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Underestimated chlorophyll *a* fluorescence measurements on *Buxus microphylla* red winter leaves

J.G. PENG[*], X.R JIANG[*], J. XU[**], L. GUO[***], R.F. GAO[#], and Y. LIU[*,+]

College of Landscape Architecture, Beijing Key Laboratory of Ornamental Plants Germplasm Innovation and Molecular Breeding, Beijing Laboratory of Urban and Rural Ecological Environment, Beijing Forestry University, Beijing, China[*]
College of Architecture and Urban Planning, Guangzhou University, Guangzhou, China[**]
Beijing Botanical Garden, Beijing, China[***]
College of Biological Sciences and Biotechnology, Beijing Forestry University, Beijing, China[#]

Abstract

Leaves under stressful conditions usually show downregulated maximum quantum efficiency of photosystem II [inferred from variable to maximum chlorophyll (Chl) *a* fluorescence (F_v/F_m), usually lower than 0.8], indicating photoinhibition. The usual method to evaluate the degree of photoinhibition in winter red leaves is generally by measuring the F_v/F_m on the red adaxial surface. Two phenotypes of overwintering *Buxus microphylla* 'Wintergreen' red leaves, with different measuring site and leaf thickness, were investigated in order to elucidate how red pigments in the outer leaf layer affected the Chl *a* fluorescence (F_v/F_m) and photochemical reflectance index. Our results showed that the F_v/F_m measured on leaves with the same red surface, but different leaf thickness, exhibited a slightly lower value in half leaf (separated upper and lower layers of leaves by removing the leaf edge similarly as affected by winter freezing and thawing) than that in the intact leaf (without removing the leaf edge), and the F_v/F_m measured on the red surface was significantly lower than that on the inner or backlighted green surface of the same thickness. Our results suggest that the usual measurement of F_v/F_m on red adaxial surface overestimates the actual degree of photoinhibition compared with that of the whole leaf in the winter.

Additional key words: boxwood; evergreen species; palisade tissue; red carotenoids; spongy tissue.

Introduction

Leaves of some evergreen species under high light usually turn red in the winter and the degree of redness increases with the light intensity (Hormaetxe *et al*. 2005, Hughes 2011). Some researchers believe that the leaf reddening in these plants is closely related to sustained photoinhibition triggered by excessive light (Han *et al*. 2003, 2004, Hormaetxe *et al*. 2004, Hormaetxe *et al*. 2007). Chl *a* fluorescence is a powerful tool for sensitive, rapid, and nondestructive measurement of photochemical and nonphotochemical processes (for reviews, *see* Roháček and Barták 1999, Papageorgiou and Govindjee 2004, Demmig-Adams *et al*. 2014, Papageorgiou and Govindjee 2014). This technique has been widely used in the study of plant photoinhibition (*see e.g*., Zhu *et al*. 2005, Roháček *et al*. 2008). F_v/F_m [where F_v (variable fluorescence) = F_m (maximum fluorescence) – F_0 (minimum fluorescence)] has been extensively used to monitor quantum efficiency of PSII (Govindjee 2004). Further, the maximum quantum efficiency of PSII (as inferred from F_v/F_m), and based on the saturation pulse mode, has become a standard indicator index to assess whether and to what extent leaves suffer from photoinhibition (Baker 2008). However, an increasing number of studies have found that the F_v/F_m value is not only related to the photochemical activity of the photosynthetic apparatus, but is also affected by leaf thickness and by a content and spatial distribution of chloroplasts in the measured leaf (Schreiber *et al*. 1996, Lichtenthaler *et al*. 2005, Warren 2006, Silva-Cancino *et al*. 2012).

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[+]Corresponding author; phone: (+86) 010 62336062, fax: (+86) 010 62338079, e-mail: Yanwopaper@yahoo.com
Abbreviations: Chl – chlorophyll; Car – carotenoids; F_m – maximum fluorescence; F_0 – minimum fluorescence; F_v/F_m – maximum quantum efficiency of PSII ($F_v = F_m – F_0$); R/G – red (adaxial)/green (abaxial); G/R – green (adaxial)/red (abaxial); PRI – photochemical reflectance index.
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J.G. Peng and X.R Jiang contributed equally to this work.

Previous studies have demonstrated that the leaf reddening in overwintering evergreens, such as *Buxus sempervirens* (Koiwa *et al.*1986, Silva-Cancino *et al*. 2012), *Cryptomeria japonica* (Han *et al*. 2003, 2004), and *Aloe arborescens* (Merzlyak *et al*. 2005), is closely related to transformation of functional chloroplasts to chromoplasts [where red carotenoids (Car) are located], which certainly affects the content and spatial distribution of Chl. However, the usual measurement of F_v/F_m values of these leaves is limited to the red adaxial surface, and thus the accuracy of such a method cannot be guaranteed. Some studies have been devoted to the impact of the measuring site and leaf thickness on the determination of fluorescence parameters (Schreiber *et al*. 1996, Vogelmann and Evans 2002, Lichtenthaler *et al*. 2005, Oguchi *et al*. 2011), while most studies have been performed on green leaves during the growing season and little research is available on the red leaves, especially those that were initially green, but turned red in overwintering evergreens due to the accumulation of red pigments (Car and/or anthocyanins) (Hormaetxe *et al*. 2004, Zeliou *et al*. 2009, Hughes 2011, Hughes *et al*. 2012).

There are several factors which affect the photochemical activity of red leaves in the winter evergreens: (*1*) the variable direction of light illuminating the adaxial (mainly palisade tissue) and abaxial (mainly spongy tissue) side; (*2*) red pigments primarily accumulating in the outer layers of leaves under direct light, while the inner or backlighted layers maintain the green color (Hormaetxe *et al*. 2005, Silva-Cancino *et al*. 2012); (*3*) leaves in some evergreens suffer from repeated freezing and thawing cycles which result in the separation of upper and lower leaf layers (Hormaetxe *et al*. 2005, Hacker and Neuner 2007). However, in the previous studies on photoinhibition of red leaves in overwintering evergreens, F_v/F_m was determined only on the adaxial surface of the leaves (Han *et al*. 2003, 2004, Hormaetxe *et al*. 2004, 2007), ignoring the possible impact of the above factors on the F_v/F_m measurements.

Boxwood is a species of evergreen perennial shrubs with remarkably wide ecological niche, which plays an important role in winter landscaping (Duc *et al*. 2000, Domenico *et al*. 2012), while their sun-exposed leaves usually turn red in winter due to accumulation of red Car (Ida *et al*. 1995, Hormaetxe *et al*. 2004). Besides, the upper layer and lower layer of these leaves usually become separated under extremely low temperatures during the winter (Hormaetxe *et al*. 2005). Therefore, we chose *Buxus microphylla* as a model species in order to study the effect of leaf thickness and measuring site on the determination of Chl *a* fluorescence parameters. In the present study, Chl *a* fluorescence and photochemical reflectance index were investigated in two phenotypes of red leaves with different measuring site and leaf thickness in overwintering *B. microphylla* 'Wintergreen'. Our aim was to reveal how the red pigments synthesized in the outer leaf layer affect the F_v/F_m measurement. Our results suggest that the usual measurement of F_v/F_m on red adaxial surface is largely underestimated compared with that of the whole leaf.

Materials and methods

Plant material, experimental site and treatments: The experiment was conducted on *B. microphylla* 'Wintergreen' which was grown in the Beijing Botanical Garden Seedling Center, China (40°01′N, 116°19′E). A total of twelve shrubs were planted in an east-west array in an open field with an average crown size of 1.3 × 1.3 m and a height of 1.4 m. Two phenotypes of sun-exposed leaves with red adaxial and green abaxial surface (R/G) and green adaxial and red abaxial surface (G/R) were studied (Fig. 1). Chl *a* fluorescence measurements were conducted on the upper surface of leaves (A–C, A–B), on the lower surface (C–A, C–B) and on the inner separated surface (B–A, B–C), respectively. Small twigs were collected between 08:00–08:30 h in December (22 December 2015, 0.6°C) and in January (21 January 2016, –6.3°C), and then placed in a loosely sealed container with ice packets and a wet paper towel, before being taken to the laboratory. All sampled leaves were healthy and taken from the middle of the current-year branches. In order to prevent the photochemical activity of leaves recovering under warm room temperature, all leaves were dark-adapted in an ice box with ambient temperature controlled between 3–5°C. Chl *a* fluorescence and reflectance measurements were performed between 09:30–10:00 h and the microscopic observations between 10:00–11:00 h.

Microscopic observations: The transverse hand-cut sections were taken from fresh leaves (both R/G and G/R) of *B. microphylla* 'Wintergreen'. The histological location of the red pigments and the thickness of the intact leaf (A–C and C–A) and half leaf (upper layer: A–B and B–A; lower layer: C–B and B–C) were examined. Five images at 2,048 × 1,536 pixel resolution and 40× magnification (4× objective and 10× ocular lens) were captured with the *LEICA DM500-ICC50 HD* camera (*Leica Microsystems*, Wetzlar, Germany). The photographs of the leaf sections were rotated and adjusted for brightness and image sharpness using *Photoshop CS3* (*Adobe Systems*, San Jose, CA, USA).

Chl *a* fluorescence parameters were measured with *PAM 2500* (*Heinz Walz*, Effeltrich, Germany) by the saturation pulse method. The measuring light was 0.1 μmol(photon) m^{-2} s^{-1} (to determine the minimum fluorescence yield, F_0) and the saturation pulse light used was 10,000 μmol (photon) m^{-2} s^{-1} (to determine the maximum fluorescence

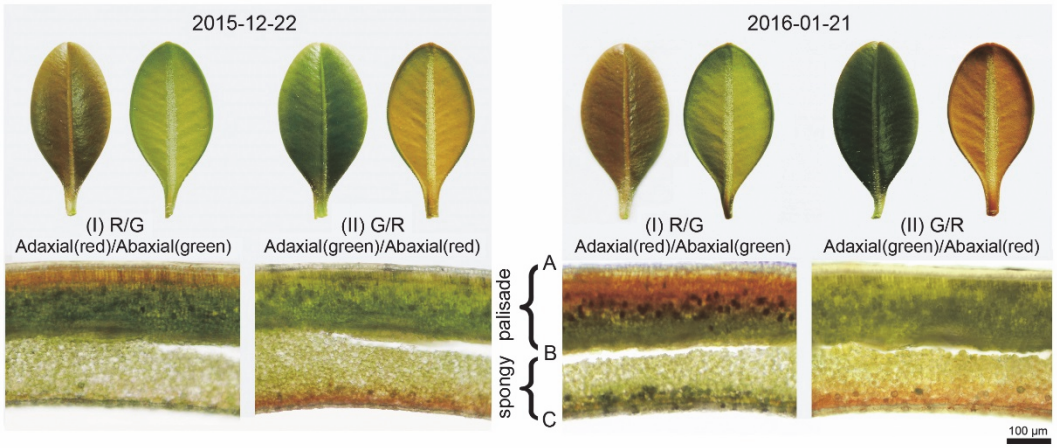

Fig. 1. Two phenotypes of winter red leaves of *Buxus microphylla* 'Wintergreen': (I) R/G, red adaxial and green abaxial surface; (II) G/R, green adxial and red abaxial surface. A, B and C respects three different surfaces in the handy-cut cross, the adaxial surface, inner separated surface and abaxial surface, respectively.

yield, F_m). At least six leaves of each treatment from different shrubs were measured and a 20-min dark adaptation was given before the measurement. All measurements were conducted under weak light [< 5 μmol(photon) $m^{-2} s^{-1}$]. The half leaf was taken from the intact leaf by removing the leaf edge (width < 1mm) and the upper layer (palisade tissue) and lower layer (spongy tissue) separated naturally (intact leaf "A–C" was divided into half leaf "A–B" and "B–C" and "C–A" was divided into "C–B" and "B–A"). To maintain a consistent distance and angle between the light source and the leaves in each measurement, leaf clips (*DLC-8*) were used to fix the leaf before the measurements. Because the saturation pulse light could affect chloroplasts in leaves and the detached leaf layers could dehydrate fast, when exposed to air, each treatment (A–C, C–A, A–B, B–A, B–C, and C–B) was taken from a different leaf and the measurement was performed as soon as possible. The maximum quantum yield of PSII efficiency was inferred from measurements of $F_v/F_m = (F_m - F_0)/F_m$.

Photochemical reflectance index (PRI): The same leaves, used for Chl *a* fluorescence determination, were used for reflectance measurements. An array spectrometer *AvaSpec-HS1024-TEC* (*AVantes*, Apeldoorn, The Netherlands) was used with an integrating sphere (fiber-optic pore diameter = 0.8 cm) as described by Bernini *et al.* (2009). The photochemical reflectance index (PRI) was calculated as: $PRI = (R_{531} - R_{570})/(R_{531} + R_{570})$ (Gamon and Surfus 1999).

Statistical analyses: Chl *a* fluorescence parameters and PRI were analyzed with *OriginPro 9.0* (*OriginLab Corp*., MA, USA). One-way analysis of variance (*ANOVA*) was performed to examine the differences in the parameters. Multiple comparisons were analyzed using *Tukey*'s HSD tests. To ascertain the correlation between PRI and fluorescence parameters (F_0, F_m, and F_v/F_m), the correlation coefficient (R^2) of the linear regression equation was compared, using the *ANOVA F*-test. We presented our results as the means ± SD, obtained from six replicates. All statistical analyses were conducted using the *SPSS 18.0* statistical software package (*SPSS Inc*., Chicago, USA).

Results

Microscopic observations: Both the leaf phenotypes of R/G and G/R in *B. microphylla* 'Wintergreen' accumulated red pigments (adaxial and abaxial layer, respectively) under direct light in the winter months more in January than in December, while the inner and backlighted layers remained green (Fig. 1). Leaf thickness determination showed that the thickness of the two phenotypes was similar in both the intact leaf (A–C or C–A) and the half leaf (A–B or B–A, C–B or B–C), but for the same phenotype, the thickness of the upper layer (palisade tissue) was greater than that of the lower layer (spongy tissue) (Table 1).

Chl *a* fluorescence characteristics: Overall, the fluorescence parameters F_0, F_m, and F_v/F_m from the two phenotypes (R/G and G/R) of *B. microphylla* 'Wintergreen' showed a similar trend in December (2015) and January (2016) (Fig. 2). For leaves with the same thickness, but measured on different leaf surfaces, R/G showed insignificant differences in F_0, F_m, and F_v/F_m between A–C and C–A in December, but in January, all parameters were significantly lower in A–C than those in C–A. Besides, both in December and January, all these parameters were much lower in A–B than those in B–A. For the results of G/R, both in December and January, all

Table 1. The leaf thickness [μm] of the two phenotypes [R/G – red (adaxial)/green (abaxial) and G/R – green (adaxial)/red (abaxial)] of sun leaves in *Buxus microphylla* 'Wintergreen' on 2015–12–22 and 2016–01–21. Data are presented as means ± SD ($n \geq 5$).

Treatment		Leaf thickness [μm]			
		2015–12–22		2016–01–21	
		R/G	G/R	R/G	G/R
Intact leaf	A–C(C–A)	324.1 ± 3.9	323.1 ± 5.8	323.6 ± 4.5	322.8 ± 6.3
Half leaf	A–B(B–A)	187.2 ± 6.2	186.8 ± 7.2	187.8 ± 5.5	186.9 ± 4.8
Half leaf	C–B(B–C)	137.2 ± 4.3	136.9 ± 5.3	136.5 ± 4.7	137.4 ± 5.1

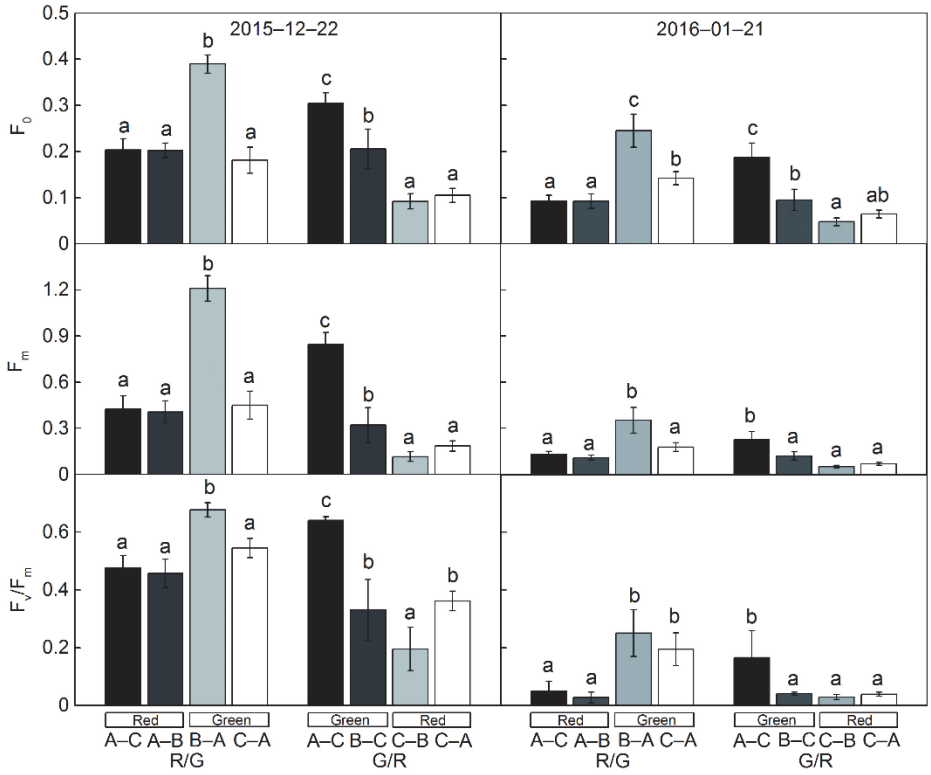

Fig. 2. The chlorophyll *a* fluorescence parameters (F_0, F_m, and F_v/F_m) for the two phenotypes of red leaves on 2015–12–22 and 2016–01–21. Different *lowercase letters* (a, b, c) indicate statistically significant differences ($P \leq 0.05$) between treatments in the same phenotype (in the same month) using *Tukey*'s HSD tests. The bars represent the means ± SD ($n = 6$). The red and green bars under the histograms represent the color of the measuring sites (red and green, respectively). R/G – red (adaxial)/green (abaxial), G/R – green (adaxial)/red (abaxial).

these fluorescence parameters were lower in C–A than those in A–C, and lower in C–B than in B–C, where no significant differences were observed in F_v/F_m between B–C and C–B. Therefore, for the leaves of the same thickness, but measured on different leaf surfaces, fluorescence parameters determined on red surface were lower than those on green surface.

For leaves with the same measuring red leaf surface but different leaf thickness, R/G showed that the F_0 was almost similar in A–B and A–C for leaves both in December and January. Although the F_m and F_v/F_m were slightly lower in A–B than those in A–C, differences were not significant. For the results of G/R, all fluorescence parameters were lower in C–B than those in C–A both in December and January. The differences were not statistically significant at the 5% level, except that of the F_v/F_m of C–B which was significantly lower than that of C–A in December. Therefore, overall, fluorescence parameters were slightly lower (but not significantly) in the half leaf than those in the intact leaf.

PRI: The results of PRI gave similar pattern as those for the fluorescence parameters (Fig. 3). For leaves of R/G, no significant differences were observed in PRI between A–C and C–A in December, but in January, the PRI was significantly lower in A–C than in C–A. Also, the PRI was significantly lower in the red layer than green layer (R/G: A–B<B–A; G/R: C–A<A–C, C–B< B–C). Besides, the PRI were almost similar in the half leaf and intact leaf (R/G: A–B and A-C; G/R: C-B and C-A).

Discussion

Chl *a* fluorescence is emitted from Chl molecules mostly in antenna of PSII; its intensity is related to the content and distribution of Chl, as well as to various other reactions (Govindjee *et al*. 1986, Schreiber *et al*. 1996, Papageorgiou and Govindjee 2004, Lichtenthaler *et al*. 2005).

To explore the relationship between the F_v/F_m and the measuring site, two phenotypes with the same leaf thickness were compared. Our results showed that both in R/G and G/R, the values of F_v/F_m were: red leaf < green leaf (R/G: A–C<C–A, A–B< B–A; G/R: C–A<A–C, C–B <B–C) (Fig. 2), suggesting that whether the red pigments accumulated on the adaxial or abaxial surface, they would downregulate the level of F_v/F_m. Earlier, Jiang *et al*. (2015) had demonstrated that the leaf reddening of *B*. *microphylla* resulted from the accumulation of Car and another species of *Buxus* (*B*. *sempervirens*) accumulated red Car on the upper mesophyll layers, which was closely related to the transformation of chloroplasts to chromoplasts (Hormaetxe *et al*. 2004, Silva-Cancino *et al*. 2012). This could certainly affect the content and distribution of Chls and accordingly, the levels of F_0 and F_m (Schreiber 1997, Campbell *et al*. 1998). Therefore, the functional chloroplast transformed into chromoplasts are definitely one of the important reasons that leads to lowering F_v/F_m at red measuring sites compared to the green ones in *B*. *microphylla* 'Wintergreen'.

Several studies have suggested that Chl *a* fluorescence measurements on green leaves may not truly reflect the actual status of the whole leaf but only the part of Chl in the upper layer (Schreiber *et al*. 1996, Warren 2006). In order to investigate the relationship between the F_v/F_m and leaf thickness in red leaves, the intact leaf and half leaf with the same measuring red site were compared. Although our results showed that the F_v/F_m was slightly lower in the half leaf [R/G (A–B), G/R (C–B)] than that in the intact leaf [R/G (A–C), G/R (C–A)], these

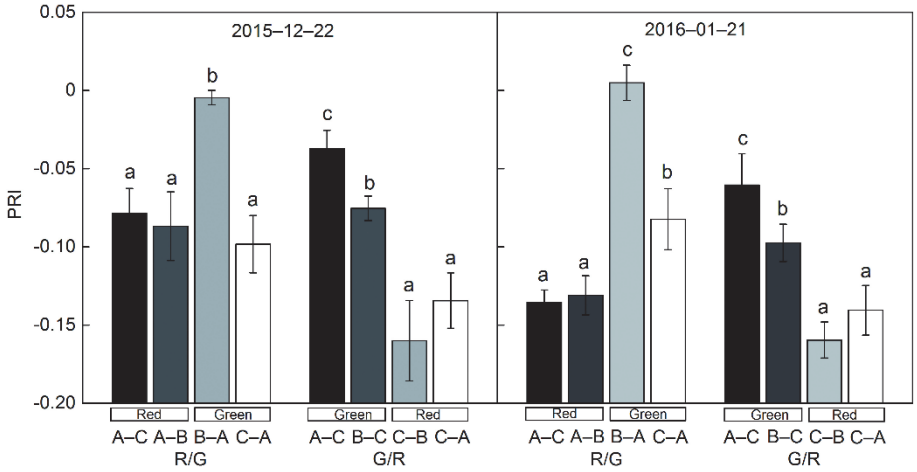

Fig. 3. The photochemical reflectance index (PRI) for the two phenotypes of red leaves on 2015–12–22 and 2016–01–21. Different *lowercase letters* (a, b, c) indicate statistically significant differences ($P \leq 0.05$) between treatments in the same phenotype (in the same month) using *Tukey*'s HSD tests. The bars represent the means ± SD ($n = 6$). The red and green bars under the histograms represent the color of the measuring sites (red and green, respectively). R/G – red (adaxial)/green (abaxial), G/R – green (adaxial)/red (abaxial).

Table 2. Correlation coefficients (R^2) between PRI and chlorophyll fluorescence parameters (F_0, F_m, and F_v/F_m) in *Buxus microphylla* 'Wintergreen' for the two phenotypes [R/G – red (adaxial)/green (abaxial) and G/R – green (adaxial)/red (abaxial)] of winter red leaves ($n \geq 12$). The asterisks indicate the statistical significance at ** $P \leq 0.01$; and *$0.01 < P \leq 0.05$ using the *ANOVA F*-test.

Treatment	Parameter	R/G						G/R					
		F_0		F_m		F_v/F_m		F_0		F_m		F_v/F_m	
		R^2	*P* value	R^2	*P* value	R^2	*P* value	R^2	*P* value	R^2	*P* value	R^2	*P* value
A–C	PRI	0.88	<0.01**	0.91	<0.01**	0.82	<0.01**	0.95	<0.01**	0.51	0.01*	0.23	0.08
A–B		0.90	<0.01**	0.91	<0.01**	0.36	0.06	–	–	–	–	–	–
B–A		0.96	<0.01**	0.89	<0.01**	0.82	<0.01**	–	–	–	–	–	–
B–C		–	–	–	–	–	–	0.83	<0.01**	0.74	<0.01**	0.57	<0.01**
C–B		–	–	–	–	–	–	0.87	<0.01**	0.68	<0.01**	0.15	0.14
C–A		0.92	<0.01**	0.63	<0.01**	0.21	0.08	0.97	<0.01**	0.91	<0.01**	0.23	0.08

differences were not statistically significant at the 5% level. Our results suggest that whether the measuring red site was the adaxial surface or abaxial surface, the F_v/F_m mainly provided information on chloroplasts on the measuring side. Besides, we noted that the amount of light reaching the inner chloroplasts become lesser as the leaf thickness increased (Oguchi *et al*. 2011, Vogelmann and Han 2000) and the photochemical activity was higher in the inner layers (or on the backlighted side) than in the outer layers (or the illuminated side) (Fig. 2, also *see* Silva-Canno *et al*. 2012). Therefore, these results confirmed that the F_v/F_m values obtained from the outer red surface could not truly provide information on all the chloroplasts in the leaves. Combined with the above analysis of the impact of measurement site on the F_v/F_m value, we believe that when red pigments accumulate on the measurement site, the measured F_v/F_m value is likely underestimated seriously when compared with the actual value in intact leaves.

Furthermore, our study also showed that both in December (2015) and January (2016), the F_0, F_m, and F_v/F_m in all treatments declined synchronously (Fig. 2) compared with those in leaves from the growing season (data not shown), which was possibly correlated with the sustained and locked-in nonphotochemical dissipation under extremely stressful conditions during the winter (Adams *et al*. 2014, Demmig-Adams *et al*. 2014, Verhoeven 2014). The PRI negatively correlated with the content of xanthophyll-cycle pigments (Weng *et al*. 2006). Our results showed that the trend of PRI was similar to that of F_0, F_m, and F_v/F_m in that the PRI with the same leaf thickness, but different measuring site, performed as: red leaf < green leaf, and no significant differences were observed in the PRI between the half leaf and intact leaf at the same measuring red site but different leaf thickness (Fig. 3). The correlation analysis on the PRI and fluorescence parameters showed that the F_0 and F_m and part of F_v/F_m were significantly and positively correlated with PRI (Table 2), suggesting that the decline of F_v/F_m in red phenotypes of *B. microphylla* 'Wintergreen' was also related to the upregulation of the xanthophyll cycle-dependent nonphotochemical dissipation. The PRI of the red phenotypes was always lower than that of the green phenotypes, implying that the accumulation of red pigments might participate in the synthesis of xanthophyll-cycle pigments which deserves further study.

In summary, the above findings suggest that the red pigments accumulating in the outer layers of *B. microphylla* 'Wintergreen' could definitely decrease the F_v/F_m by changing the content and the distribution of chlorophylls. The xanthophyll cycle-dependent nonphotochemical dissipation may also be involved in the downregulation of F_v/F_m in the winter leaves. The usual measurement of F_v/F_m on the red adaxial site would greatly overestimate the actual level of photoinhibition compared with that of the whole leaf. Therefore, we urge that the color of measuring site must be seriously considered when measuring the F_v/F_m of the red leaves in winter evergreens, especially in those species where the red carotenoids are present.

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