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Observations on the Relationship Between Above- and Below-Ground Anthocyanin Production in *Galax urceolata* (Poir.) Brummitt Growing in Sun-Exposed and Shaded Locations

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ABSTRACT *Galax urceolata* (Diapensiaceae) is a common evergreen herb of southern Appalachian forests. During the fall and winter, leaves of plants in high light produce substantial amounts of anthocyanins. Oddly, rhizomes in these plants also accumulate anthocyanins. The purpose of this observational study was to identify seasonal trends in anthocyanin production in above- and below-ground tissues of *Galax*. We measured anthocyanins and chlorophyll in *Galax* using standard extraction and spectrophotometric procedures from plants in sun-exposed and shaded locations; one population at Mount Jefferson State Natural Area in fall 2007 and two at Grandfather Mountain in fall/winter 2008–09. Rhizome carbohydrates (soluble sugars and starch) were measured from the Grandfather populations using high performance liquid chromatography. We found significantly more anthocyanins in leaves and rhizomes of plants from sun-exposed locations compared to plants from shaded locations, but no differences in carbohydrate concentrations. Starch levels declined significantly through the fall/winter of 2008–09, while soluble sugars, such as sucrose, raffinose, and fructose, increased. Rhizomal anthocyanins were distributed throughout the entire cross-section except for the vascular tissues, whereas in petioles and leaves, they were restricted to the epidermal or subepidermal layers. Rhizomal anthocyanins often concentrated around lateral roots as they penetrated the cortex. These results contradict the paradigm that light is always required for anthocyanin production, and suggest the possibility of some form of communication between leaves and rhizomes with respect to anthocyanin content, although the nature of that signal is unknown. At this time, the adaptive significance of below-ground anthocyanins in *Galax* remains unresolved.

INTRODUCTION *Galax urceolata* (Poir.) Brummit is an evergreen herb, with a distribution centered in the southern Appalachians. *Galax* shows an interesting pattern of color change during the autumn and winter months. When the leaves of this plant are exposed to high irradiance combined with low temperatures in the latter seasons of the year, it produces abundant anthocyanins, which ultimately turn the exposed leaves a deep burgundy red (McCarron 1995, Hughes et al. 2005, Hughes and Smith 2007). At other times of the year, anthocyanin production is either absent, or present in only small amounts in leaves, usually around sites of injury. Winter reddening is fairly common in some evergreen plants (Parker 1962, Grace et al. 1998) as well as herbaceous plants subjected to low temperatures (Solecka et al. 1999). Interestingly, red *Galax* leaves return to green after the anthocyanins dissipate in early spring when temperatures moderate. Leaves of this plant typically last two full growing seasons before senescing in their third year (McCarron 1995).

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Several hypotheses have been proposed to explain the physiological roles anthocyanins in leaves, including photoprotection from excess light (Feild et al. 2001; Hoch et al. 2001, 2003; Steyn et al. 2002; Gould et al. 2002a, 2002b; Gould 2004; Hughes et al. 2005; Hughes and Smith 2007), attenuation of UV-B radiation (Hatier and Gould 2008) and as antioxidants (Yamasaki et al. 1997; Gould and Lee 2002; Gould et al. 2002a, 2002b; Lee and Gould 2002; Neill et al. 2002a, 2002b; Neill and Gould 2003; Tattini et al. 2005; Kytridis and Manetas 2006). Others have suggested that anthocyanins buildup in tissues after sugars accumulate; for example in autumn leaves after low temperatures slow phloem transport (Murakami et al. 2008). Previous studies have shown a clear role for photoprotection by anthocyanins in the leaves of *Galax* (Hughes et al. 2005, Hughes and Smith 2007) as well as weak support for their ability to act as antioxidants (Hughes et al. 2005).

However, the fact that this species also accumulates substantial amounts of anthocyanins in the rhizome (which is often very short and caudex-like) is less well understood or appreciated. Since the necessity for photoprotection is essentially absent in belowground tissues, the role of these pigments in rhizomatous tissue is puzzling and deserving of more study. Previous work on belowground anthocyanin accumulation has been concerned primarily with documenting anthocyanin content and speciation in certain crop plants such as potatoes (*Solanum tuberosum* L.) and sweet potatoes (*Ipomoea batatas* Poir.) (Terahara et al. 2004, Kano and Mano 2002, Eichhorn and Winterhalter 2005) with the goal of maximizing their antioxidant potential in the human diet. The antioxidant potential of anthocyanins in *Galax* is currently unknown. No relationships between antioxidant potential or photoprotection with anthocyanin content were found for *aerial* roots of *Metrosideros excelsa* Sol. ex Gaertn. (Solangaarachchi and Gould 2001).

Because so little is known about belowground anthocyanins in *Galax*, we decided to study seasonal trends in above- and belowground anthocyanin accumulation for plants from sun-exposed and shaded locations. We hypothesized that plants in high light would produce significantly more anthocyanins in all tissues, both above- and below-ground. Specifically, this observational study was designed to answer the following four questions:

1) What is the anatomical distribution of anthocyanins in the rhizomes, petioles and leaves of *Galax* plants?
2) What is the temporal trend in leaf, petiolar, and rhizomal anthocyanin content before, during, and after autumnal senescence of the overstory?
3) Do anthocyanin amounts in rhizomes differ between plants growing in sun-exposed versus shaded locations?
4) Is there a relationship between anthocyanin formation and carbohydrate concentrations in *Galax* rhizomes?

MATERIALS AND METHODS

Plant Material and Light Readings

We sampled plants (ramets) of *Galax urceolata* from three separate populations, in two geographic locations, over two different seasons. Ramets were obtained in the fall of 2007 from a population along the Rhododendron Trail in Mount Jefferson State Natural Area, Ashe County, North Carolina (hereafter referred to as MJ). This mountainous site lies equidistant from the towns of Jefferson and West Jefferson (36°24′8.60″N, 81°27′50.1″W, elev 1,393 m). Three sampling trips were made during the fall of 2007: the first on 12 October (prior to significant leaf fall), the second on 27 October (significant leaf fall was underway) and the last on 13 November (leaf fall completed). Each collection consisted of 10 or more ramets from two separate locations; one from a sun-exposed location and the other from a shaded one. Those individuals residing in the shade plot grew beneath a dense shrub layer consisting of Catawba rosebay (*Rhododendron catawbiense* Michx.) and mountain laurel (*Kalmia latifolia* L.). High light individuals were located near the border of the park trail and experienced little to no shading. Twelve light readings (photosynthetically active radiation) were taken with a Li-Cor 250A light meter equipped with a 190 quantum sensor (Li-Cor, Inc., Lincoln, Nebraska) at approximately 12:00 pm from each of the two locations at each sampling.

These light readings were then averaged for each location. It is important to note that plants from both sun-exposed and shaded sites were still green on the date of the first sampling (12 October). Prior work with this species by the first author has shown that rhizomes between plants rarely if ever exceed 0.5 m in length, so to minimize clonal sampling, collected ramets were spaced no closer than 1 m from each other.

Two additional populations of plants were sampled in the fall/winter of 2008/2009 from near the summit of Grandfather Mountain (hereafter referred to as GM), located in Avery County, North Carolina, approximately 27 km southwest of Mt. Jefferson. One population was located along Black Rock Trail (36°05′42.5″N, 81°49′38.3″W, elev 1,520 m), and the other along Bridge Trail (36°05′40.4″N, 81°49′50.1″W, elev 1,537), each approximately 0.5 km from one another. The overstory consisted of red spruce (*Picea rubens* Sarg.), some Fraser fir [*Abies fraseri* (Pursh) Poir.], American beech (*Fagus grandifolia* Ehrh. var. *grandifolia*), yellow birch (*Betula alleghaniensis* Britton), cucumber magnolia [*Magnolia acuminata* (L.) L. var. *acuminata*], and mountain ash (*Sorbus americana* Marshall). Five ramets were obtained from sun-exposed sites and five from shaded sites for each population on the following dates: 27 September (prior to leaf fall) and 7 November (leaf fall completed) in the fall of 2008 and 9 March in the winter of 2009. As at MJ, there were no red plants on the first sampling date. Light readings were taken as described above. The first set of light readings was made on 30 September rather than on 27 September due to inclement weather on the day when the ramets were collected.

On occasion, shade plants at both sites were subjected to light flecks of fairly high intensity. However, only green plants were sampled from shaded locations to insure that these were truly low light plants, while conversely, only red plants were sampled from sun-exposed locations.

Anatomical Distribution of Anthocyanins in Galax Plants

Using plants from MJ, fresh cross-sections of leaves, petioles and rhizomes (and also longitudinal sections of rhizomes) were made by hand and viewed using both dissecting and compound microscopes. The anatomical distribution of the anthocyanins was noted for each organ. Only plants from MJ were sectioned.

Pigment Extraction

Plants were gently washed to remove any excess organic material. Three to four leaf disks (0.28 cm^2) were excised per leaf using a standard hole punch. Portions of rhizomes were either cut into 1 cm long cylinders and halved or quartered for more even freezing and extraction (MJ samples) or cut into three small disks, approximately 1–2 mm thick (GM samples) prior to extraction. Diameter measurements were made for both sets of samples. For MJ plants only, petioles were cut into ~2 mm sections for pigment extraction. Each sample was fully submerged in liquid nitrogen to lyse cells and facilitate anthocyanin extraction. Immersion in liquid nitrogen proved to be not necessary for the GM samples. For both sites, we observed complete extraction of pigments, so any bias due to preparation technique is probably minimal.

Anthocyanins were extracted for at least 24 hours in a HCl-methanol solution (6 mM $HCl:H_2O$:MeOH, 7:23:70 v/v) in the dark at 5°C (Hughes et al. 2005). A total of 10 individuals were sampled per location (sun-exposed vs shaded) on each date for the MJ plants while at GM, sample sizes were reduced to five plants and petioles were not analyzed.

Following extraction, anthocyanin content was measured spectrophotometrically as A_{530}-$0.24A_{653}$ (Murray and Hackett 1991) using a UV-VIS spectrophotometer (Shimadzu Scientific Instruments, Columbia, Maryland). Post-extraction tissue was then dried at 50–60°C for at least 24 hours and absorbances expressed as A_{530}/gdw.

For GM plants, leaf chlorophyll was extracted in the dark at 5°C for a minimum of 24 hr in N, N-dimethylformamide, using the same leaves from the anthocyanin extractions. Absorbances were measured at 647 nm and 663 nm after zeroing at 750 nm and values were expressed as mg chl/cm^2 using the equations of Porra et al. (1989).

Carbohydrate Analyses

Rhizomes collected from GM were immediately placed in plastic bags and stored on ice

until transport back to the lab that same day. Approximately 1 gm fresh weight of rhizome was placed in a Whirl-Pak® plastic bag and immediately frozen in liquid nitrogen. Samples were then transferred and stored at −80°C in a freezer until being shipped overnight in dry ice to the United States Forest Service Northern Research Station in South Burlington, Vermont.

Upon arrival, rhizome samples were immediately stored at −80°C until carbohydrate analyses were performed. In preparation for these analyses, the outer layer of rhizome tissue was removed and the remaining tissue was placed in test tubes containing 5 mL of 80% ethanol, evacuated in a vacuum oven at −52 kPa for 15 minutes, and then boiled in a water bath for 15 minutes. Samples were then chopped using a razor blade and homogenized in 80% ethanol using a Brinkman Instruments polytron (Westbury, New York). The pulverized sample was subsequently extracted in 5 mL of fresh 80% ethanol, boiled (15 min) and centrifuged at 3,000 rpm (10 min). This extraction procedure was repeated a second time (Wong et al. 2003). The final ethanol extract was used to determine soluble sugar concentrations and the tissue pellet was used to quantify starch concentrations.

Concentrations of soluble glucose, fructose, raffinose, stachyose, sucrose and xylose were determined from the ethanol extracts according to the methods of Hinesley et al. (1992). A subsample of the extract was dried at 37°C in a limited volume insert vial, reconstituted in 200 μl 0.1 mM Ca EDTA and filtered through a 0.45 μm syringe filter. Samples were analyzed using high performance liquid chromatography on an Alliance 2695 Separations Module and a 410 Refractive Index Detector (Waters Corp., Milford, Massachusetts) equipped with a Sugar-Pak column at 90°C and using 0.1 mm Ca EDTA as the solvent at a flow rate of 0.6 ml min^{-1}. Soluble sugars were identified using known standards, quantified with Waters Empower™ software and expressed as mg/g dry mass.

Starch was quantified using methods slightly modified from Hendrix (1993). The pellet from the ethanol extract was gelatinized with 0.2 N KOH, boiled for 30 minutes in a water bath, and neutralized with 1 M acetic acid. The solubilized starch was then hydrolyzed to glucose using amyloglucosidase (10115, Sigma-Aldrich, St. Louis, MO) in 0.1 M acetate buffer (pH 4.5) and incubated at 55°C for 30 min. The reaction was terminated by boiling for 4 min, after which the supernatant was centrifuged at 3,000 rpm for 10 min. Glucose was quantified from the supernatant using a liquid glucose reagent set (Pointe Scientific, Inc., Canton, Michigan). Absorbance of samples and glucose standards were measured at 340 nm with an ELx800UV microplate reader (Bio-Tek Instruments, Inc., Winooski, Vermont). Final starch calculations were determined using glucose standard curves and expressed as mg/g dry mass.

Statistical Analyses

T-tests were used to determine if irradiance differed between plants growing in sun-exposed and shaded sites at MJ. Differences in anthocyanin amounts on each date for plants from MJ were assessed using the non-parametric two-sample Wilcoxon test (SAS V. 9.1, Cary, North Carolina) due to non-normality in the data. For GM data analyses, analyses of variance (ANOVA) were used to analyze irradiances, anthocyanins, chlorophyll, and carbohydrate concentrations, with trail, light and date as fixed factors. Since trail effects proved to be non-significant they were eliminated from later analyses. Since different plants were sampled on each date in both sites, repeated measures analyses were not necessary. Equality of variances was confirmed using Levene's test, and since ANOVA is robust with respect to normality, we felt verified doing a parametric analysis on these data, even though on occasion, there were instances of non-normality. All differences were assumed significant if $p < 0.05$.

RESULTS

Light Conditions for Sun-exposed and Shaded Locations

Mean irradiances were much higher for plants from sun-exposed sites than from shaded sites ($p < 0.001$ on all dates) at both MJ and GM. They also increased through the seasons due to the loss of the overstory canopy (Table 1). By the last sampling date, irradiance values were 83% lower in the shade at MJ and 90–98% lower at GM, depending on the trail.

Table 1. Mean irradiances (photosynthetically active radiation, μmol m^{-2} sec^{-1}) ± SE for sun-exposed and shaded locations taken near noon on cloudless days. [$n = 12$ for Mount Jefferson Natural Area (fall 2007) and $n = 3$ to 7 for Grandfather Mountain (fall/winter 2008/2009)]

	Mt. Jefferson State Park	
	Location	
Date	Sun-exposed	Shaded
12 October	723 ± 87	15 ± 2
27 October	767 ± 104	16 ± 1
13 November	933 ± 82	159 ± 45

	Grandfather Mountain			
	Trails			
	Black Rock		Bridge	
	Location			
Date	Sun-exposed	Shaded	Sun-exposed	Shaded
30 September*	146 ± 22	12 ± 1	68 ± 7	33 ± 2
7 November	246 ± 59	21 ± 3	323 ± 64	11 ± 1
9 March	1,168 ± 57	24 ± 1	828 ± 46	82 ± 40

Note: differences between sun and shade locations for all populations on all dates significant at $p < 0.001$ based on *t-tests* and ANOVA.

*Plants were collected on 27 September, but inclement weather prevented light readings from being made that day.

Distribution of Anthocyanins in Galax

Figures 1A and 1B show the difference in pigmentation between sun-exposed and shaded ramets of *Galax* at MJ during fall 2007 while Figures 1C and 1D show the same for plants from GM. There is a distinct and visible difference in anthocyanin production between the sun-exposed and shaded ramets at both sites and the lack of anthocyanin production is an indicator that those ramets were in shaded conditions (based on controlled studies conducted earlier; Hughes et al. (2005).

There were different patterns of anthocyanin distribution in each of the three examined organs (Figure 2). Leaves accumulated anthocyanins in upper palisade mesophyll cells (Figure 2A), but not in epidermal cells, consistent with Hughes et al. (2005). Petioles had occasional anthocyanin production in the epidermal and subepidermal cell layers (Figure 2B). Rhizomes exhibited a broader pattern of anthocyanin distribution (Figure 2C and 2D), occurring densely throughout the entire cross-section but noticeably absent from the cambial and vascular tissues.

We also observed anthocyanins forming patterns reminiscent of halos around holes in leaves as an apparent response to wounding (Figure 3A). In addition, there was abundant accumulation of anthocyanins in the area immediately surrounding adventitious roots as they penetrated through the cortex of the rhizome (Figure 3B, 3C).

Trends in Anthocyanin Production and Chlorophyll Amounts

Prior to leaf fall (i.e., the first collection date) all leaves were green and there were no differences in anthocyanin amounts between sun and shade leaves at either the MJ or GM sites. Leaves in sun-exposed locations at MJ began producing anthocyanins right after leaf fall (Figure 4A) whereas those in the shade did not. Petiolar anthocyanin increased through the fall, but there were no significant differences between sun and shade plants when expressed on a weight basis (Figure 4B). However, on a volume basis shade petioles had significantly higher anthocyanin amounts than sun petioles on the first and last sampling dates (data not shown). This was partly due to the fact that petiolar biomass density (g/m^3) was consistently higher in shade than in the sun ($p < 0.001$ on first two dates, $p = 0.061$ on last date).

Accumulation of anthocyanins in rhizomes from MJ was similar to that in leaves collected

from the same site when expressed on a weight basis, with no significant difference between sun and shade plants on the first collection date, but significant differences on the last two sampling dates (Figure 4C). Results were essentially the same on a volume basis (not shown) and biomass density did not differ between sun and shade rhizomes.

Anthocyanin accumulation patterns were roughly similar for plants from GM, despite being sampled in a different season, and over a longer time period (the result of bad weather, which greatly delayed the date of the last sampling event). Leaves in the sun showed a significant accumulation of anthocyanins beginning in early November, and continuing to the last date (Figure 5B). Chlorophyll, in contrast, tended to decrease in November and March for leaves in the sun whereas amounts peaked in November for those in the shade before dropping slightly in March (Figure 5A). Rhizomes, on the other hand, did not show any significant differences between sun-exposed and shaded locations until the last date in March (Figure 5C) when those from the sun-exposed sites had about 20% more anthocyanins on a dry weight basis. On a volume basis, however, anthocyanins increased from 1.54/cm^3 in September to 2.21/cm^3 in March ($p < 0.0001$). Rhizome densities (mg/cm^3) did not differ due to light but did decline significantly on each date (0.29 $\pm$ 0.016 September, 0.22 $\pm$ 0.004 November and 0.17 $\pm$ 0.008 March; $p < 0.0001$).

Trends in Carbohydrate Production in Rhizomes

There were no differences in carbohydrate concentrations between plants from sun-exposed and shaded sites at GM on any date, except for glucose (Figure 6). There were, though, distinct seasonal patterns in terms of allocation among the various fractions. Starch declined significantly from September through March, while various soluble fractions, such as stachyose, raffinose, and fructose, tended to increase. The largest increase occurred for stachyose, which increased over two-fold from September to November, after which it stabilized. The other major sugar that showed an increase was sucrose, but the pattern differed from that for stachyose. Sucrose levels peaked in November, and decreased substantially by the following March (Figure 6). Both raffinose and fructose, in contrast, continued to increase from September to March. Glucose, on the other hand, showed no seasonal pattern in rhizomes from sun-exposed locations, while it increased over time in those from shaded locations.

DISCUSSION

Light Conditions

Because it was not possible to predict which leaves would turn red and which would stay green at the beginning of each growing season, we used the presence or absence of leaf anthocyanins as a proxy for the seasonal light conditions. We did, however, measure the instantaneous light irradiances throughout each area over the two seasons, verifying that they were substantially higher where we found red leaves, and lower where we found green leaves. Because sun-exposed and shaded locations were in close proximity to each other, we also felt that intra-site differences in nutrients and soil water were unlikely to have caused a differential induction of anthocyanins under existing field conditions.

Anatomical Distribution of Anthocyanins in Galax

The location of anthocyanins in the subepidermal layer of leaves is consistent with a photoprotective role as shown in studies on *Galax* by Hughes et al. (2005) and Hughes and Smith (2007). Their function in petioles, though, is not as clear, even though their epidermal and subepidermal layer locations are suggestive of just such a role. Gould et al. (2010) showed a strong photoprotective role for stem anthocyanins in a variety of herbaceous and woody species so it is possible they function analogously here also. However, it is perplexing that petioles in shade had greater amounts of anthocyanins than those in the sun. Perhaps varying angles of inclination and shading of the petioles by the leaf blade influence their anthocyanin content.

The pattern of anthocyanin distribution in rhizomes though, is quite different. Anthocyanins were found throughout the cross-section in nearly all cells except those of the vascular tissue and cambium. They also accumulated around adventitious roots that push their way through cortical cells. However, because rhizomes are located underground, it is obvious


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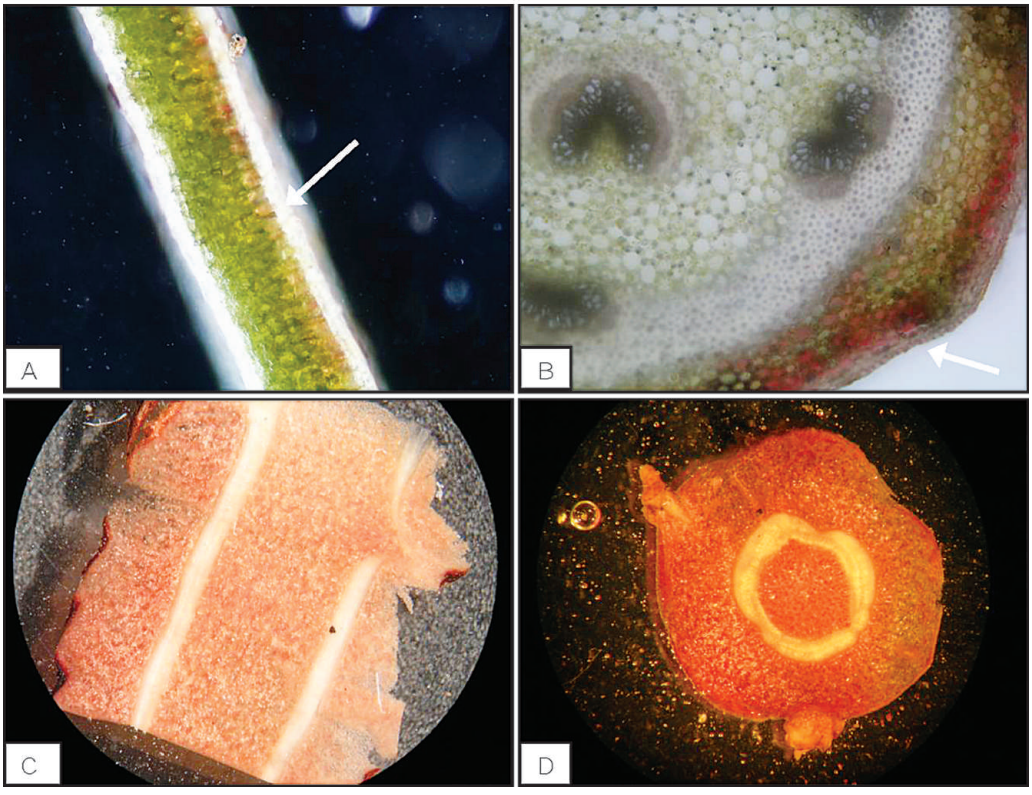
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that they cannot play a photoprotective role in these organs.

Coordination of Rhizome and Leaf Anthocyanin Accumulation

The timing of anthocyanin accumulation in rhizomes seems somewhat variable with respect to that in leaves. For the plants at MJ, the elevation of anthocyanins in leaves and rhizomes occurred nearly simultaneously, whereas at GM, significant increases in plants growing in the sun lagged one sampling period behind that of the leaves. However, at both sites, the increases in rhizome anthocyanins never preceded that in the leaves. This appears to be the first instance of coordination between aboveground and below-ground anthocyanin accumulation in a plant. To our knowledge, there is no such coordination in other plants. Sweet potato [*Ipomoea batatas* (L.) Lam.] tubers, for example, accumulate large amounts of anthocyanins when grown in high light, but their leaves and stems do not (Lalusin et al. 2006).

With respect to our third question in the introduction, *Galax* growing in sun-exposed locations whose leaves turned red in the winter also accumulated more anthocyanins in their rhizomes than plants growing nearby in shaded locations but whose leaves remained green. This confirms our prediction that sun plants accumulate more anthocyanins, and verifies our anecdotal observations that plants which accumulate foliar anthocyanins also accumulate significantly higher amounts below-ground in their rhizomes. However, it should be emphasized that even plants in the shade had anthocyanins in their rhizomes (at least at GM); growing in high light simply elevated anthocyanins levels above those already present in low light. In contrast, at MJ, where shade was probably more constant throughout the sampling peri-

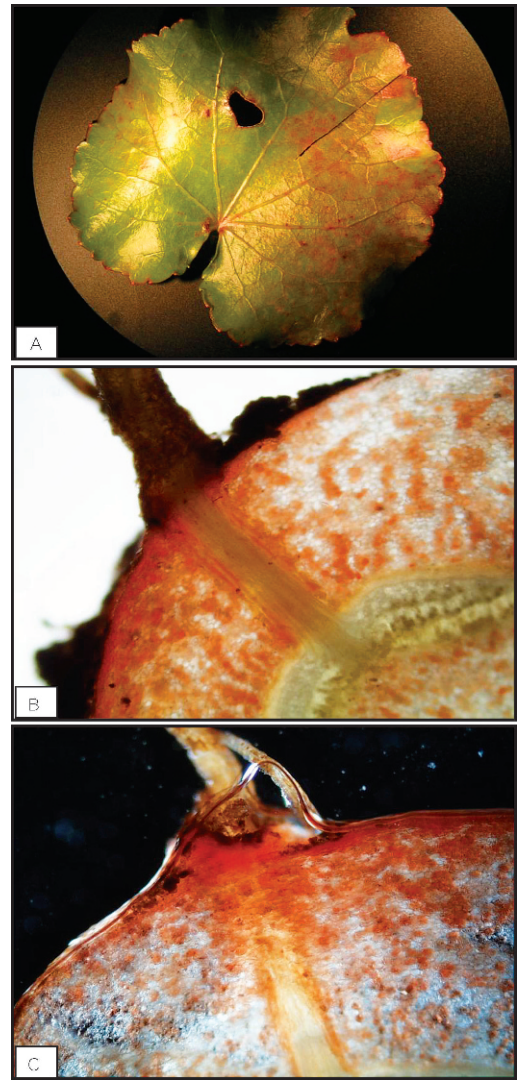

Figure 3. Anthocyanin accumulation in response to cell damage. A) Prominent anthocyanin presence around areas of mechanical wounding in leaf tissue (see large lesion), and (B and C) anthocyanin presence around zones of lateral root growth through the cortex. All samples from Mount Jefferson Natural Area population.

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Figure 1. Visible difference in anthocyanin production between (A) sun-exposed and (B) shaded plants on the last collection date, November 13, 2007 at Mt. Jefferson Natural Area, and (C) sun-exposed and (D) shaded plants on Grandfather Mountain on the last sampling date, 9 March 2009.

Figure 2. Anthocyanin distribution in: A) leaf cross section (white arrow shows absence of anthocyanins in epidermal layer), B) petiole cross section (white arrow shows presence of anthocyanins in epidermal layer), C) rhizome longitudinal section (after leaf fall), and D) rhizome cross section (after leaf fall). All samples from Mount Jefferson Natural Area population.

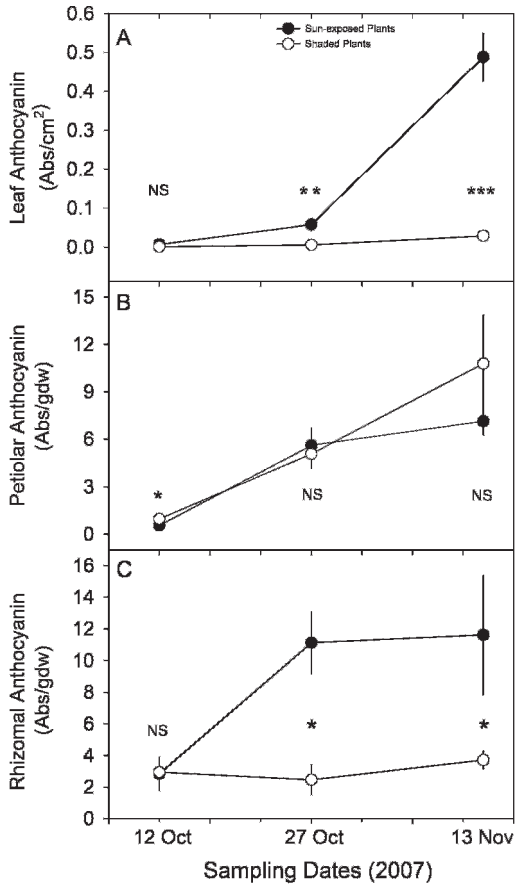

Figure 4. Mean anthocyanin content for plants growing in sun-exposed (red leaved plants) and shaded (green leaved plants) locations at Mount Jefferson Natural Area (fall 2007) for (A) leaves, (B) petioles, and (C) rhizomes. Asterisks indicate significant differences based on the exact solution to the nonparametric Wilcoxon test. (NS, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). Data points are means ± SE ($n = 10$ except for leaves in high light, where $n = 9$). Lack of error bars indicates that they were smaller than the symbol on the graph.

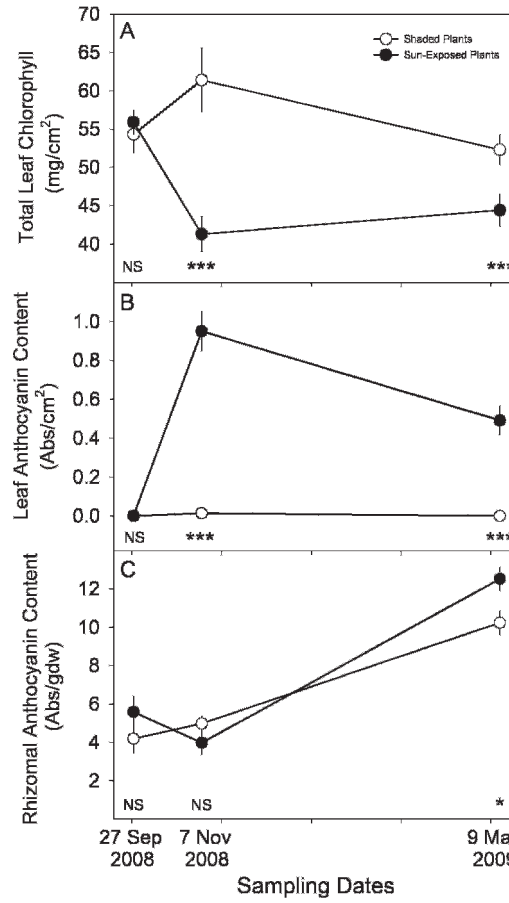

Figure 5. Chlorophyll and anthocyanin content for plants growing in sun-exposed (red leaved plants) and shaded (green leaved plants) locations on Grandfather Mountain (fall/winter 2008/2009) for (A) leaf chlorophyll, (B) leaf anthocyanins and (C) rhizome anthocyanins. Asterisks indicate significant differences ($p < 0.05$). Data points are means ± SE ($n = 10$).

od, there was very little accumulation of anthocyanins in the shade rhizomes.

Speculations on the Physiological Functions of Rhizomal Anthocyanins

The fact that anthocyanins are found in abundance in *Galax* rhizomes (Figure 2), and accumulate to greater amounts only in those plants subject to high light, raises some interesting questions about their synthesis and functionality. Obviously, rhizomal anthocyanins in *Galax* cannot serve a photoprotective role as they appear to do in leaves (Hughes et al. 2005, Hughes and Smith 2007). However, they could function as osmotica to prevent freezing or drought injury (Chalker-Scott 1999, 2002), or as antioxidants (Neill and Gould 2003) to protect against stress-induced reactive oxygen species (ROS) that arise from either pathogen attack (Lorenc-Kukula 2005) or environmental stresses such as cold temperatures (Chalker-Scott 1999). It is unlikely that ROS produced in high light leaves are transported to the rhizomes (at least on warm days when the soils are not frozen) since most ROS, with the exception of hydrogen peroxide, are short-lived species, (Gould et al. 2002a). However, ROS may be

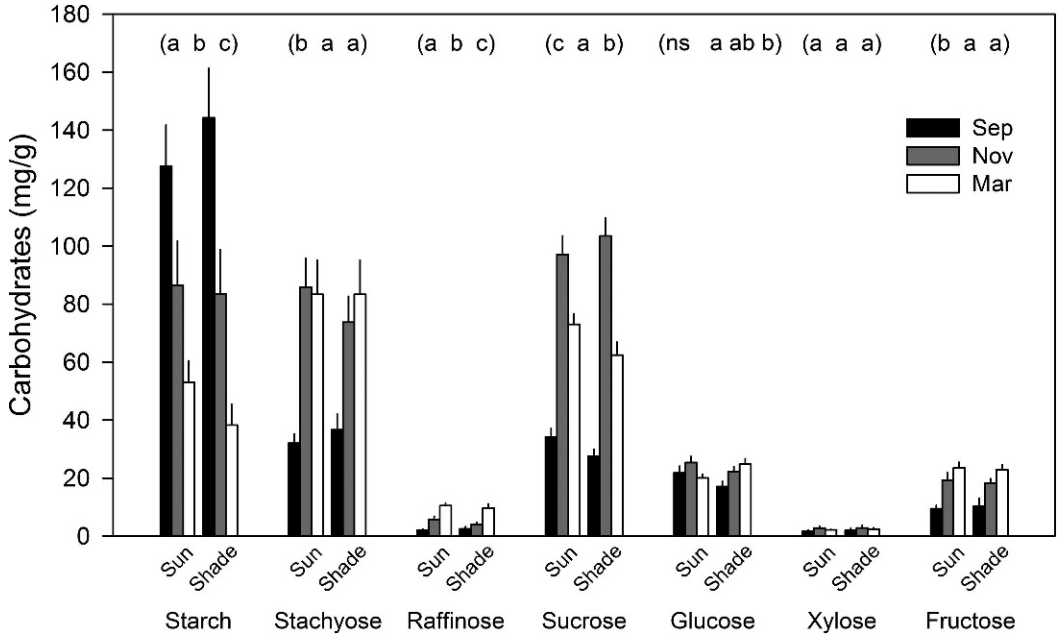

Figure 6. Mean carbohydrate concentrations in rhizomes for plants growing in sun-exposed and shaded locations on Grandfather Mountain (fall/winter 2008/2009). Bars represent mean ± SE (n = 10). Letters above each group indicate seasonal differences within that group for both sun and shade rhizomes as determined by the Tukey-Kramer HSD test, since there were no instances of significant sun - shade effects, except for glucose. For glucose, NS indicates no significant seasonal differences for sun plants, while the bars show significant differences for the shade plants.

generated *in situ* by cold stress, and subsequently detoxified by anthocyanins. Whether rhizomes of plants in sun-exposed locations are subject to greater attack by ROS than those in shaded locations, and whether such rhizomes have a greater antioxidant capacity, are yet to be determined.

Rhizomes of *Galax* are shallowly located in forest soils, and during cold winter months, may be subject to temperature fluctuations sufficiently large to cause membrane damage to cells, particularly if the plants are located in relatively open and sun-exposed habitats where night time dissipation of infra-red radiation would be maximal. Anthocyanins may serve as a cryoprotectant by osmotically lowering the freezing point and preventing ice nucleation and subsequent membrane damage within rhizomes (Chalker-Scott 1999, Hughes et al. 2005). It is not known though, if rhizomes of plants in sun-exposed locations are exposed to more extreme temperature fluctuations than those in shaded locations.

There are optimality problems with an osmotically based function for the anthocyanins, however. First, other compounds could more easily be used to prevent ice nucleation, such as the low molecular weight compounds proline and glycinebetaine (Beck et al. 2007). Second, sucrose itself is an excellent osmoticum, and could serve this function without the additional metabolic costs of synthesizing large anthocyanin molecules. Finally, sucrose can be cytosolic, whereas anthocyanins are primarily vacuolar and would be less effective in preventing damage to the cytosol or plasma membrane. Nonetheless, some researchers have speculated that anthocyanins may protect the tonoplast from freezing injury (Leng et al. 2000). Freezing tolerance studies of rhizomes from sun-exposed and shaded habitats should be done to see if there are differences between the two groups.

It seems more plausible that rhizomal anthocyanins in *Galax* are acting as antioxidants, like they do in sweet potato (Philpott et al. 2004). During cold periods, metabolic imbalances between the relatively temperature-independent mitochondrial and chloroplastic electron transport chains and the more

temperature-dependent enzymatic processes in the Krebs and Calvin cycles, can lead to *in situ* formation of ROS (Møller 2001, Logan 2006, Suzuki and Mittler 2006). The widespread distribution of anthocyanins across tissues in the rhizome (Figures 2C and 2D) would be consistent with the requirement that they be in close proximity to the source of the ROS for them to function efficiently as antioxidants. However, most ROS are extravacuolar, and would still be spatially separated within the cell from anthocyanins sequestered in the vacuole (Schaberg et al. 2008). Since anthocyanin synthesis occurs in the cytoplasm (Winefield 2002) ROS could be scavenged there prior to transport into the vacuole (Neill et al. 2002a). Hydrogen peroxide is a particularly stable ROS that is able to be transported across the tonoplast into the vacuole where it could be detoxified *in situ* by vacuolar anthocyanins (Yamasaki et al. 1997). A close relationship between antioxidant capacity and anthocyanin and phenolic content has been found in the stems of two mulberries (*Morus* spp.) (Sývacý and Sökmen 2004), which is suggestive of, but not proof of, their antioxidant capability. Since rhizomes of both sun-exposed and shaded plants could be subject to ROS attack in cold weather, this might explain why anthocyanins are present in both groups (at least at GM). The additional accumulation of anthocyanins in the rhizomes of sun-exposed plants may help them cope with higher levels of ROS. Conversely, higher light may simply stimulate more anthocyanin synthesis but the additional amount does not actually provide any further benefit to the plant. Finally, the idea that rhizomal anthocyanins might protect against below-ground pathogens (Schaefer et al. 2008), which can also induce ROS, begs the question of why rhizomes of sun-exposed plants would be more susceptible to attack than rhizomes of shaded plants.

We find it particularly interesting that anthocyanins are produced in visibly higher amounts in cortical cells immediately surrounding emerging adventitious roots (Figures 3B and 3C). Because the extension of such roots involves the wounding and destruction of those cortical cells, it is logical to suppose that those cells also generate ROS as a response to this damage, with the most likely candidate being H_2O_2. It is interesting to note that Liszkay et al. (2004) have shown that even normal root growth and extension can result in extensive ROS production, so this seems a reasonable conclusion for adventitious root growth also. Furthermore, Gould et al. (2002a) have clearly demonstrated that wounding in leaves results in oxidative injury resulting from excessive production of H_2O_2 and that the extent of damage is limited by the immediate production of anthocyanins around the sites of injury. In fact, we too have seen anthocyanins concentrated around herbivorized or damaged parts of *Galax* leaves (Figure 3A).

Speculations on the Synthesis of Rhizomal Anthocyanins and the Potential Role of Sucrose

Since most studies claim that anthocyanin synthesis is light dependent (Mancinelli 1985), the question arises as to whether rhizomal anthocyanins in *Galax* are formed *in situ* by some light-independent process, or whether they, or their precursors, are transported from leaf to rhizome. Although the current consensus is that anthocyanins have a specific light requirement, there have been occasional reports of anthocyanin synthesis in the absence of light (Blank 1947). Beggs and Wellmann (1985) reported that some varieties of corn (*Zea mays* L.) can synthesize anthocyanins in the dark, while sweet potatoes can accumulate anthocyanins in their tubers underground where there is little or no light (Lalusin et al. 2006). Since anthocyanins in some plants can be synthesized in the dark, it suggests that some cue other than light might up-regulate the pathway.

One candidate stimulant is sucrose, which has been known for over 100 yr to stimulate anthocyanin production in plants (Onslow 1925). Alston (1958) reported that he could induce anthocyanins in spotted snapweed (*Impatiens balsamina* L.) hypocotyls in the dark if a 2% sucrose solution was added to White's medium. Stem girdling has also been shown to simultaneously increase leaf sugar (including sucrose) and anthocyanin concentrations in autumnal sugar maple (*Acer saccharum* Marsh.) leaves (Murakami et al. 2008). Other recent studies show that sucrose specifically induces a variety of genes in the biosynthetic pathway, including the *PAP1*

gene (Solfanelli et al. 2006), as well as the chalcone and anthocyanidin synthase genes (Hara et al. 2003). Furthermore, this induction appears to require sucrose specifically, and does not result simply from osmotic stress in response to high sugar concentrations (Solfanelli et al. 2006). Since *Galax* can photosynthesize in the winter when temperatures temporarily moderate (McCarron 1995, Hughes et al. 2010) and can accumulate substantial amounts of photosynthates in the leaves (Hughes et al. 2005), it is possible that it transports sucrose to the rhizome at those times, which then either turns on an *in situ* light-independent pathway for anthocyanin synthesis, or, substitutes for the light-induction step in the typical light-dependent pathway. It is interesting that in this study, sucrose concentrations in the rhizomes rose substantially and starch levels declined, just prior to the increases in rhizomal anthocyanins. We hypothesize that rhizomal anthocyanin synthesis in *Galax* may be triggered by high sucrose levels, which themselves result from starch hydrolysis as the season progresses. The lack of a significant difference in sucrose levels between rhizomes in sun-exposed and shaded plants may reflect the greater incorporation of excess sucrose into anthocyanin production in the sun than in the shade, such that after synthesis, the remaining sucrose levels do not differ. Future manipulative studies, such as feeding plants sucrose under controlled light conditions, would help elucidate the role of this sugar in anthocyanin synthesis in the rhizome.

There are several additional possibilities with regard to anthocyanin accumulation in the rhizomes. One is that anthocyanins could be transported intact in the phloem from the leaves, but the absence of any pigmentation in the vascular system of *Galax* argues against that hypothesis. Further argument against this transport hypothesis is the fact that plants with green leaves, and hence no foliar anthocyanins to transport, can still produce anthocyanins in their rhizomes. A second hypothesis is that colorless leucoanthocyanidins could be transported to the rhizome, and then converted via the enzyme anthocyanidin synthase to the colored anthocyanidins and eventually anthocyanins (Springob et al. 2003). Recently, Buer et al. (2007) have demonstrated long-distance symplastic transport of flavonoids from shoot to root in *Arabidopsis*. Alternatively, a third hypothesis is that light induces production of stable mRNA transcripts or transcriptional regulator proteins in leaves, which are then transported to the rhizomes where they switch on biosynthetic or regulatory genes, thereby enabling *in situ* production of anthocyanins in the dark. The recent discovery that low molecular weight proteins are involved in the flowering response of plants and transported in the phloem from the leaves to the apical meristems lends special credence to this particular hypothesis (Corbesier et al. 2007, Tamaki et al. 2007). However, it will take much additional experimentation to differentiate among these various hypotheses.

Conclusions

We have shown that in three separate populations of *Galax*, levels of rhizomal anthocyanins in red-leaved plants growing in sun-exposed locations in the winter are higher than those for green-leaved plants growing in shaded locations. This accumulation may be due to either light-independent synthesis or transport of precursors or genetic regulators from the leaves. Carbohydrate analyses are consistent with a sugar stimulation of synthesis, as levels of starch decline, and soluble sugars, especially sucrose, rise through the season. There is anatomical evidence that suggests these anthocyanins may act as antioxidants in the rhizomes, although cryoprotective, herbivory or pathogen deterrent functions cannot be explicitly ruled out. Additional mechanistically-based studies may eventually elucidate the adaptive significance of these rhizomal anthocyanins.

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LITERATURE CITED

Alston, R.E. 1958. Leuco-anthocyanin synthesis in dark-grown seedlings of *Impatiens balsamina*. Amer. J. Bot. 45:289–294.

Beck, E.H., S. Fettig, C. Knake, K. Hartig, and T. Bhattarai. 2007. Specific and unspecific responses of plants to cold and drought stress. J. Biosciences 32:501–510.

Beggs, C.J. and E. Wellmann. 1985. Analysis of light-controlled anthocyanin formation in coleoptiles of *Zea mays* L. — the role of UV-B, blue, red and far-red light. Photochem. Photobiol. 4:481–486.

Blank, R. 1947. The anthocyanin pigments of plants. Bot. Rev. 13:241–317.

Buer, C.S., G.K. Muday, and M.A. Djordjevic. 2007. Flavonoids are differentially taken up and transported long distances in *Arabidopsis*. Plant Physiol. 145:478–490.

Chalker-Scott, L. 1999. Environmental significance of anthocyanins in plant stress responses. Photochem. Photobiol. 70:1–9.

Chalker-Scott, L. 2002. Do anthocyanins function as osmoregulators in leaf tissues? *In:* Gould, K.S. and D.W. Lee (eds.). Why leaves turn red: anthocyanins in vegetative organs. Advances in Botanical Research, Academic Press, London, United Kingdom.

Corbesier, L., C. Vincent, S. Jang, F. Fornara, Q. Fan, I. Searle, A. Giakountis, S. Farrona, L. Gissot, C. Turnbull, and G. Couplands. 2007. FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. Science 316:1030–1033.

Eichhorn, S. and P. Winterhalter. 2005. Anthocyanins from pigmented potato (*Solanum tuberosum* L.) varieties. Food Res. Intl. 38:943–948.

Feild, T.S., D.W. Lee, and N.M. Holbrook. 2001. Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. Plant Physiol. 127:566–574.

Gould, K.S. 2004. Nature's Swiss Army knife: the diverse protective roles of anthocyanins in leaves. J. Biomed. Biotechnol. 5:214–320.

Gould, K.S. and D.W. Lee (eds.). 2002. Anthocyanins and leaves. The function of anthocyanins in vegetative organs. Advances in Botanical Research, Volume 37. Academic Press, London, United Kingdom.

Gould, K.S., D.A. Dudle, and H.S. Neufeld. 2010. Why some stems are red: cauline anthocyanins shield photosystem II against high light stress. J. Exp. Bot. 61:2707–2717.

Gould, K.S., J. Mckelvie, and K.R. Markham. 2002a. Do anthocyanins function as antioxidants in leaves? Imaging of H_2O_2 in red and green leaves after mechanical injury. Plant, Cell and Environment 25:1261–1269.

Gould, K.S., T.C. Vogelmann, T. Han, and M.J. Clearwater. 2002b. Profiles of photosynthesis within red and green leaves of *Quintinia serrata*. Physiologia Plantarum 116:127–133.

Grace, S.C., B.A. Logan, and W.W. Adams, III. 1998. Seasonal differences in foliar content of chlorogenic acid, a phenylpropanoid antioxidant, in *Mahonia repens*. Plant, Cell and Environment 21:513–521.

Hara, M., K. Oki, K. Hoshino, and T. Kuboi. 2003. Enhancement of anthocyanin biosynthesis by sugar in radish (*Raphanus sativus*) hypocotyl. Plant Sci. 164:259–265.

Hatier, J-H.B. and K.S. Gould. 2008. Foliar anthocyanins as modulators of stress signals. J. Theoret. Biol. 253:625–627.

Hendrix, D.L. 1993. Rapid extraction and analysis of nonstructural carbohydrates in plant tissues. Crop Sci. 33:1306–1311.

Hinesley, S.B., D.M. Pharr, L.K. Snelling, and S.R. Funderbunk. 1992. Foliar raffinose and sucrose in 4 conifer species: Relationship to seasonal temperature. J. Amer. Soc. Hort. Sci. 117:852–855.

Hoch, W.A., E.L. Singsaas, and B.H. McCown. 2003. Resorption protection. Anthocyanins facilitate nutrient recovery in autumn by

shielding leaves from potentially damaging light levels. Plant Physiol. 133:1296–1305.

Hoch, W.A., E.L. Zeldin, and B.H. McCown. 2001. Physiological significance of anthocyanins during autumnal leaf senescence. Tree Physiol. 21:1–8.

Hughes, N.M. and W.K. Smith. 2007. Attenuation of incident light in *Galax urceolata* (Diapensiaceae): Concerted influence of adaxial and abaxial anthocyanic layers on photoprotection. Amer. J. Bot. 94:784–790.

Hughes, N.M., H.S. Neufeld, and K.O. Burkey. 2005. Functional role of anthocyanins in high-light winter leaves of the evergreen herb *Galax urceolata*. New Phytol. 168:575–587.

Hughes, N.M., K. Reinhardt, T.S. Field, A.R. Gerardi, and W.K. Smith. 2010. Association between winter anthocyanin production and drought stress in angiosperm evergreen species. J. Exp. Bot. doi:10.1093/jxb/erq042.

Kano, Y. and K. Mano. 2002. The effects of night soil-temperatures on diurnal changes in carbohydrate contents of roots and stems of sweet potatoes (*Ipomoea batatas* Poir.). Jap. J. Soc. Hort. Sci. 71:747–751.

Kytridis, V-P. and Y. Manetas. 2006. Mesophyll versus epidermal anthocyanins as potential in vivo antioxidants: evidence linking the putative antioxidant role to the proximity of the oxy-radical source. J. Exp. Bot. 57:2203–2210.

Lalusin, A.G., M. Ohta, and T. Fujimara. 2006. Temporal and spatial expression of genes involved in anthocyanin biosynthesis during sweet potato (*Ipomoea batatas* [L.] Lam.) root development. Intl. J. Plant Sci. 167:249–256.

Lee, D.W. and K.S. Gould. 2002. Why leaves turn red. Amer. Scientist 990:524–530.

Leng, P., H. Itamura, H. Yamamura, and X.M. Deng. 2000. Anthocyanin accumulation in apple and peach shoots during cold acclimation. Scientia Horticulturae 83:43–50.

Liszkay, A., E. van der Zalm, and P. Schopfer. 2004. Production of reactive oxygen intermediates ($O_2^{\cdot -}$, H_2O_2, and $^{\cdot}OH$) by maize roots and their role in wall loosening and elongation growth. Plant Physiol. 136: 3114–3123.

Logan, B.A. 2006. Oxygen metabolism and stress physiology. p. 539–553. *In:* Wise, R.R. and J.K. Hoober (eds.). The structure and function of plastids. Springer, The Netherlands.

Lorenc-Kukula, K. 2005. Ecotopic expression of anthocyanin 5-O-glucosyltransferase in potato tuber causes increased resistance to bacteria. J. Agric. Food Chem. 53:272–281.

Mancinelli, A.L. 1985. Light-dependent anthocyanin synthesis — a model system for the study of plant photomorphogenesis. Bot. Rev. 51:107–157.

McCarron, J.K. 1995. Ecophysiological adaptations of *Galax aphylla* to the understory of southern Appalachian forests. M.S. thesis, Appalachian State University, Boone, North Carolina.

Møller, I.M. 2001. Plant mitochondria and oxidative stress: Electron transport, NADPH turnover, and metabolism of reactive oxygen species. Ann. Rev. Plant Physiol. Plant Molec. Biol. 52:561–591.

Murakami, P.F., P.G. Schaberg, and J.B. Shane. 2008. Stem girdling manipulates leaf sugar concentrations and anthocyanin expression in sugar maple trees during autumn. Tree Physiol. 28:1467–1473.

Murray, J.R. and W.P. Hackett. 1991. Dihydroflavonol reductase activity in relation to differential anthocyanin accumulation in juvenile and mature phase in *Hedera helix* L. Plant Physiol. 97:343–351.

Neill, S.O. and K.S. Gould. 2003. Anthocyanins in leaves: light attenuators or antioxidants? Funct. Plant Biol. 30:865–873.

Neill, S.O., K.S. Gould, P.A. Kilmartin, K.A. Mitchell, and K.R. Markham. 2002a. Antioxidant activities of red versus green leaves in *Elatostema rugosum*. Plant, Cell and Environment 25:539–548.

Neill, S.O., K.S. Gould, P.A. Kilmartin, K.A. Mitchell, and K.R. Markham. 2002b. Antioxidant capacities of green and cyanic leaves in the sun species, *Quintinia serrata*. Funct. Plant Biol. 29:1437–1443.

Onslow, M.W. 1925. The anthocyanin pigments of plants, 2nd ed. Cambridge

University Press, Cambridge, United Kingdom.

Parker, J. 1962. Relationships among cold hardiness, water-soluble protein, anthocyanins, and free sugars in *Hedera helix* L. Plant Physiol. 37:809–813.

Philpott, M., K.S. Gould, C. Lim, and L.R. Ferguson. 2004. In situ and in vitro antioxidant activity of sweetpotato anthocyanins. J. Agric. Food Chem. 52:1511–1513.

Porra, R.J., W.A. Thompson, and P.E. Kriedemann. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta 975:384–394.

Schaberg, P.G., Murakami, P.F, M.R. Turner, H.K. Heitz, and G.J. Hawley. 2008. Association of red coloration with senescence of sugar maple leaves in autumn. Trees 22:573–578.

Schaefer, H.M., M. Rentzsch, and M. Breuer. 2008. Anthocyanins reduce fungal growth in fruits. Natural Product Communication 3:1267–1272.

Solangaarachchi, S.M. and K.S. Gould. 2001. Anthocyanin pigmentation in the adventitious roots of *Metrosideros excelsa* (Myrtaceae). New Zealand J. Bot. 39:161–166.

Solecka, D., A-M. Boudet, and A. Kacperska. 1999. Phenylpropanoid and anthocyanin changes in low-temperature treated winter oilseed rape leaves. Plant Physiol. Biochem. 37:491–496.

Solfanelli, C., A. Poggi, E. Loreti, A. Alpi, and P. Perata. 2006. Sucrose-specific induction of the anthocyanin biosynthetic pathway in *Arabidopsis*. Plant Physiol. 140:637–646.

Springob, K., J. Nakajima, M. Yamazaki, and K. Saito. 2003. Recent advances in the biosynthesis and accumulation of anthocyanins. Natural Products Reports 20:288–303.

Steyn, W.J., S.J.E. Wand, D.M. Holcroft, and G. Jacobs. 2002. Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. New Phytologist 155:349–361.

Suzuki, N. and R. Mittler. 2006. Reactive oxygen species and temperature stresses: A delicate balance between signaling and destruction. Physiologia Plantarum 126: 45–51.

Sývacý, A. and M. Sökmen. 2004. Seasonal changes in antioxidant activity, total phenolic and anthocyanin constituent of the stems of two *Morus* species (*Morus alba* L. and *Morus nigra* L.). Plant Growth Regulation 44:251–256.

Tamaki, S., S. Matsuo, H.L. Wong, S. Yokoi, and K. Shimamoto. 2007. Hd3a protein is a mobile flowering signal in rice. Science 316:1033–1036.

Tattini, M., L. Guidi, L. Morassi-Bonzi, P. Pinelli, D. Remorini, E. degl'Innocenti, C. Giordana, R. Massai, and G. Agati. 2005. On the role of flavonoids in the integrated mechanisms of response of *Ligustrum vulgare* and *Phillyrea latifolia* to high solar radiation. New Phytologist 167:457–470.

Terahara, N., I. Konczak, H. Ono, M. Yoshimoto, and O. Yamakawa. 2004. Characterization of acylated anthocyanins in callus induced from storage root of purple-fleshed sweet potato, *Ipomoea batatas* L. J. Biomed. Biotechnol. 5:279–286.

Winefield, C. 2002. The final steps in anthocyanin formation: A story of modification and sequestration. Adv. Bot. Res. 37:55–74.

Wong, B.L., K.L. Bagget, and A.H. Rye. 2003. Seasonal patterns of reserve and soluble carbohydrates in mature sugar maple (*Acer saccharum*). Can. J. Bot. 81:780–788.

Yamasaki, H., Y. Sakihama, and N. Ikehara. 1997. Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H_2O_2. Plant Physiol. 115:1405–1412.