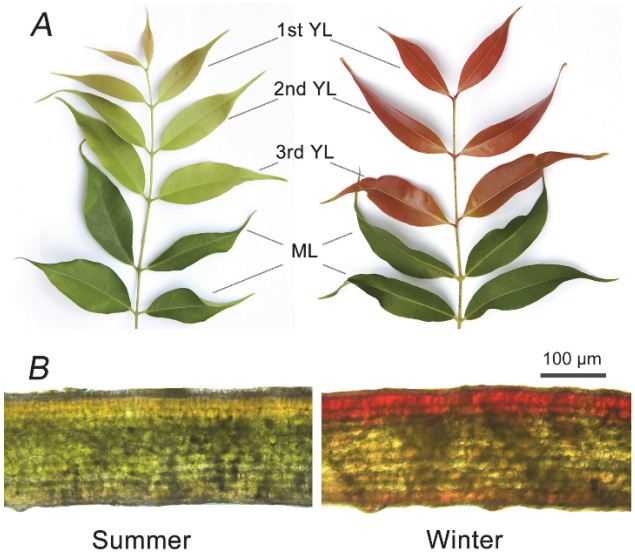

Fig. 1. The appearance of *Acmena acuminatissima* leaves (*A*) and cross-section of green and red young (first, second, and third) leaves (*B*) in summer and winter. YL – young leaves, ML – mature leaves.

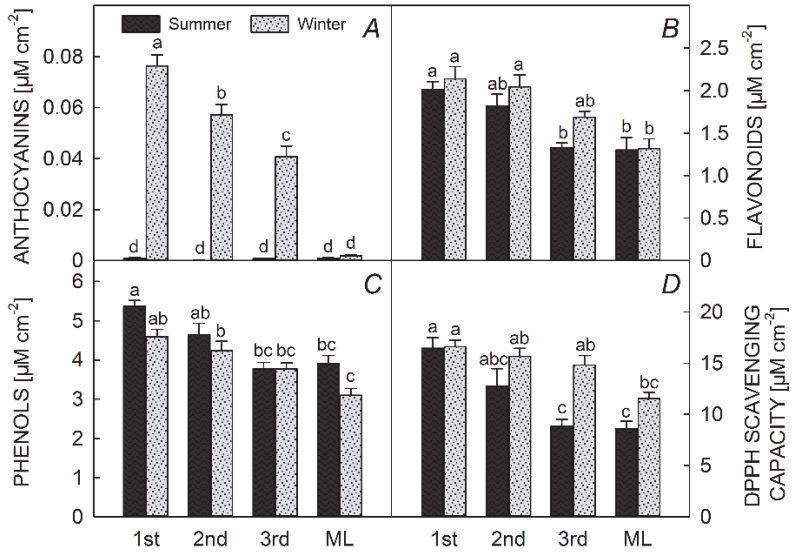

Fig. 2. Contents of anthocyanins (*A*), flavonoids (*B*), and phenols (*C*) and DPPH-scavenging capacity (*D*) in various (first, second, and third) leaves of *Acmena acuminatissima* in summer and winter. ML – mature leaves. Data are means ± SE values (n = 5). *Different letters* above bars indicate statistical significance (P<0.05).

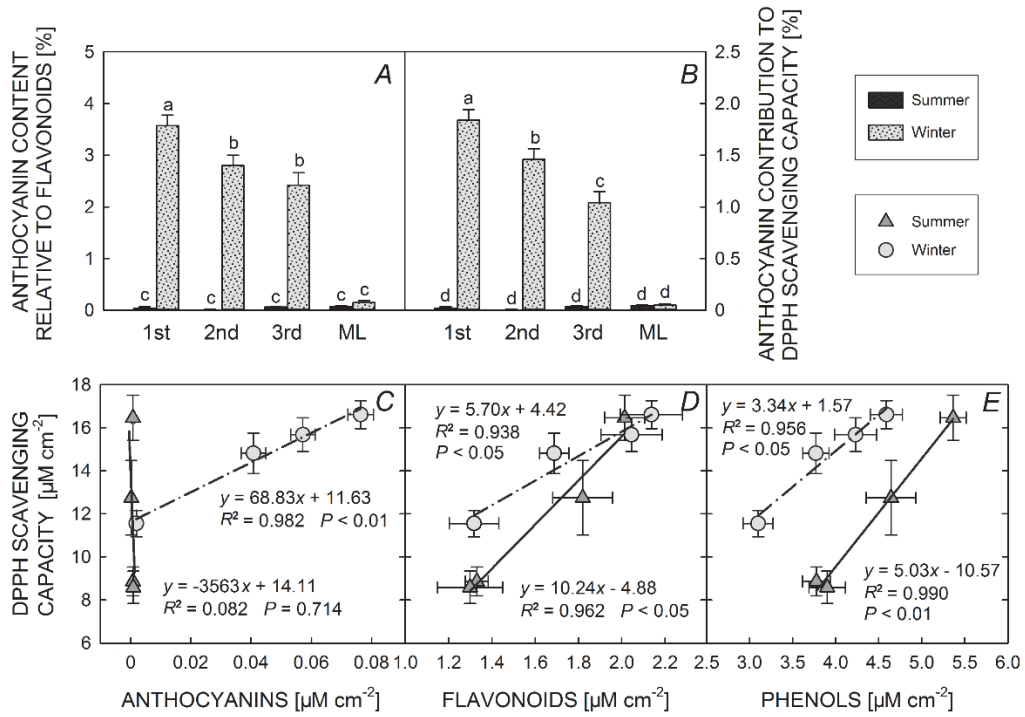

Fig. 3. The relative proportion of anthocyanins to flavonoids (*A*) and of anthocyanin contribution to DPPH-scavenging capacity (*B*), and correlations among DPPH-scavenging capacity and antioxidants (*C–E*) in various (first, second, and third) leaves of *Acmena acuminatissima* in summer and winter. ML – mature leaves. Data are means ± SE values (n = 5). *Different letters* above bars indicate statistical significance (P<0.05).

Chl fluorescence: Summer young leaves showed a slightly lower F_v/F_m than that of mature leaves (Fig. 7*A*). Compared with the value in summer, F_v/F_m was dramatically reduced in both young and mature leaves under winter conditions, and it was significantly lower in the young leaves than in mature leaves. The light-response curves showed that young leaves evidently had a lower Φ_{PSII} and ETR than that of the mature leaves in summer (Fig. 7*B*,*C*). However, an opposite tendency was observed in NPQ between the young and mature leaves in summer: young leaves had dramatically higher NPQ than mature leaves (Fig. 7*D*). In winter, the Φ_{PSII}, ETR, and NPQ decreased markedly, and they did not show differences between young and mature leaves except for Φ_{PSII} at PPFD lower than 200 μmol m^{-2} s^{-1}.

Discussion

In order to acclimate to winter conditions, leaves of *A. acuminatissima* underwent significant changes in visual appearance and physiological performance. Winter young leaves exhibited a striking redness, due to the presence of anthocyanins (Figs. 1,2). The anthocyains were mainly accumulated in the uppermost two layers of mesophyll cells of young leaves (Fig. 1*B*). This tissue distribution accords with the hypothesis that anthocyanins afford photoprotection by functioning as a light screen, which has been previously tested in *Begonia pavonina*, *Triolena hirsuta* (Gould *et al*. 1995), *Lactuca sativa* (Neill and Gould 2003), *Galax urceolata* (Hughes *et al*. 2005), *Iris pumila* (Tucić *et al*. 2009), *Begonia semperflorens* (Zhang *et al*. 2010), and *Castanopsis fissa* (Zhang *et al*. 2016). In addition to attenuating visible light, anthocyanins can also play a role in photoprotection in leaves by scavenging reactive oxygen species (Neill *et al*. 2002, Neill and Gould 2003). Although significantly greater amounts of anthocyanins were accumulated in young leaves during winter, there was not much difference in total flavonoid, phenol concentrations, and DPPH-scavenging capacity in the young leaves between winter and summer (Fig. 2). This is attributable to the fact that anthocyanins comprise only a minor proportion (less than 4%) of total flavonoids and contributed little (less than 2%) to DPPH-antioxidant capacity (Fig. 3*A*,*B*). Possibly, total flavonoids and phenols contribute a major proportion of DPPH-antioxidative capacity, because a positive correlation between DPPH-scavenging capacity and total flavonoids or phenols was found in the various leaves either in summer or winter (Fig. 3*D*,*E*). Thus, our results strongly suggest that anthocyanins primarily function as a light attenuator and only secondary as antioxidants in winter young leaves of *A. acuminatissima*. This is in agreement with previous studies showing that anthocyanins provide little enhancement of the photoprotection available in sugar maple (*Acer saccharum*) through free radical scavenging (van den Berg and Perkins 2007).

Anthocyanins were transiently accumulated in young leaves and they were degraded entirely as the leaves matured. This shows that the presence of anthocyanins is linked to photosynthetic immaturity (Hughes *et al*. 2007). Compared to mature leaves, young leaves of *A. acuminatissima* displayed significantly reduced amounts of Chl and Rubisco (Figs. 4,5). As a result, photosynthetic capacity in the young leaves was dramatically lower than that in mature leaves in both summer and winter (Fig. 6*A*). In addition, the young leaves also manifested lower g_s and E than the mature leaves (Fig. 6), suggesting that stomata were immature in the young leaves. In fact, due to increased dark respiration, C_i was higher in the young than that in mature leaves (Fig. 6*B*), indicating that limitation of g_s plays a relatively minor role in influencing photosynthetic capacity in young leaves.

It has been shown that flavonoids have a strong protective function against excess visible light (Havaux and Kloppstech 2001). In both summer and winter, young leaves had higher flavonoid and phenol concentrations and DPPH-scavenging capacity than that of mature leaves (Fig. 2), indicating that a stronger photoprotection was required for the immature photosynthetic apparatus in the young leaves. In winter, possibly due to limited uptake of nutrients (Laine *et al*. 1994), accumulation of Rubisco along leaf developmental direction in *A. acuminatissima* was limited (Fig. 5), whereas accumulation of Chl was almost unaffected (Fig. 4). This might exacerbate the imbalance between photon absorption by the photochemical apparatus and its utilization by Rubisco-dependent CO_2 assimilation in young leaves. That might explain why anthocyanins were accumulated in the winter young leaves: anthocyanins shade the photochemical apparatus from excess light, thereby maintaining the balance. This conclusion is consistent with a previous observation in *Castanopsis fissa* (Zhang *et al*. 2016). The shading effect of anthocyanins in the winter young leaves could be reflected by a smaller Chl *a*/*b* ratio (Fig. 4*D*), a characteristic related to acclimation to a low-light environment. The Chl *a*/*b* ratio in winter mature leaves was also slightly smaller than in summer mature leaves, probably because the irradiance in winter was lower than in summer.

In winter, lower temperatures are common in subtropical climates and can severely limit photosynthesis in many plants (Kratsch and Wise 2000). The response of photosynthetic performance to winter was different between the young and mature leaves. In winter, P_N obviously declined in the mature leaves, whereas it showed little change in young leaves. However, the gross photosynthetic rate decreased in young leaves in winter because a marked reduction of dark respiration resulted in a low C_i (Fig. 6*B*).

Compared to gas exchange, the changing patterns of Chl fluorescence parameters in the various leaves differed between the two seasons (Fig. 7). In summer, the young leaves exhibited evidently lower Φ_{PSII} and ETR than that of mature leaves (Fig. 7), which corresponded with photosynthetic rate. For example, due to the combined effects of high light and cold temperatures, winter markedly lowered F_v/F_m, Φ_{PSII}, and ETR in both young and mature leaves (Fig. 7). Moreover, Φ_{PSII} and ETR did not show differences between young and mature leaves (Fig. 7). Since ETR is calculated as $\Phi_{PSII} \times PPFD \times 0.85 \times 0.5$, light absorbed nonphotosynthetically by anthocycanins acting as a light screen is counted as a part of ETR. Therefore, at any given PPFD, the actual value of ETR in young winter leaves would have been smaller than in mature winter leaves, even though the apparent ETR (Fig. 7*C*) was similar. Indeed, the gross rate of CO_2 assimilation was smaller in the young winter leaves than that in mature winter leaves (Fig. 6*A*), partly because less photosynthetic radiation reached the chloroplasts in young winter leaves.

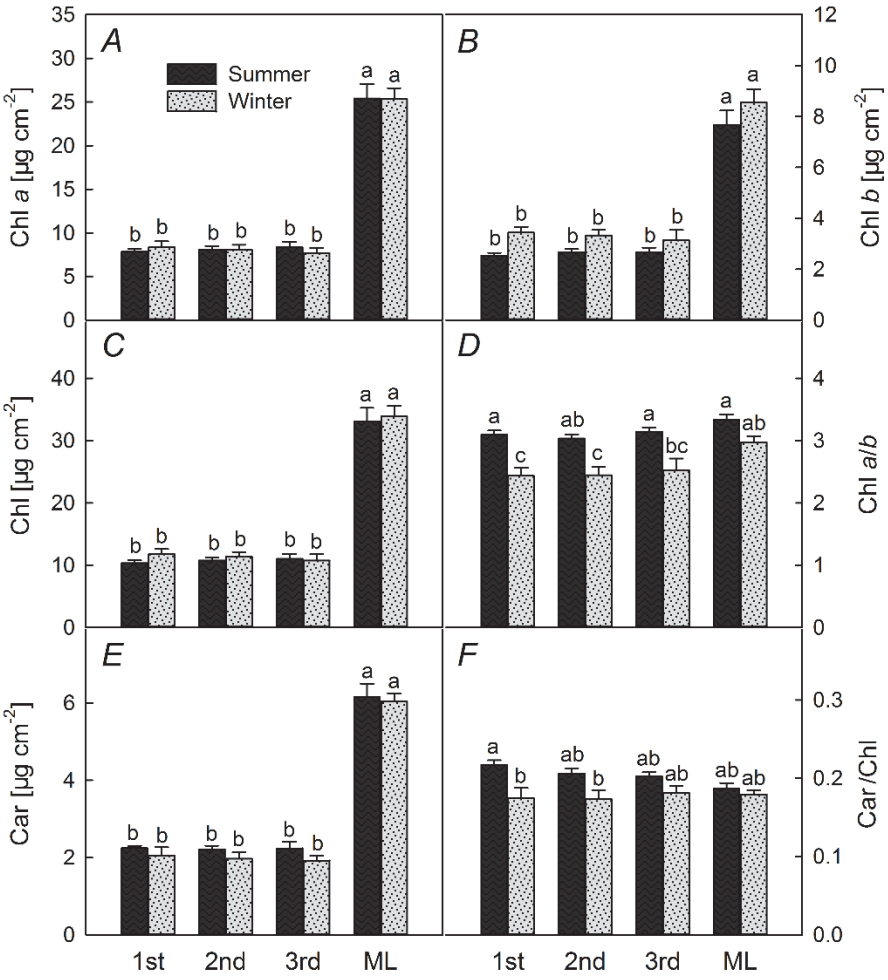

Fig. 4. Photosynthetic pigments in various (first, second, and third) leaves of *Acmena acuminatissima* in summer and winter. Chl *a* (*A*), Chl *b* (*B*), total chlorophylls (Chl *a*+*b*) (*C*), Chl *a*/*b* (*D*), carotenoids (Car) (*E*). and Car/Chl (*F*) are illustrated. Data are means ± SE values (n = 5). ML – mature leaves. *Different letters* above bars indicate statistical significance (P<0.05).

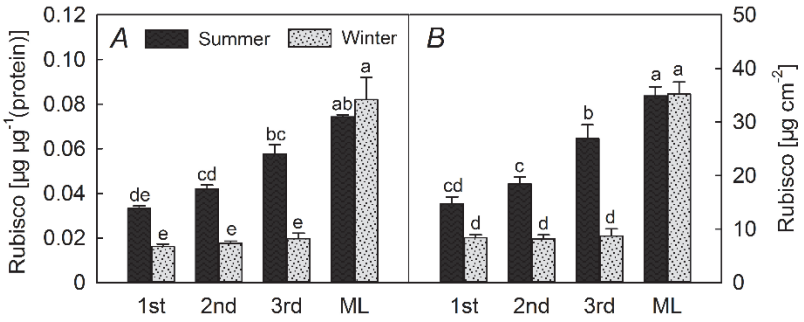

Fig. 5. Rubisco in various (first, second, and third) leaves of *Acmena acuminatissima* in summer and winter. The Rubisco large subunit is expressed as mass per unit of total soluble protein (*A*) or mass per unit of leaf area (*B*). Data are means ± SE values (n = 4). ML – mature leaves. *Different letters* above bars indicate statistical significance (P<0.05).

In summer, the young leaves exhibited higher NPQ than winter mature leaves (Fig. 7), consistently with the higher Car/Chl ratio displayed by the leaves (Fig. 4). Increased NPQ in summer young leaves might meet the requirement of photoprotection. The response of NPQ to low temperatures is complex and may vary among varieties and species, irradiances, and stress durations (Kościelniak and Biesaga-Kościelniak 2006, Mai *et al*. 2010, Wang *et al*. 2016). In winter, NPQ dramatically declined in both young and mature leaves of *A. acuminatissima*, and young leaves did not derive any advantage from NPQ over the mature leaves. A hypothesis to explain the NPQ decrease would be inactivation of enzymes associated with the xanthophyll cycle (Müller-Moulé *et al*. 2002). Loss of the contribution of NPQ to photoprotection means that the mere stronger DPPH-scavenging capacity probably could not provide sufficient photoprotection in winter young leaves. This could explain why anthocyanins were accumulated in the winter young leaves: since NPQ showed a reverse change trend to that of anthocyanins in the young leaves in two contrasting seasons, the accumulation of anthocyanins in young leaves is to compensate for the insufficient photoprotection mediated by NPQ. *A. acuminatissima* is a dominant species in subtropical forests of the late successional stages, and strong shade resistance and high responsive NPQ may

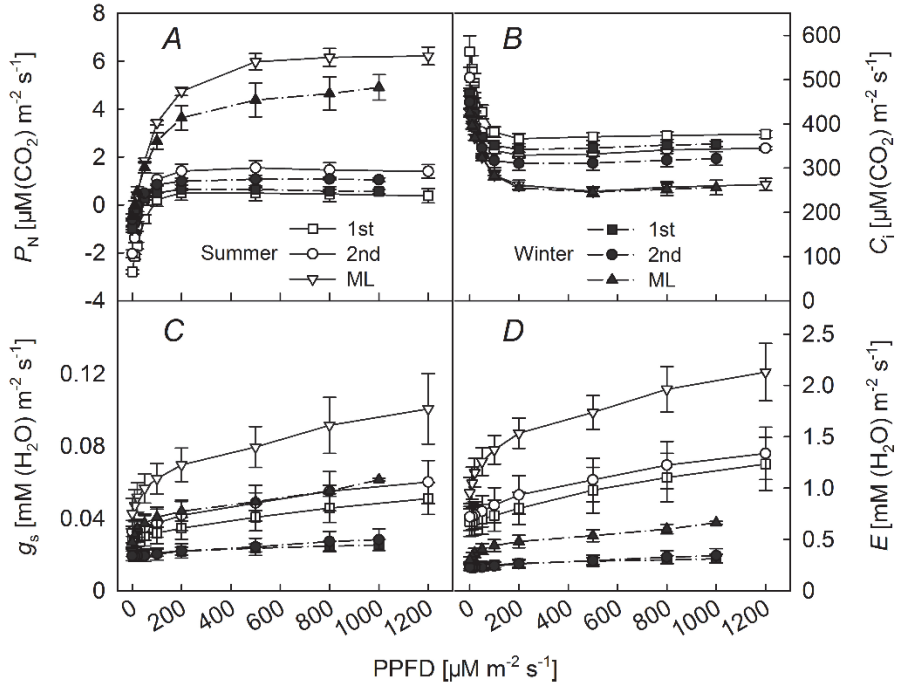

Fig. 6. Light-response curves of net photosynthetic rate (P_N, *A*), intercellular CO_2 concentration (C_i, *B*), stomatal conductance (g_s, *C*), and transpiration rate (*E*, *D*) in various (first, second, and third) leaves of *Acmena acuminatissima* in summer and winter. ML – mature leaves. Data are means ± SE values (n = 4).

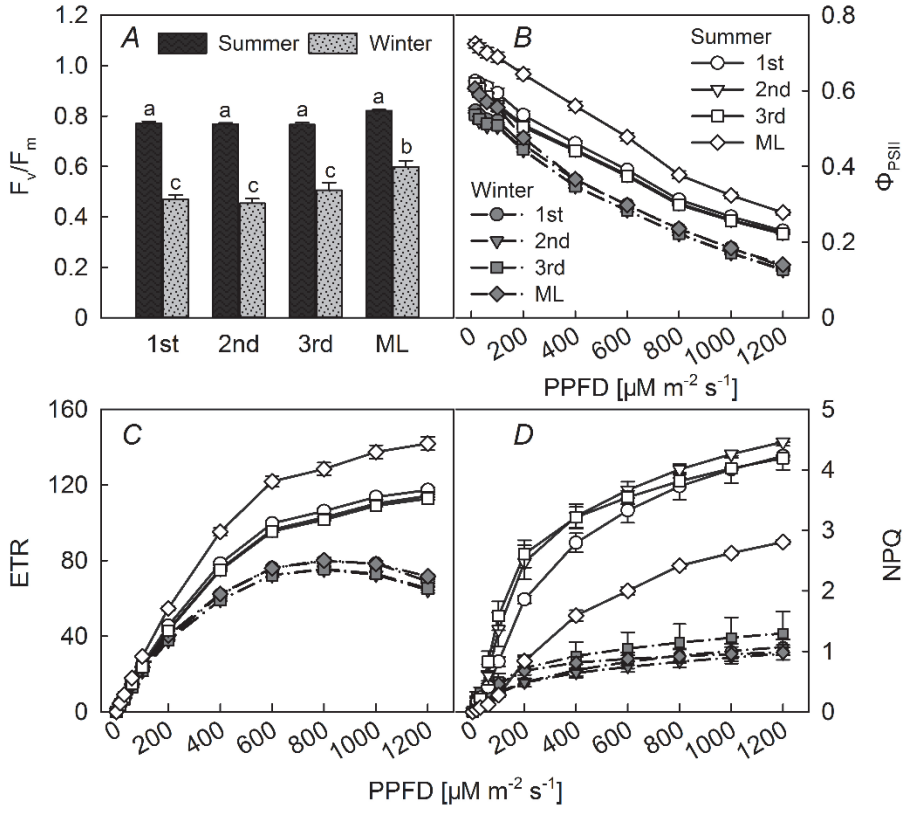

Fig. 7. Maximum photochemical efficiency (F_v/F_m, *A*), light-response curves of actual photochemical yield (Φ_{PSII}, *B*), electron transport rate (ETR, *C*), and nonphotochemical quenching (NPQ, *D*) in various (first, second, and third) leaves of *Acmena acuminatissima* in summer and winter. Data are means ± SE values (n = 5). ML – mature leaves. *Different letters* above bars indicate statistical significance (P<0.05).

contribute to its success in occupying a dominant status in the subtropical forests (Zhang *et al*. 2015). Moreover, *A. acuminatissima* is one of the few trees in subtropical forests that can grow new leaves in the winter. This pattern of leaf growth may also play an important role in helping *A. acuminatissima* to establish a dominant population in subtropical forests. Quite possibly, this leaf growth strategy in winter may be facilitated by the photoprotection provided by anthocyanins in young leaves from the challenges of stress conditions in winter.

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