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RESEARCH PAPER

Xanthophyll cycle pigment and antioxidant profiles of winter-red (anthocyanic) and winter-green (acyanic) angiosperm evergreen species

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Abstract

Leaves of many angiosperm evergreen species change colour from green to red during winter, corresponding with the synthesis of anthocyanin pigments. The ecophysiological function of winter colour change (if any), and why it occurs in some species and not others, are not yet understood. It was hypothesized that anthocyanins play a compensatory photoprotective role in species with limited capacity for energy dissipation. Seasonal xanthophyll pigment content, chlorophyll fluorescence, leaf nitrogen, and low molecular weight antioxidants (LMWA) of five winter-red and five winter-green angiosperm evergreen species were compared. Our results showed no difference in seasonal xanthophyll pigment content (V+A+Z g^{-1} leaf dry mass) or LMWA between winter-red and winter-green species, indicating red-leafed species are not deficient in their capacity for non-photochemical energy dissipation via these mechanisms. Winter-red and winter-green species also did not differ in percentage leaf nitrogen, corroborating previous studies showing no difference in seasonal photosynthesis under saturating irradiance. Consistent with a photoprotective function of anthocyanin, winter-red species had significantly lower xanthophyll content per unit chlorophyll and less sustained photoinhibition than winter-green species (i.e. higher pre-dawn F_v/F_m and a lower proportion of de-epoxidized xanthophylls retained overnight). Red-leafed species also maintained a higher maximum quantum yield efficiency of PSII at midday (F'_v/F'_m) during winter, and showed characteristics of shade acclimation (positive correlation between anthocyanin and chlorophyll content, and negative correlation with chlorophyll *a*/*b*). These results suggest that the capacity for photon energy dissipation (photochemical and non-photochemical) is not limited in red-leafed species, and that anthocyanins more likely function as an alternative photoprotective strategy to increased VAZ/Chl during winter.

Key words: Anthocyanin, antioxidant, ascorbate, chlorophyll, evergreen, photoinhibition, photoprotection, red leaves, winter, xanthophyll.

Abbreviations: A_{sat}, maximum photosynthesis under saturating irradiance; AO, ascorbate oxidase; APX, ascorbate peroxidase; Chl, chlorophyll; DPPH, α,α-diphenyl-β-picrylhydrazyl; DTT, dithiothreitol; DTPA, diethylenetriaminepentaacetic acid; F_m, maximal chl fluorescence emitted when reaction centres are fully reduced in the dark-acclimated state; F'_m, maximal chl fluorescence emitted when reaction centres are fully reduced in the light-acclimated state; F_o, minimum chl fluorescence emitted in the dark-acclimated state; F'_o, minimum chl fluorescence emitted in the light-acclimated state; F_v, variable fluorescence in the dark-acclimated state—calculated as (F_m–F_o); F'_v, variable fluorescence in the light-adapted state—calculated as (F'_m-F'_o; F_v/F_m, maximum quantum yield efficiency of PSII in the dark-adapted state—calculated as (F_m–F_o)/F_m); F'_v/F'_m, maximum quantum yield efficiency of PSII in the light-adapted state—calculated as (F'_m–F'_o)/F'_m); LHC, light harvesting complex; LMWA, low molecular weight antioxidants; NPQ, non-photochemical quenching—calculated as (F_m–F'_m)/F'_m); PAR, photosynthetically active radiation; PSII, photosystem II; ROS, reactive oxygen species; VAZ, violaxanthin+antheraxanthin+zeaxanthin; AZ/VAZ, (antheraxanthin+zeaxanthin)/(violaxanthin+antheraxanthin+zeaxanthin).

Introduction

Photoprotection during winter

Evergreen plants have evolved a broad range of physiological adaptations enabling extended photosynthetic carbon gain during the winter months (for reviews see Tranquillini, 1964; Nilsen, 1992; Öquist and Huner, 2003; Adams *et al.*, 2004). Adaptations that allow photosynthetic tissues to avoid and/or dissipate excess light energy during the cold, winter months are especially important for reducing photo-oxidative damage (Krause, 1994). Briefly, low temperatures inhibit the carboxylation reactions of the Calvin–Benson cycle but do not affect photon capture and electron transport; this imbalance in energy absorption versus photochemical-processing results in a greater proportion of closed reaction centres, increased energy and electron transfer to molecular oxygen by chlorophyll, production of radical oxygen species (ROS), and ultimately photo-oxidative damage (Baker, 1994; Hüner *et al.*, 1998; Mittler, 2002). Therefore, evergreen species with diminished capacity for carbon fixation during winter must up-regulate photoprotective mechanisms to alleviate the potentially harmful imbalance between the capture and processing of photon energy (Verhoeven *et al.*, 1999; Adams *et al.*, 2002, 2004).

Non-radiative energy dissipation is a strategy used by all plants in which excess excitation energy is diverted away from P_{680} in photosystem II (PSII) and dissipated as heat. This can be accomplished via several mechanisms—the physical dissociation of the light harvesting complex (LHC) from photosystem II (PSII), de-activation of the D1 protein in PSII, and the xanthophyll cycle (Björkman and Demmig-Adams, 1994; Ottander *et al.*, 1995; Adams *et al.*, 2001, 2002; Rosenqvist and van Kooten, 2003). Because these processes are competitive with photochemistry, they are collectively termed non-photochemical quenching (NPQ). As might be expected, evergreen plants commonly increase all components of NPQ during winter, with the greatest increases in plants exposed to the highest irradiances (Logan *et al.*, 1998; Cavender-Bares *et al.*, 1999, 2005; Verhoeven *et al.*, 1999, 2005; Close *et al.*, 2003; Adams *et al.*, 2004). Antioxidants represent a second line of defence by which plants may curtail photo-oxidative damage once ROS have formed (Grace and Logan, 1996; Kytridis and Manetas, 2006). Up-regulation of antioxidants (e.g. Mehler-peroxidase pathway) has been reported in cases of high light stress (Grace and Logan, 1996; Logan *et al.*, 1998; Garcia-Plazaola *et al.*, 2004), and may also vary seasonally, concomitant with increased vulnerability to photo-oxidative stress (Esterbauer and Grill, 1978; Anderson *et al.*, 1992; Polle *et al.*, 1996; Garcia-Plazaola *et al.*, 1999).

While NPQ and antioxidants appear to be ubiquitous in the plant kingdom, the synthesis of anthocyanin pigments during periods of high light stress is not. Anthocyanins are vacuolar pigments responsible for the red, purple, and blue colouration of plant tissues in many plant species, and have been implicated as playing a photoprotective role in photosynthetic tissues (for reviews see Chalker-Scott, 1999; Gould, 2004; Archetti *et al.*, 2009; Hughes, 2011). Their presence results in a conspicuous red to purple colouration of leaves, and has been reported in leaves under high light in combination with cold stress (Close *et al.*, 2002; Hughes and Smith, 2007*a*, *b*; Kytridis *et al.*, 2008), drought stress (Spyropoulos and Mavormmatis, 1978; Sherwin and Farrant, 1998; Yang *et al.*, 2000), and photosynthetically-vulnerable stages of leaf ontogeny (Feild *et al.*, 2001; Lee *et al.*, 2003; Karageorgou and Manetas, 2006; Hughes *et al.*, 2007). *In vivo,* the anthocyanic layer intercepts up to 43% incoming photosynthetically active radiation (PAR), primarily in the 500–600 nm waveband (Pietrini and Massacci, 1998). This 'sunscreen' effect has been shown to reduce photoinhibition of photosynthesis in subjacent cells (Feild *et al.*, 2001; Hughes *et al.*, 2005; Liakopoulos *et al.*, 2006; Hughes and Smith, 2007*b*). Increasing evidence also suggests anthocyanins function as *in vivo* antioxidants, neutralizing hydrogen peroxide that crosses the vacuolar tonoplast (Gould *et al.*, 2002; Kytridis and Manetas, 2006). Given the increased vulnerability to photo-oxidative damage inherent in winter photosynthesis, it is not surprising that many angiosperm evergreen species synthesize anthocyanins in winter leaves. Why, then, do only some species exhibit this winter colour change, while others do not?

Winter redness versus greenness

Previous studies attempting to define a common stress factor that unifies species undergoing winter-reddening have thus far been unsuccessful (see Hughes, 2011, for a review). Hughes and Smith (2007*a*) tested whether a limited capacity for winter photosynthesis could be linked to winter reddening, as reduced energy sinks might render plants more vulnerable to increased light stress, thus warranting additional protection from anthocyanin pigments. However, no difference in seasonal photosynthetic carbon gain (i.e. photosynthetic gas exchange under saturating irradiance) was observed between winter-red and winter-green species in the Appalachian mountains, USA, and, the highest winter photosynthesis reported in the study was in a winter-red species (*Lonicera japonica*). Hughes *et al.* (2010) examined the possible relationship between winter anthocyanin production and drought tolerance, as low leaf water potentials are known to induce anthocyanin synthesis (Spyropoulos and Mavormmatis, 1978; Sherwin and Farrant, 1998; Yang *et al.*, 2000). Here too, red and green-leafed species overlapped considerably in daily water potentials and their degree of physiological acclimation to drought stress (e.g. cell wall hardening, osmotic adjustment). Kytridis *et al.* (2008) examined winter-red versus winter-green morphotypes of the Mediterranean evergreen *Cistus creticus.* This intra-specific study showed that anthocyanic phenotypes had smaller pools of xanthophyll pigments throughout the year compared with green phenotypes, as well as decreased nitrogen and photosynthetic inferiority. Their results suggested that anthocyanins play a compensatory, photoprotective role when capacity for non-photochemical quenching and/or photosynthesis is limited. While some components of this idea have been tested at

the inter-specific level (photosynthetic capacity; Hughes and Smith, 2007*a*), others (xanthophyll cycle pigment pool size, leaf nitrogen, NPQ) have not. Comparisons of these, and other, photoprotective mechanisms at the inter-species level could therefore be helpful not only for testing the photoprotection hypothesis for winter colour change, but also for determining why some species exhibit winter colour change while others do not.

In the current study, ten angiosperm evergreen species were examined, including five species which synthesize anthocyanin in winter leaves, and five which do not. These species were previously characterized for seasonal photosynthetic capacity in Hughes and Smith (2007*a*). Xanthophyll cycle pigments, chlorophyll fluorescence, and low molecular weight antioxidants (LMWA) are compared here during both summer (before colour change) and winter (after). If anthocyanins function in photoprotection, then signs of acclimation to decreased light stress should be observed in winter-red species compared with winter-green, but only during winter, when anthocyanins were present. Seasonal comparisons were also used to determine whether winter-red species were intrinsically deficient in other photoprotective constituents, such as xanthophyll cycle pigments and/or antioxidants, relative to winter-green species.

Materials and methods

Sites and species

Field sites and species used were the same as those described in Hughes and Smith (2007*a*). Winter red-leafed species included two clonal groundcover herbaceous species—*Galax urceolata* (Poir.) Brummitt and *Gaultheria procumbens* (L.), one vine species—*Lonicerajaponica* (Thunb.), and two shrub species—*Leucothoe fontanesiana* (Steud.) Sleumer and *Rhododendron* sp. (a horticultural azalea). Winter green-leafed species included one vine species—*Vinca minor* (L.), and four shrub species—*Rhododendron catawbiense* (Michx.), *Kalmia latifolia* (L.), *Rhododendron maximum* (L.), and *Rhododendron* sp. (a horticultural azalea). Sun-exposed, first year leaves were used in all measurements. In shrub species (all Ericaceae), first year leaves comprised the most apical whorl. Measurements were taken on south-facing branches of shoots: at midday [1300 h and 1500 h on sunny days (<10% cloud cover)], and pre-dawn (0100 and 0300 h). Field temperatures were derived from a local field station, approximately 8 km from the study site, archived online at http://www.wunderground.com/weatherstation/WXDailyHistory.asp?ID=KNCCROSS1.

Pigment analyses

Leaf tissues were collected at pre-dawn on one summer day (5 September 2007: high, 26 °C; low, 9 °C) and at pre-dawn and midday on one winter day (25 February 2008: high, 13 °C, low, 0 °C). Six 1.1 cm^2 hole-punches were derived from one leaf of five separate individuals, and immediately frozen in foil envelopes in liquid nitrogen; tissues were stored at –80 °C until analysis. Chlorophylls and carotenoids were extracted from one leaf disc, anthocyanins were extracted from a second disc, and % water content was determined with a third (in order to estimate % dry mass for leaf tissues of other samples).

For chlorophyll and carotenoid extractions, the fresh mass of the frozen disc was measured first. The disc was then immediately ground in 2 ml 80% acetone and centrifuged for 2 min at 13 000 rpm. The pellet was re-extracted in 1 ml 100% acetone and centrifuged for 2 min at 13 000 rpm. The pooled supernatant was filtered through 0.45 micron nylon filters (Millex- HV, Millipore Filter Corp., Bedford, MA). Chromatography was carried out on an Agilent 1200 Series system with a diode array detector and quaternary pump. Pigments were separated on an Allosphere ODS-1 column (5 μm particle size, 250×4.6 mm). The flow rate for pigment separation was 2 ml m^{-1} with a 20 μl injection. Solvent programmes were adapted from Gilmore and Yamamoto (1991). Solvent A (acetonitrile:methanol:0.1 M TRIS pH 8.0) (76:17:7 by vol.) was run for 6 min, a 2 min gradient was run from Solvent A to Solvent B (4:1 v/v, methanol:hexane), then Solvent B was run for 4 min, before a 12 min column equilibrium of Solvent A. Peaks were detected at 445 nm and assessed using ChemStation Software (Agilent Technol., Palo Alto, CA).

Anthocyanins were extracted in 1 ml of 1% HCl in methanol, and quantified spectrophotometrically as A_{530}–0.24A_{653} with an extinction coefficient of 30 000 l mol^{-1} cm^{-1} (Murray and Hackett, 1991) using a Hewlett Packard 8453 UV-VIS spectrophotometer (Hewlett Packard, Palo Alto, CA). All pigment contents were expressed on a dry mass basis (rather than leaf area) because leaves of different species varied substantially in leaf thickness.

Chlorophyll fluorescence

Chlorophyll *a* fluorescence measurements were derived on the same days and times as tissues were sampled for pigment analyses, though different individual leaves were used. For same-day measurements (i.e. F_v/F_m and Fv'/Fm'), individual leaves were tagged and re-sampled. A PAM Fluorescence System (Hansatech Institute, model FMS-2, Cambridge, UK) emitting a 2 s long, 3 mmol m^{-2} s^{-1}, amber (594 nm) saturating pulse was used to derive dark-adapted pre-dawn maximum quantum yield efficiency of PSII, or F_v/F_m. F_v was calculated as F_m–F_o. At midday, quantum yield efficiency of PSII in the light, or Fv'/Fm' (or (Fm' – Fo')/ Fm')), was measured by applying the measurement clip to sun-exposed leaves, and quickly applying the measuring clip (resulting in 1–2 s of darkness as the measurement head was applied). From F_v/F_m and Fv'/Fm', Stern-Volmer non-photochemical quenching (NPQ) at midday was calculated for individual leaves as (Fm – Fm')/ Fm') (Rosenqvist and van Kooten, 2003). One leaf from five to seven separate individual plants was sampled for each species.

Antioxidants

Total low molecular weight antioxidants (LMWA) and ascorbic acid (total and reduced) were quantified at midday on two summer days (2 August 2007: high, 25 °C; low, 14 °C; and 5 September 2007: high, 26 °C; low, 9 °C) and one winter day (27 February 2007: high, 10 °C; low, –3 °C). Whole leaves were excised from branches, placed in cryogenic bags, and immersed in liquid nitrogen within 10 sec of excision; tissues were frozen at –80 °C until analysed.

LMWA were quantified using the stable radical α,α-diphenyl-β-picrylhydrazyl (DPPH), as described in Hughes *et al.* (2005). Briefly, 20 mg of freeze-dried tissue was extracted in 4 ml of MeOH:H_2O:acetic acid (70:23:7 by vol.) for 18–24 h at 4 °C in the dark. The solution was centrifuged for 2 min and the supernatant was assayed at varying concentrations to determine the concentration of leaf extract (μg dry wt ml^{-1}) needed to neutralize 180 μM DPPH by 50% (IC_{50}).

Levels of ascorbate (an important antioxidant in the Mehler-peroxidase pathway) were also measured, as ascorbate levels are known to be particularly sensitive to light stress (Grace and Logan, 1996). Reduced ascorbate was quantified using an ascorbate oxidase (AO) assay and the proportion of the ascorbate pool in an oxidized state was quantified by assaying with dithiothreitol (DTT) (Luwe *et al.*, 1993; Burkey *et al.*, 2006). Two hundred mg of frozen, ground, fresh tissue were extracted in 1:10 volume (mg:ml) of 6% (w/v) meta-phosphoric acid, 0.2 mM diethylenetriaminepentaacetic

acid (DTPA) extraction buffer for 10 min on ice. The extraction solution and tissue were then centrifuged at 20 000 *g* for 10 min at 4 °C. A 0.5 ml aliquot of the supernatant was centrifuged and used for analyses. Independent experiments based on the addition of known quantities of commercial ascorbic acid during tissue extraction showed that 87±11% (mean ±standard deviation for the ten species) was recovered in the reduced form, thus demonstrating this protocol efficiently extracted ascorbate without significant changes in redox state.

For quantification of reduced ascorbate, 5 µl of the extraction solution was combined with 0.995 ml of KPi buffer (pH 7.0 100 mM KH_2PO_4 in ddH_2O), and A_{265} nm was measured. Two µl of 1 U μl^{-1} AO (1000 U ascorbate oxidase in 1.0 mlL KPi) was then added, and after 2 min, reduced ascorbate was quantified as ΔA_{265} using the extinction coefficient at 265 nm of $\epsilon 265nm = 14.3\ mM^{-1}\ cm^{-1}$. For assay of oxidized ascorbate 5 µµl of 200 mM DTT was used in the place of AO in the described protocol, and reaction time was 5 min instead of 2 min.

Leaf nitrogen

Ten mg of homogenized, freeze-dried tissue left over from LMWA analyses were analysed for percentage leaf nitrogen and carbon:-nitrogen ratios using a CHN 2400 Elemental Analyzer (Perkin Elmer Corporation, Norwalk, CT). A NIST (National Institute of Standards and Technology) standard was also run every 22 samples to ensure accuracy of measurements.

Statistics

Normality for all data was assessed using the Shapiro–Wilks test, and determined as $P > 0.05$. Log_{10} and square root transformations were used to normalize data when $P < 0.05$. Photopigment content for winter-red and winter-green species was compared using a nested MANOVA (species nested within colour). Separate tests were run for summer pre-dawn, winter pre-dawn, and winter midday measurements. Fluorescence parameters (F_v/F_m, Fv'/Fm', and NPQ) were analysed similarly. Pre-dawn summer and winter pigment levels were compared within individual species using a two-tailed, Student's *t* test assuming equal variance. Antioxidants (LMWA, ascorbate, and per cent reduced ascorbate) for red-leafed versus green-leafed species were each analysed using a Kruskal–Wallis test, due to a non-normal distribution of the data. Seasonal changes in antioxidant levels within species were made using a Student's *t* test, and compared using late summer (September) versus winter levels. The correlations between anthocyanin, chlorophyll content, and chlorophyll *a*/*b* were determined using regression analyses, pooling data from both pre-dawn and midday tissues.

To determine roughly whether our diverse phylogenetic sampling muddled the results, analyses were repeated using only species within the Ericaceae, which included three green-leafed species (*Rhododendron* spp., *Kalmia latifolia*), and three red-leafed species (*Gaultheria procumbens, Leucothoe fontanesiana,* and *Rhododendron* sp.).

Results

Pigment analyses

Mean values for each species are listed in Table 1; averages for leaf-colour groups are illustrated in Fig. 1. During summer, when leaves of all species were green, there was no significant winter-colour effect on xanthophyll cycle pigment pool size (violaxanthin+antheraxanthin+zeaxanthin, or VAZ) on a per chlorophyll basis ($P=0.11$). On a per unit dry mass basis, winter-red species had a marginally higher VAZ content ($P=0.07$). Per cent de-epoxidation of xanthophyll pigments (AZ/VAZ) did not differ between winter-red and winter-green species during summer ($P=0.99$). Winter-red species had significantly greater amounts of total chlorophyll (chlorophyll +*b*) ($P=0.003$) and β-carotene ($P<0.001$) during summer, but there was no significant winter-colour effect on chlorophyll *a*/*b* ($P=0.47$) (Fig. 1B, D, respectively). Similar results were derived when non-Ericaceae were excluded from data analyses (data not shown), suggesting that the results were probably not significantly influenced by the diverse phylogeny of our sampled community.

During winter, after colour change had occurred, there was no significant winter-colour effect on VAZ content on a dry mass basis either at pre-dawn or midday ($P=0.42$ and $P=0.36$, respectively), but winter-green species had significantly higher VAZ on a chlorophyll content basis ($P<0.001$) at both pre-dawn and midday (Fig. 1A, C, D). Red-leafed species had significantly higher chlorophyll content than winter-green species at both pre-dawn and midday ($P=0.038$ and $P<0.001$, respectively), and significantly lower chl *a*/*b* ratios ($P<0.0001$ at pre-dawn and midday). Per cent xanthophyll de-epoxidation (AZ/VAZ) was significantly higher in green-leafed species at pre-dawn during winter ($P<0.0001$) but not during midday ($P=0.57$). Anthocyanin content had a significant negative correlation with chlorophyll *a*/*b* ($r^2=0.26$, $P=0.003$) and a significant positive correlation with total chlorophyll ($r^2=0.13$, $P=0.04$) (Fig. 2).

During the winter, two of the five winter-green species showed significant increases ($P<0.05$) in xanthophyll cycle pigment content on a dry mass basis relative to summer (*R. catawbiense* and *R. maximum*), while one showed a significant decrease (*V. minor*); none of the winter-red species exhibited significant changes in VAZ between seasons (Table 1). All species except *R. catawbiense* and *R. maximum* showed significant declines in average total chlorophyll content between summer and winter (ranging from 35% to 60%). All winter-green species except *V. minor* had significant increases in chlorophyll *a*/*b* from summer to winter, while no winter-red species exhibited any significant change (Fig. 1D). Four of the five winter-green species showed significant increases in average VAZ on a per chlorophyll basis during winter relative to summer, as did all winter-red species (the exception was *V. minor*) (Table 1).

Chlorophyll fluorescence

Consistent with the pre-dawn xanthophyll (AZ/VAZ) results, pre-dawn F_v/F_m values of winter-red species were significantly higher than those of winter-green species during winter ($P<0.0001$) (Fig. 3A), but not during summer ($P=0.18$) (data not shown). At midday during winter, red-leafed species also had significantly higher Fv'/Fm', and higher NPQ than green-leafed species ($P<0.0001$ for both; Fig. 3C, E).

Antioxidants

There was no significant association between winter leaf colour and total low molecular weight antioxidants (as measured by DPPH assay) during either mid-summer

Table 1. Mean seasonal (summer and winter) pigment data by species, with standard deviation
Values represent means of 4–6 individuals. PD, pre-dawn; MD, midday. All carotenoids = nmol/g dry tissue; chl a+b (μmol/g dry tissue); VAZ/Chl (mmol/mol) with SD; anthocyanin nmol g^{-1}.

Red Species	Season	Time	Neox.	Lutein	β-caro.	VAZ	(AZ)/(VAZ)	Chl a + b	Chl a/b	VAZ/Chl	Anthocya.
Leucothoe	Summer	PD	203 (48)	421 (110)	422 (94)	370 (35)	.147 (.059)	7.13 (1.8)	1.89 (.05)	.054 (.01)	-
fontanesiana	Winter	PD	141 (57)	322 (110)	148 (59)	285 (110)	.413 (.079)	3.25 (1.2)	1.73 (.18)	.088 (.01)	3. 2 (1.2)
		MD	129 (38)	351 (100)	186 (59)	253 (20)	.903 (.052)	3.90 (1.5)	1.90 (.31)	.072 (.03)	2.67 (2.0)
Galax	Summer	PD	256 (37)	580 (99)	493 (87)	329 (68)	.269 (.095)	9.52 (1.3)	1.77 (.09)	.034 (.01)	-
urceolata	Winter	PD	206 (48)	519 (130)	233 (56)	360 (110)	.433 (.130)	6.24 (1.3)	1.63 (.06)	.057 (.01)	4.86 (2.9)
		MD	188 (43)	467 (130)	196 (11)	321 (50)	.888 (.078)	5.20 (1.3)	1.71 (.18)	.064 (.02)	6.00 (1.4)
Lonicera	Summer	PD	165 (37)	312 (35)	355 (34)	364 (74)	.139 (.017)	5.80 (0.4)	2.04 (.30)	.064 (.02)	-
japonica	Winter	PD	96 (18)	315 (56)	264 (52)	298 (38)	.315 (.053)	3.75 (0.5)	2.18 (.16)	.081 (.01)	3.18 (.66)
		MD	94 (40)	320 (110)	240 (120)	242 (85)	.865 (.057)	3.54 (1.6)	2.19 (.04)	.070 (.01)	2.97 (.77)
Rhodo.	Summer	PD	182 (68)	451 (150)	439 (180)	312 (92)	.186 (.030)	6.83 (2.6)	1.94 (.17)	.047 (.01)	-
sp.	Winter	PD	106 (26)	348 (20)	198 (25)	347 (27)	.335 (.046)	2.95 (2.1)	2.06 (.18)	.118 (.01)	5.4 (0.25)
		MD	112 (26)	330 (29)	180 (11)	278 (50)	.779 (.070)	2.93 (3.8)	2.04 (.14)	.095 (.01)	3.6 (1.2)
Gaultheria	Summer	PD	165 (48)	376 (97)	394 (110)	436 (130)	.241 (.010)	6.01 (1.6)	1.94 (.12)	.073 (.01)	-
procumbens	Winter	PD	143 (55)	338 (110)	176 (45)	328 (80)	.408 (.140)	3.64 (1.0)	1.95 (.14)	.092 (.01)	5.28 (.80)
		MD	107 (15)	285 (15)	160 (22)	274 (9.7)	.840 (.040)	3.37 (.05)	1.97 (.09)	.083 (.01)	3.45 (1.4)
Rhodo.	Summer	PD	103 (44)	287 (87)	206 (99)	249 (57)	.216 (.046)	3.89 (1.5)	1.99 (.19)	.072 (.04)	-
catawbiense	Winter	PD	108 (17)	347 (70)	182 (17)	380 (81)	.669 (.120)	3.38 (0.5)	2.63 (.25)	.110 (.02)	-
		MD	84 (10)	295 (26)	148 (22)	356 (47)	.938 (.018)	2.83 (0.5)	3.16 (.32)	.127 (.01)	-
Rhodo.	Summer	PD	147 (12)	462 (65)	266 (89)	379 (31)	.198 (.034)	5.10 (0.6)	1.92 (.07)	.075 (.01)	-
sp.	Winter	PD	89 (24)	296 (43)	200 (41)	379 (40)	.719 (.064)	2.97 (0.6)	2.55 (.31)	.130 (.02)	-
		MD	58 (12)	237 (29)	90.9 (49)	331 (35)	.859 (.086)	2.16 (0.3)	2.89 (.38)	.150 (.01)	-
Kalmia	Summer	PD	167 (22)	430 (70)	402 (74)	422 (92)	.164 (.045)	6.14 (0.9)	2.09 (.07)	.068 (.01)	-
latifolia	Winter	PD	94 (33)	448 (77)	207 (49)	408 (140)	.507 (.060)	3.18 (1.0)	2.80 (.60)	.129 (.02)	-
		MD	86 (10)	410 (28)	180 (5.9)	337 (47)	.800 (.090)	2.94 (0.2)	2.82 (.31)	.114 (.01)	-
Vinca	Summer	PD	233 (65)	563 (110)	371 (70)	321 (73)	.210 (.050)	8.21 (2.2)	1.81 (.04)	.042 (.02)	-
minor	Winter	PD	130 (29)	356 (56)	180 (36)	219 (32)	.480 (.090)	4.15 (1.0)	1.63 (.11)	.056 (.02)	-
		MD	67 (8)	224 (29)	77 (18)	168 (36)	.850 (.040)	1.84 (0.4)	1.51(.11)	.091 (.00)	-
Rhodo.	Summer	PD	102 (10)	299 (51)	225 (35)	211 (58)	.190 (.076)	2.97 (0.6)	2.43 (.23)	.117 (.02)	-
maximum	Winter	PD	101 (19)	389 (68)	199 (53)	344 (62)	.553 (.057)	3.63 (0.3)	1.89 (.089)	.057 (.01)	-
		MD	83.8 (22)	305 (54)	177 (14)	253 (17)	.763 (.014)	2.55 (0.4)	2.28 (.39)	.10 (.01)	-

(P=0.80), late summer (P=0.78), or winter (P=0.68) (Fig. 4A). Within species, LMWA significantly increased during winter (compared with late summer) in only four species—the green-leafed *R. catawbiense,* and the red-leafed *L. fontanesiana*, *G. procumbens*, and *Rhododendron* spp. (P <0.02 for each); others maintained similar LMWA levels throughout the year.

Summer (green) leaves of winter-red and winter-green species did not significantly differ in ascorbate content during mid-summer (P=0.29), but by late summer, winter-red species had significantly lower ascorbate contents than winter-green species (P <0.01) (Fig. 4B). During winter, red and green-leafed species did not differ in ascorbate content (P=0.50). Within species, ascorbate levels significantly increased during winter in only three species—the red-leafed *L. japonica*, *G. urceolata*, and *Rhododendron* species (P <0.01 for each); others maintained similar ascorbate levels throughout the year. When ascorbate pools were analysed in terms of redox status, winter-red species had a significantly lower percentage of reduced ascorbate, on average, compared with winter-green species during mid and late summer (P <0.01 and P <0.02, respectively). During winter, red-leafed species also had a lower percentage of reduced ascorbate, on average, although this difference was only marginally significant (P=0.11) (Fig. 4C).

Discussion

Do anthocyanins function in photoprotection?

If foliar anthocyanins intercept a significant proportion of incident photosynthetically active radiation (PAR), species which undergo winter reddening should exhibit less photoinhibitory stress and, perhaps, even symptoms of shade acclimation, relative to species which remain green, but only during seasons when anthocyanins are present. Consistent with this hypothesis, red-leafed species had lower xanthophyll content on a per chlorophyll basis and less sustained photoinhibition (i.e. higher pre-dawn F_v/F_m and lower AZ/VAZ retained overnight) during winter relative to green-leafed species. These trends were not observed during the summer, when all leaves were green (Figs 1, 3). The xanthophyll cycle involves enzymatic conversion of violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z) in the PSII antennae complex in response toe accumulation of

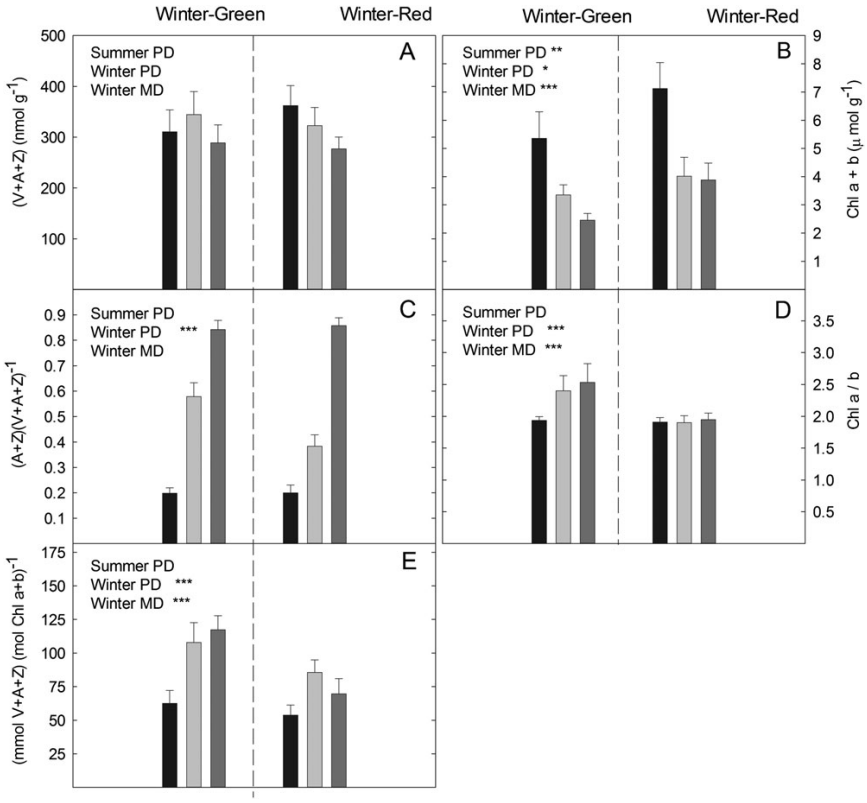

Fig. 1. Mean seasonal pigment data for pre-dawn (PD) and midday (MD). Black bars illustrate mean summer pre-dawn values, grey bars illustrate mean winter pre-dawn values, light bars illustrate mean winter midday values. Bars represent means of winter-red or winter-green species data for 4–5 individuals; error bars represent standard error. Significant differences between winter-green (left group) and winter-red (right group) species are denoted with asterisks (*0.1>*P*>0.01; **0.01>*P*>0.001; ***$P<0.001$).

protons in the lumen (Demmig-Adams and Adams, 1996; Eskling *et al.*, 1997; Gilmore, 1997). Acidification of the lumen also induces binding of the PsbS protein to the de-epoxidated xanthophylls (A and Z), which helps trigger dissipation of the excitation energy as heat (Eskling *et al.*, 1997; Li *et al.*, 2002). Therefore, higher proportions of AZ/VAZ generally indicate increases in engaged photoprotection, and retained AZ/VAZ overnight reflects sustained photoprotection, usually in response to long-term stress (Demmig-Adams and Adams, 1996; Gilmore, 1997; Verhoeven *et al.*, 1999). In addition to lower AZ/VAZ in winter-red leaves, there was also a significant, positive correlation between anthocyanin content and chlorophyll content, and a significant, negative correlation with chlorophyll *a*/*b* (Fig. 2). This suggests that anthocyanins caused a shading effect in associated tissues, as high chlorophyll content and low chlorophyll *a*/*b* are symptoms of shade acclimation (Cui *et al.*, 1991; Grace and Logan, 1996; Demmig-Adams, 1998). Additional evidence for shade acclimation in these species was given in Hughes and Smith (2007*a*), where light-response curves of winter-red species showed greater quantum yield of photosynthesis at low PAR, and a lower light saturation point, relative to winter-green species during winter, but not during summer, when all leaves were green.

There are some caveats, however, that should be considered before concluding that anthocyanins function in photoprotection. For example, although red-leafed species did show symptoms of shade acclimation during winter, some of these (e.g. higher total chlorophyll) were also observed during the summer when all leaves were green (Fig. 1B). Why winter-red species would exhibit higher chlorophyll content year-round is unclear. However, the significant correlation between anthocyanin, chlorophyll, and chlorophyll *a*/*b* contents observed during winter does suggest that anthocyanins enhanced pre-existing differences that may have been present prior to colour change (Fig. 2). Another point to consider is that, although red-leafed species did have significantly lower AZ/VAZ retained overnight during winter (indicating less sustained photoinhibition), the proportion of AZ/VAZ at midday was similar between winter-red and winter-green species (Fig. 1C). As might be expected, the increase in NPQ between pre-dawn and midday (measured via chlorophyll fluorescence) was also significantly greater for red-leafed species than green during winter (Fig. 3E). These combined results suggest that red-leafed species utilized more of the rapidly and/or intermediately reversible components of non-photochemical quenching between pre-dawn and midday than green-leafed

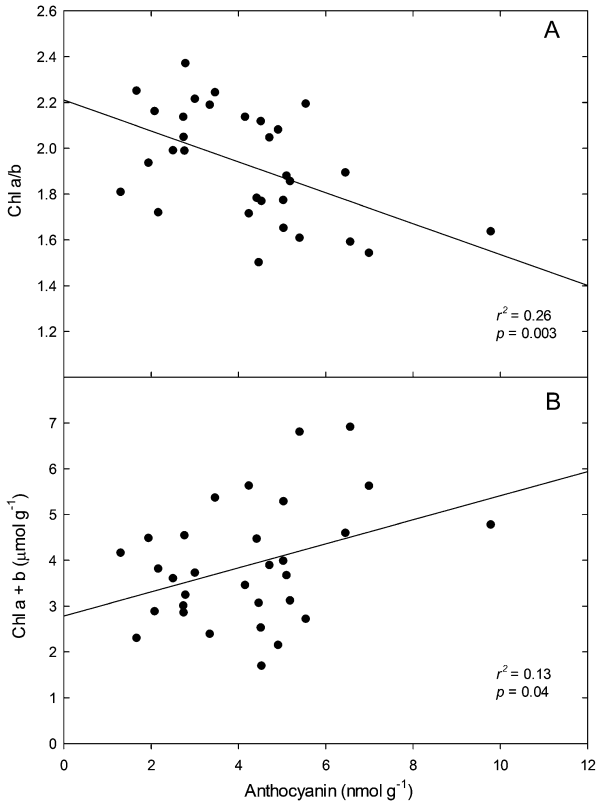

Fig. 2. Correlation between anthocyanin and chlorophyll *a*/*b* (upper) and total chlorophyll (lower). Points represent individuals, and 5–8 individuals are present for each of five winter-red, evergreen angiosperm species. Points represent pigment values from combined pre-dawn and midday winter measurements.

species. This may be a consequence of a smaller proportion of VAZ/Chl in red-leafed species (Table 1; Fig. 1), resulting in a higher proportion of de-epoxidation required for adequate photoprotection. The sustained de-epoxidation of xanthophylls at night in winter-green species but not winter-red may also be attributed to greater damage to PSII, and consequent formation of protective chlorophyll/carotenoid protein complexes (Gilmore and Ball, 2000).

Regarding the antioxidant analyses, if anthocyanins were functioning in photoprotection (either through light-attenuation or antioxidant activity), it would be expected that red-leafed species would have smaller LMWA pools during winter than green-leafed species because free radicals would (presumably) be less prolific. This hypothesis was based on the fundamental assumption that winter stress would result in an up-regulation of antioxidants relative to summer, in response to increased vulnerability to photoinhibition and photo-oxidative damage (Esterbauer and Grill, 1978; Anderson *et al.*, 1992; Polle *et al.*, 1996). However, in the species tested here, LMWA levels remained relatively constant throughout the year, and only significantly increased in four of the 11 species measured (Fig. 4A). When ascorbate (an important reductant in the water–water cycle) was analysed specifically, similar results were observed (Fig. 4B); ascorbate levels were only observed to increase significantly during winter in three of the 11 species tested relative to late summer values. Verhoeven *et al.* (2005) also reported no significant increases in seasonal ascorbate between summer and winter in *Taxus*×*media*, and suggested that the water–water cycle was not as important in winter-acclimation as NPQ because antioxidant activity is largely impeded by cold temperatures.

It might also be anticipated that LMWA would be higher when anthocyanins were present (i.e. winter versus summer leaves of winter-red species), as anthocyanins are a component of the LMWA pool. The fact that LMWA content significantly increased in only half of the red-leafed species measured during winter (compared with late summer) suggests that the contribution of anthocyanin to the LMWA pool is not substantial enough to be detected against the larger background pool of other LMWA. Thus, antioxidant data were not as informative as expected in this study, and could not be used to infer a photoprotective function for anthocyanins.

Do winter-red species have a lower capacity for other photoprotective mechanisms?

Previous studies have suggested that individuals or species which undergo winter colour change correspond to those with the greatest need for photoprotection during winter, due to either (i) seasonal reductions in capacity for photosynthesis and/or (ii) a reduced capacity for other photoprotective strategies (e.g. non-photochemical quenching, antioxidants) (Hughes and Smith, 2007*a*; Kytridis *et al.*, 2008). The former component (i) was tested in Hughes and Smith (2007*a*) on the same species studied here, where it was shown that winter-red species exhibited a similar range of photosynthetic capacities as winter-green species during both summer and winter (based on photosynthetic gas exchange measurements). It was also demonstrated here that winter-red species are not deficient in leaf nitrogen relative to winter-green species (in contrast to red versus green morphotypes of *C. creticus* reported by Kytridis *et al.*, 2008) (Table 2). It therefore appears that, on average, winter-red species do not have a significant reduced capacity for winter photosynthesis.

In the current study, the second component (ii) of this hypothesis was tested —that winter-red species compensate for a diminished capacity for non-photochemical quenching and/or antioxidants with anthocyanins. As described previously, during winter, red- and green-leafed species exhibited no significant difference in VAZ/dry mass, and during summer, winter-red species had marginally significantly *larger* pools of xanthophyll cycle pigments (VAZ/dry mass) on average (P=0.07). These results indicate that red-leafed species are most probably not limited in their capacity for synthesis of xanthophyll cycle pigments, relative to winter-green species. When xanthophyll content was expressed on a per chlorophyll basis, however, VAZ/Chl of red-leafed

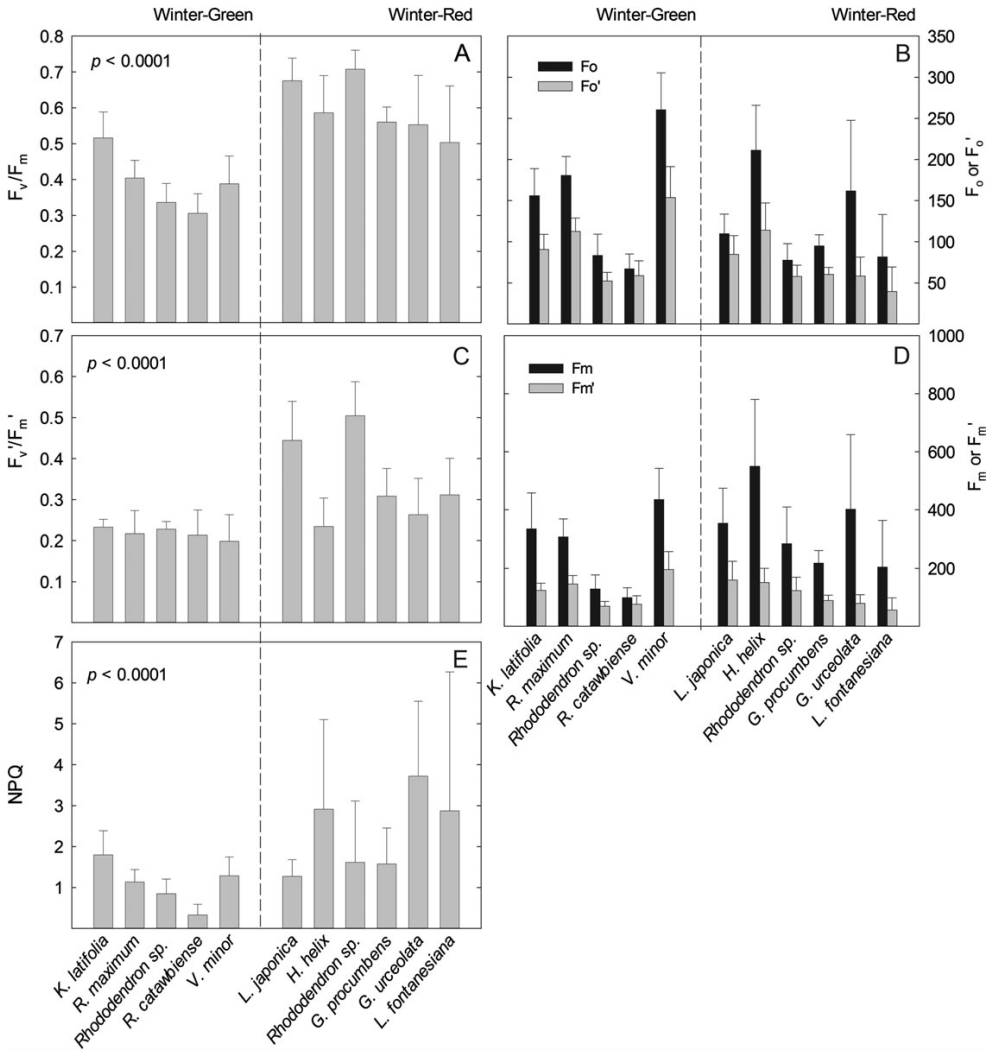

Fig. 3. Winter chlorophyll *a* fluorescence measurements at pre-dawn (F_v/F_m) and midday (Fv'/Fm' and NPQ). Bars represent means of 5–7 replicates +SD.

species was significantly *lower* than green-leafed species during winter (similar to the findings of Kytridis *et al.*, 2008). However, this was apparently due to greater chlorophyll content in red-leafed species relative to green-leafed species, rather than lower VAZ (Fig. 1; Table 1). Antioxidant analyses also showed that winter-red species were not intrinsically deficient in LMWA during summer or winter, as winter-red species exhibited a similar range of ascorbate and LMWA levels in both seasons compared to the winter-green species (Fig. 4). It is also worth noting that the species with both the lowest VAZ content (on a dry mass basis) and LMWA content during winter was green-leafed (*V. minor*), indicating that even the lowest levels of these photoprotective components combined did not result in winter reddening of leaves.

From these results, it is clear that winter-red species cannot be unified based simply on a reduced capacity for xanthophyll cycle or LMWA production. Yet, according to our proposed explanation for winter colour change, a reduced capacity for energy sinks in general (i.e. either photoprotection *or* photosynthesis) should render an evergreen species to be in greater need for photoprotection by anthocyanins during winter. Unfortunately, testing this assumption by simply combining photosynthesis and photoprotection data is complicated by the known trade-off between photosynthetic capacity and relative need for photoprotection (Osmond, 1981; Powles, 1984). For example, a species with high photosynthetic capacity (A_{sat}) should also exhibit a relatively low engagement of photoprotection due to a greater capacity for energy dissipation via photosynthetic photochemistry and carboxylation. In such a case, low AZ/VAZ, VAZ, or LMWA might be considered more a reduced need than a deficiency in photoprotection. True deficiencies in such a trade-off system are therefore difficult to determine, especially using inter-species comparisons as a relative scale

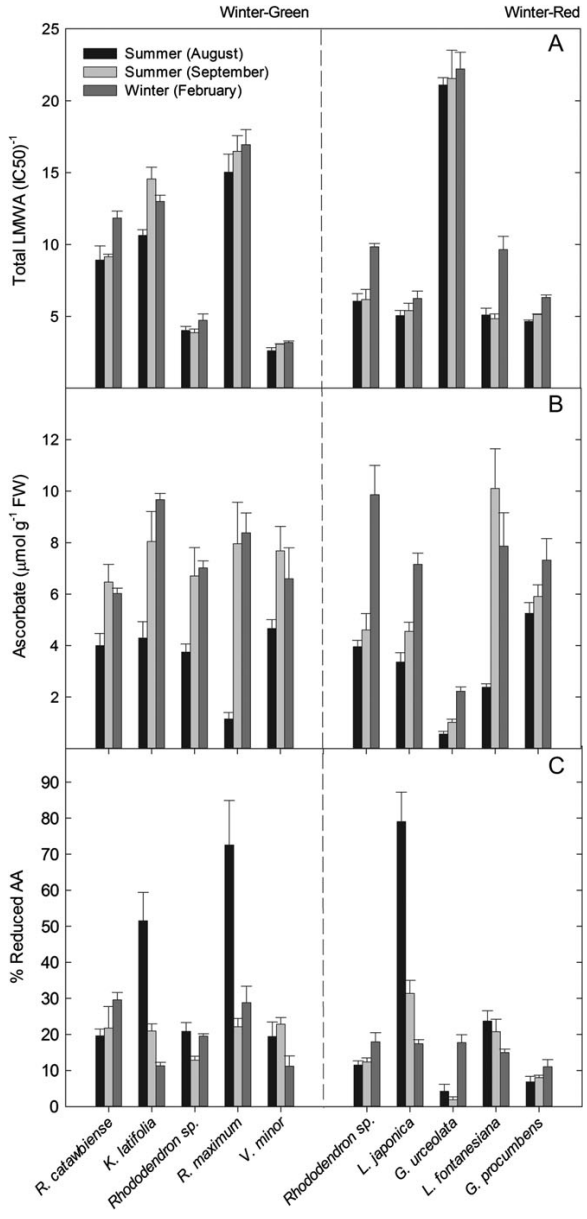

Fig. 4. Seasonal antioxidant data for winter-green species (left of the dashed line) and winter-red species (right). (Top) total low molecular weight antioxidants, measured by DPPH assay; (middle) ascorbate levels per gram fresh weight; (bottom) per cent reduced ascorbate. Bars represent means of five replicates, +SD.

for what is to be considered 'low' or 'high'. Even simple assumptions, such as that photosynthetic capacity should be inversely proportional to photoprotective engagement, can be difficult to demonstrate at the inter-specific level. When mean winter A_{sat} on a warm day (from Hughes and Smith, 2007*a*) was compared with mean winter LMWA, VAZ, and VAZ/Chl, significant correlations could not be demonstrated (e.g. species with highest winter A_{sat} were not necessarily the species with lowest LMWA, VAZ, or VAZ/Chl) (data not shown). However, our photosynthesis and photoprotection data were derived from different field seasons, leaves, and weather conditions (Hughes and Smith, 2007*a* versus the current study). Therefore, while the current study does clearly show that winter-red species have a similar capacity for synthesizing similar quantities of LMWA and xanthophyll cycle pigments as winter-green species, it cannot be demonstrated at this time that red species represent *either* species with low photosynthetic capacity *or* reduced capacity for photoprotection. What can be concluded at this point is that either explanation alone is insufficient to explain winter reddening.

Table 2. Mean winter leaf nitrogen content per unit dry mass (with SD) for winter-green (top group) versus winter-red (lower group) species

Values represent means of five individuals.

Species	%N	C:N
Kalmia latifolia	1.4 (0.2)	36 (3)
Rhododendron maximum	0.99 (0.1)	52 (6)
Rhododendron sp. (azalea)	1.3 (0.1)	38 (3)
Vinca minor	1.2 (0.1)	41 (3)
Lonicera japonica	1.3 (0.3)	39 (8)
Hedera helix	1.2 (0.1)	40 (3)
Galax urceolata	1.3 (.1)	40 (3)
Rhododendron sp. (azalea)	1.3 (0.1)	40 (3)
Gaultheria procumbens	1.2 (0.1)	39 (3)
Leucothoe fontanesiana	1.2 (0.04)	42 (2)

Conclusion

In summary, our results were generally consistent with a photoprotective function of anthocyanin pigments in winter leaves of angiosperm evergreens. Red-leafed species showed signs of increased shade acclimation, and a less-sustained photoinhibition of photosynthesis, relative to green-leafed species. However, why some species exhibit winter colour change while others do not could not be determined, as red and green species could not be classified based solely on the capacity for xanthophyll cycle pigments or for LMWA. Our previous work also could not establish a link between winter colour change and either winter photosynthetic capacity (Hughes and Smith, 2007*a*) or drought tolerance (Hughes *et al.*, 2010). In all three studies, winter-red and winter-green species appeared to have overlapping physiologies and biochemistries, rendering a single, physiological predictor of winter reddening elusive. We maintain that comparative physiological studies are still valuable to pursue, however, especially those utilizing red and non-red morphotypes of a single species (Kytridis *et al.*, 2008). These latter systems may be especially valuable if the proximate causes of winter colour change are subtle. The possibility is also acknowledged that the cause(s) of winter reddening may be different for different species, and that winter colour change could be due to a variety (or

combination) of factors. In this regard, more inter-specific surveys involving additional species could be valuable for determining which factors contribute to colour change among different species and lineages.

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