FUNCTIONAL ROLE OF ANTHOCYANINS IN HIGH LIGHT WINTER LEAVES OF THE EVERGREEN HERB, *GALAX URCEOLATA*

A Thesis

by

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ABSTRACT

FUNCTIONAL ROLE OF ANTHOCYANINS IN HIGH LIGHT WINTER LEAVES OF THE EVERGREEN HERB, *GALAX URCEOLATA*. (May 2004)

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Galax urceolata is an evergreen understory herb whose leaves exhibit a dramatic change in color from green to red under high light, low temperature conditions. This color change has been attributed to anthocyanin synthesis in the outermost mesophyll cells, though its functional significance has yet to be investigated. In this study, four hypotheses for anthocyanin synthesis in Galax were proposed and tested. The first hypothesis was that anthocyanins function as light-attenuators, protecting subjacent mesophyll cells from excess irradiance by absorbing blue-green light. To test this hypothesis, the ratio of variable to maximal fluorescence (F_v/F_m) was used as an estimator of maximum PSII efficiency in red and green leaves during and after prolonged exposure to white, red, and green light. As predicted, red leaves were found to exhibit significantly less of a decline in F_v/F_m than green leaves when exposed to green and white light, but comparable declines when the leaves were exposed to red light (which anthocyanins absorb poorly).

When white light was shone on anthocyanin-free abaxial surfaces of red and green leaves, declines in F_v/F_m were comparable between the two groups. The second hypothesis tested was that anthocyanins increase leaf temperature, which could function to alleviate photooxidative stress in high light winter plants by helping reinstate the balance between light capture and cold temperature-retarded carbon fixation. The data gathered did not support this hypothesis. The third hypothesis, that anthocyanins act as antioxidants, purported that these molecules function to neutralize reactive oxygen species formed during periods of photooxidative stress, and was tested by comparing the α,α-diphenyl-β-picrylhydrazyl (DPPH) radical-scavenging activities of red and green leaves. Results showed no difference in antioxidant activities between the two groups, and no correlation between antioxidant activity and anthocyanin concentration. The fourth hypothesis addressed the possibility that anthocyanins are synthesized as a result of an imbalance of the source:sink ratio, being formed merely as an end product of carbon overflow into the phenylpropanoid pathway when carbon fixation exceeds its utilization or export. This hypothesis was also not supported, as non-structural carbohydrate levels did not differ between red and green leaves during the winter. However, soluble sugar levels were found to be 2-fold higher during the winter than the summer in both groups, suggesting that high levels of soluble sugars may serve as a biochemical cue necessary to initiate anthocyanin synthesis in the presence of high light during winter months. In summary, this study suggests that anthocyanins do not affect leaf temperature, antioxidant activity, or function as a carbon overflow in Galax, though they do appear to significantly curtail photooxidative stress by acting as light-attenuators.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my family for their love, inspiration, and support. Specifically, I would like to thank my dad for teaching me the power of attitude, the necessity of compassion and humor in life, and the philosophy of never doing a job any less than at your best. I would also like to thank my mom for being there to supplement my lack of political knowledge and culinary skills, but mostly for just being such a great mom and person. Many thanks also to my brother Johnny, for always being able to make me laugh, and Mammom- thank you for your advice, and for being there to talk on my long drives home.

On a less mushy, but equally sincere, note, I would like to thank Dr. Howie Neufeld, my advisor and mentor, for coming up with this thesis topic, for exploiting every means necessary for it to come to fruition, and most of all, for helping me become a scientist.

I would also like to take this opportunity to thank all the professors and researchers at the University of Georgia, Appalachian State University, and the USDA who allowed me to work in their facilities on my research- namely, Kent Burkey, Hugh Earl, Matthew Rowe, Mark Venable, and Fitz Booker. I would also like to acknowledge the generosity of Alan Davison, Guillermo Goldstein, and Joe Sullivan, who loaned us much of the equipment used in this study. Special thanks also to L. Renee Tucker and Barbara Jones for their invaluable help with the bioassays involved in this study.

Lastly, I would like to thank my very good friends- Alison, Bruce, Heather, Justin, Jessica, Pet, and Jamie. Thank you for all for your love and friendship.

DEDICATION

This thesis is dedicated in loving memory to my grandfather, Lee Hughes. I wish I liked plants this much when you were still here- I think you could have taught me a thing or two.

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INTRODUCTION

Galax urceolata is an evergreen understory herb native to the Appalachian Mountains. When exposed to extended periods of cold temperatures and high light, Galax leaves produce anthocyanins in their peripheral (outermost) mesophyll cells, causing leaves to turn a deep shade of red. Leaves that remain shaded, however, stay green. In the spring, anthocyanins dissipate corresponding with the onset of warm temperatures and return of the deciduous canopy. Leaves may subsequently persist for 2 to 3 additional growing seasons (McCarron 1995). Because the appearance of anthocyanins coincides predictably with cold temperatures and high light, one might suspect that these pigments are performing some type of regulated function within Galax leaf tissues. Indeed, recent studies on anthocyanins in senescing autumn leaves (Feild *et al.* 2001; Hoch *et al.* 2001), seed pods (Smillie and Hetherington 1999), lettuce (Neill and Gould 2003), *Arabidopsis* (Landry *et al.* 1995), and leaves of some tropical species (Gould *et al.* 2000; Neill *et al.* 2002a) have provided much support for the prospect that anthocyanins play more than a merely benign role in leaf tissues, showing instead that they may confer a significant degree of protection against photooxidative damage in light stressed plants by acting as antioxidants and/or light attenuators. With this known, it is surprising that so few studies have been conducted on functional roles of anthocyanins in temperate evergreen plants, since these plants must seasonally endure the stress of excess irradiance compounded by

low temperatures (Grace *et al.* 1998; Kaku *et al.* 1992; Parker 1962). The purpose of this study was to examine the apparent effects of anthocyanins on leaf biochemistry and physiology of the broadleaf evergreen herb *Galax urceolata*, in light of both traditional and less-explored hypotheses of color change, as described below.

One hypothesis that has received much attention in the recent literature proposes that anthocyanins located within the epidermal, palisade, and/or peripheral mesophyll layers of leaves exposed to excess irradiance act as light attenuators, absorbing blue-green light that could otherwise be absorbed by chlorophyll *b* in the subjacent mesophyll (Feild *et al.* 2001; Gould *et al.* 1995; Hoch *et al.* 2001; Lee and Gould 2002; Neill and Gould 2003). Anthocyanins occurring within these cell layers have been shown to shield the lower mesophyll from potentially excessive light so effectively that the lower mesophyll cells of some anthocyanin-protected species assume physiological features of shade-adapted cells (Gould *et al.* 2002). The interception of these photons would be especially advantageous in plants that absorb more light than can be effectively converted to chemical energy via photosynthesis, as is the case for many high light over-wintering species. Since light-harvesting reactions occur essentially independently of temperature (Baker 1994), an increase in light will result in an increase in energy absorbed by the photosystems, regardless of ambient temperature. However, the rate of biochemical reactions in the Calvin cycle are directly proportional to temperature, and as temperature decreases, so will the rate of carbon fixation. This imbalance between energy capture and assimilation may ultimately lead to a decline in available open reaction centers, and subsequently, increasing amounts of absorbed energy that are transferred to non-chlorophytic

molecules, such as xanthophyll pigments and, more deleteriously, oxygen in the surrounding tissue (Demmig-Adams and Adams 1996; Logan *et al.* 1998). The transfer of electrons to molecular O_2 may then drive the formation of biologically damaging reactive oxygen intermediates (ROI)s including singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl (OH^-) radicals (Mittler 2002; Ort 2001). Such an increase in ROIs may lead to tissue damage and even cell death as the reactive molecules oxidize proteins, peroxidize lipids, inhibit enzymes, and damage DNA and RNA (Mittler 2002).

The ability of anthocyanins to absorb strongly in the high energy blue-green wavelengths could conceivably confer another protective advantage for high light winter leaves. In addition to acting as a light attenuator, anthocyanins may also be functioning to increase leaf temperature. Cyanic leaves have been observed in this study and others (Feild *et al.* 2001 ; Neill and Gould 2003) to absorb significantly more light energy than acyanic conspecifics. By increasing leaf temperature, chemical reactions that had been retarded by cold temperatures may be accelerated, thus alleviating to some degree susceptibility to photoinhibition. Although previous studies have found no apparent effects of anthocyanins on leaf temperature (Lee 1986; Lee *et al.* 1987), this hypothesis was tested in this study as an experimental aside, to ensure consistency with findings of other species.

In addition to absorbing light energy, anthocyanins, as well as their precursor chlorogenic acid, have demonstrated an additional photoprotective function through their role as antioxidants (Grace *et al.* 1998; Mittler 2002; Neill *et al.* 2002a). Classified with ascorbic acid and other flavonoids as low molecular weight antioxidants (LMWA)s,

anthocyanins have been shown to scavenge the reactive oxygen molecules H_2O_2, O_2^-, $ONOO^-$, and possibly OH^- and $^1O_2$, and have been demonstrated to possess about four times greater antioxidant capacity than α-tocopherol and ascorbic acid (Lee and Gould 2002; Mittler 2002; Neill *et al.* 2002a). Their precursor, chlorogenic acid, possesses an antioxidant activity even greater than this, contributing more to the LMWA pool than any other LMWA in some species (Grace *et al.* 1998). For these reasons, it has been thought that anthocyanins, when located within photosynthetic mesophyll cells, may be functioning to neutralize excess ROIs as they are produced by organelles during times of stress (Gould *et al.* 2002). During periods of environmental stress it is not unusual for plants to increase their production of antioxidants to balance the increase in ROIs (Grace and Logan 1996; Mittler 2002; Neill *et al.* 2002a), which may be another reason why Galax produces anthocyanins under high light, cold temperature conditions.

An additional hypothesis, which has received significantly less experimental attention than the above, is the possibility that anthocyanins occur as a product of increased carbon flow through the phenylpropanoid pathway when source carbohydrate levels exceed their utilization, and/or transport of these compounds to the rhizome or other storage organs is impeded (such as by freezing of the soils and/or cessation of growth). Because phenylpropanoids are built on a carbon skeleton, with anthocyanins containing an additional glucose molecule, these compounds could conceivably be acting as a temporary carbon sink, remaining sequestered within the vacuole until transport mechanisms are restored. Furthermore, as this theory would predict, when the sink becomes no longer limiting (such as during periods of new growth or temperature

increase), anthocyanins diminish. Several studies, including this one, have observed anthocyanin synthesis to be initiated in response to increases in leaf carbohydrate content (Jeanette *et al.* 2000; Onslow 1925), and recent studies have further purported that genes for chalcone synthase (CHS-A) and the anthocyanin pathway-specific gene *Bz1* are sugar-inducible (Tsukaya *et al.* 1991; Jeanette *et al.* 2000). No studies have been conducted, however, which test the possibility that plants which seasonally produce anthocyanins do so as a means of alleviating source:sink imbalances.

In summary, the objective of this study was to investigate each of the above hypotheses to determine what function, if any, anthocyanin synthesis appears to serve in high light winter leaves of *Galax urceolata.* Individual hypotheses were tested using a combination of observational and experimental procedures to quantitatively test the assumptions of each hypothesis in turn, as described below.

MATERIALS AND METHODS

Plant Material

Sun and shade *Galax urceolata* used in this study were obtained from the understory of temperate deciduous forests on Long Arm Mountain in Jonas Ridge, NC. Leaves were either derived directly from plants in the field, or from plants that had been potted and cultivated in a greenhouse 4 months prior to experimentation. Field plants were located in five separate plots within a 1 km radius on Long Arm Mountain. Each individual plot contained both sun leaves (red during winter) and shade leaves, with shade being provided either by *Rhododendron., Tsuga,* or *Kalmia* spp. Plants to be potted were transplanted in July, and were derived from a single clone on Long Arm Mountain. Potted plants consisted of a rhizome at least 7.5 cm long, and 2-3 leaves. Potting mix was roughly 2 parts pine bark, 1 part peat, 1 part Perlite, with Osmocote plus 15-9-12 used as a fertilizer. All experiments were conducted on first year leaves, since previous studies have shown declines in photosynthetic processes of Galax leaves as they age (McCarron 1995). First year leaves were tagged in the spring to mark leaf age.

Environmental Monitoring

To characterize the light environments of field leaves in this study, photosynthetic photon flux densities (PPFD)s were measured at 1 minute intervals using a LI-250 light

meter equipped with a 190SA quantum sensor (Li-Cor, Inc., Lincoln, NE) connected to a 21x datalogger (Campbell Scientific Inc., Logan, UT). Total daily PPFD was also measured using a microvolt integrator (Delta-T Devices, Cambridge, UK). Hourly soil and air temperatures were monitored using DS1921 Thermochron iButtons (Dallas Semiconductor Inc., Dallas, TX). Leaf temperatures were recorded using copper-constantan thermocouples attached to abaxial leaf surfaces, which were connected to a 21x datalogger.

Quantification of Pigments and TNCs

To quantify relative anthocyanin content, a standard hole puncher was used to excise four 0.28 cm^2 discs from leaves. Discs were submerged in liquid nitrogen for 5 minutes prior to extraction to perforate the waxy cuticle and disrupt cell membranes. Discs were then placed in plastic vials containing 2.5 mL 6M HCl:H_2O:MeOH (7:23:70) to extract in the dark at 4°C for 24 hours. Anthocyanin levels were determined spectrophotometrically as A_{530}-0.24A_{653} to account for chlorophyll (Murray and Hackett 1991).

Chlorophyll content was determined using three leaf discs, which were placed in 3 mL N, N'-dimethylformamide (DMF) to extract for 24 hours. Quartz cuvettes were used for spectrophotometric measurements. Solutions were zeroed at 720 nm, and absorbances were measured at 664 and 647 nm using a UV-VIS spectrophotometer (Shimudzu). Chlorophyll pigment concentrations were calculated using the equations described by Porra (2002): Chl*a* = [12*A_{664}] - [3.11*A_{647}]; Chl*b* = [20.78*A_{647}]- [4.88*A_{664}]; Chl *a*+*b* = [17.67*A_{647}] + [7.12*A_{664}].

For quantification of total non-structural carbohydrates (TNC)s, fresh cut leaves were immersed in liquid nitrogen, then oven dried for at least 24 hours. Using 25 mg of dried tissue, a Boehringer Mannheim/R-Biopharm starch kit was used to determine concentrations of starches and soluble sugars.

Leaf Optics

Red and green leaf absorbance spectra were derived using leaves which had been removed from plants in the morning and kept on ice in a wet paper towel until measurement later that day. A Li-Cor 1800 spectroradiometer with external integrating sphere was used to measure reflectance and transmission of PPFD at 2 nm intervals for abaxial and adaxial surfaces of leaves. Percent absorbance was calculated as (1-transmittance - reflectance). The amount of energy absorbed, reflected, and transmitted by the leaves at each wavelength was calculated by multiplying each value by the standard energy contained at each wavelength in ambient sunlight.

Light Effects on Pigments and Biomolecules

In order to quantify the effects of light intensity on pigment and carbohydrate concentrations, shade structures (0.25 m^2) constructed from PVC pipe and neutral density cloth providing either 80%, 60%, 40%, 20%, or 0% shade were randomly placed within a large (20 m^2) naturally occurring high light Galax plot. A second series of shade treatments was also established using potted plants in a location completely free of overstory. The shade treatments were set in place during October, 2003 before leaves had

begun to turn red. Each shade treatment contained at least 10 healthy green leaves. On December 16, leaves of potted plants were harvested for quantification of pigments and TNCs using the protocols described previously; leaves from the natural plot were harvested and analyzed on January 13. F_v/F_m values of leaves under shade treatments were also measured (protocol described in next section).

To determine whether ultra-violet (UV) light was necessary to induce anthocyanin synthesis, 1 m^2 PVC shade structures covered with either mylar (to exclude UV) or teflon (UV transparent) were placed over naturally occurring high light green plots and green potted plants in the fall. Presence or absence of anthocyanins was determined in the winter by visually assessing whether leaves exhibited red coloration.

Chlorophyll Fluorescence

Chlorophyll fluorescence was used to assess relative photooxidative stress of red and green leaves in this study. Dark-adapted values of the ratio of variable fluorescence to maximum flourescence (F_v/F_m) were measured using a Handy-PEA 1000 fluorescence analyzer (Hansatech Inst., Cambridge, UK) emitting a 2 second 3 mmol m^{-2} s^{-1} saturating pulse. F_v/F_m values represent maximum light capture efficiency of PSII, and range, theoretically, from 0 to 1. An F_v/F_m value of 1 would indicate a 100% transfer of light captured by chlorophylls to the reaction center and electron transport chain, and hence, maximum possible PSII efficiency (though F_v/F_m values in the field rarely exceed 0.86) (Adams *et al.* 1994). Since PSII efficiency is known to decline in response to environmental stress (for reasons later described), changes in F_v/F_m may be used to assess

the relative degree of photooxidative stress incurred by a leaf under various environmental conditions. Therefore, F_v/F_m was used to compare physiological responses of red and green leaves to various conditions in experiments described below. For diurnal measurements of chlorophyll fluorescence, F_v/F_m of selected field leaves was measured every 2 to 3 hours on clear days from pre-dawn to dusk.

Light-response curves for red and green leaves were measured using a pulse modulated chlorophyll fluorescence monitoring system (PAM 2000, Walz, Effeltrich, Germany), using leaves removed from the field that morning (maximum temp: 70°C; minimum: 52°C). Leaves recovered to roughly equivalent F_v/F_m values within 4 hours of being moved inside, and light-response curves were measured shortly thereafter.

High-Stress Recovery

The fluorescence-based responses of red and green leaves with equal starting F_v/F_m values were monitored during and after exposure to a high light stress period to test the hypothesis that anthocyanins confer some degree of photoprotection under high light stress. Leaves were removed from individual red and green clones within 50 meters of each other between 9 and 10 am during February 2004. The petiole of each leaf was cut under water, and remained submerged throughout the experiment. Red and green leaves were placed in separate transient environments for four days prior to experimentation to equalize slow-recovery non-photochemical quenching (NPQ). This was done so that any divergence in fluorescence between the groups could be more readily attributed to anthocyanin-based differences rather than merely differences in starting sustained

xanthophyll pigment molecule ratios and/or retarded D1/D2 protein/PSII core turnover. The green leaf transient period consisted of 4 days within an outdoor protected enclosure, where leaves were exposed to 10 hours of 175 (± 25) μmol mol $m^{-2}s^{-1}$ at field temperatures (-10 ºC to 15 ºC). Light was supplemented during this time by a 1000 W metal halide lamp equipped with a UV filter and shade cloths to obtain desired PPFDs. Red leaves were simultaneously placed indoors and exposed to similar PPFDs at 18 ºC. Once values of F_v/F_m were no longer significantly different between the two groups, both sets of leaves were placed in the dark within the outdoor protected enclosure to equilibrate overnight. At 7 a.m., a high stress treatment was applied which consisted of 10 hours of high light (1150 ± 150 μmol $m^{-2}s^{-1}$ from the metal halide bulb) combined with cold temperatures (circulating outside air, which ranged from 0 ºC to 10 ºC during the day, and -15 ºC to 0 ºC during the night). A glass water bath placed between the light source and the leaves absorbed heat emitted by the bulb during this period. After 3 days in the high stress environment, plants were moved into a low stress environment (18 ºC constant temperature, 10 hours of 175 (± 25) μmol $m^{-2}s^{-1}$ provided by a 65 W incandescent flood bulb) until F_v/F_m recovered to starting values. F_v/F_m was measured at 5 pm on each day of the high stress and recovery treatments. This experiment was conducted on both abaxial and adaxial surfaces of pre-treated leaves, as well as on adaxial surfaces of leaves that had been removed from the field 1 hour before the experiment (with no pre-treatment).

This experiment was also repeated using light filtered through red (750 nm peak transmittance) and green (550 nm) glass filters (Schott Glass, Grünenplan, Germany) to

more specifically attribute the photoprotection to anthocyanin absorbance of blue-green light. PPFDs under both filters ranged from 400-800 μmol $m^{-2}s^{-1}$. Due to the wide variation of PPFDs beneath the filters, red and green leaves were arranged so that leaves within both groups experienced the entire range of PPFDs. Leaves were also rotated daily. Since anthocyanins absorb the most strongly in the green wavelengths (530 nm absorption peak), and least strongly in the red, red leaves were expected to exhibit a significantly smaller decline in F_v/F_m than green leaves when exposed to green light, but an equivalent decline when exposed to red.

Leaf Temperature

Differences in red and green leaf temperature were determined using 3 red and 3 green leaves of roughly equal size. Leaves were cut at the petiole and suspended with their petioles in water within an open rectangular container. The container was placed in full sunlight, and thermocouples were attached to leaves as previously described. This allowed the leaves to lie across an equal plane and receive equal levels of sunlight. This procedure was repeated on three different days with three different sets of leaves.

Antioxidant Activity

Low molecular weight antioxidant (LMWA) activities were evaluated using the α,α-diphenyl-β-picrylhydrazyl (DPPH) protocol described by Neill *et al.* (2002a). DPPH is a stable radical which appears dark purple when in its radical form, but turns colorless when oxidized. Twenty-five mg of freeze-dried tissue was extracted in 5 mL extraction

buffer, and the assay was run using a 180 μM DPPH solution instead of 18 μM. Molarity of the DPPH was altered due to the increase in tissue:extractant used in this study relative to Neill *et al.* (2002a). Summer leaves were collected on July 9, 2003, and winter leaves on March 30, 2004. Both sets of leaves were collected from Long Arm Mountain between 4 and 5 pm. Leaves were immediately placed in liquid nitrogen, and then freeze- dried prior to analysis. Antioxidant potential was quantified using IC_{50} values, which represent the concentration of leaf extract (in μg dry weight mL^{-1}) needed to neutralize the DPPH by 50%. Reduced ascorbic acid was also quantified for these tissues, using the protocol described by Burkey *et al.* (2003), though freeze dried tissue was used instead of fresh tissue.

Vein Severing

A scapel was used to sever major veins <1 cm from the center petiole on one side of 5 high light green field leaves in early fall to inhibit carbohydrate export, and to observe the inducibility of anthocyanin synthesis in response to increases in leaf carbohydrate concentration. After 10 days, anthocyanins and TNCs were quantified using the protocols described previously.

Statistical Analyses

Regression analyses were used to correlate light intensity with F_v/F_m values, anthocyanin, chlorophyll, and carbohydrate concentration, as well as to correlate

anthocyanin with carbohydrate concentration. Diurnal trends in fluorescence were analyzed using repeated-measures ANOVAs. Two-sample t-tests were used to compare biomolecular ratios between sun and shade plants within and between seasons. Two-sample t-tests were also used to compare total energy absorbed between 400-700 nm, energy absorbed at blue/green wavelengths (500-600 nm), and energy reflected at red wavelengths (618-700 nm) for red and green leaves. A single-factor ANOVA with Tukey's test for means comparisons was used to compare mean IC_{50} values of red, intermediately red, and green leaf groups. A regression was further used to correlate anthocyanin concentration with antioxidant activity. Anthocyanin and TNC levels of severed and un-severed sides were compared using paired t-tests.

RESULTS

Effects of Light Intensity on Anthocyanin, Chlorophyll, and TNC Levels

Of the three naturally occurring Galax plots sampled in this study, leaves that exhibited reddening were found in environments exposed to substantially more light than leaves that remained green. Total PPFDs incident upon red leaves on a clear winter day averaged approximately 135 (± 9) mmol m^{-2}, whereas average total PPFDs of green leaves averaged only 12 (± 1.5) mmol m^{-2} photons, a difference of roughly 91%. Additionally, maximum PPFDs incurred by red leaves often surpassed 1000 µmol m^{-2} s^{-1}, while PPFDs of green plots seldom exceeded 400 µmol m^{-2} s^{-1}. Winter diurnal PPFD patterns for a typical plot containing both red and green leaves are illustrated in Figure 1.

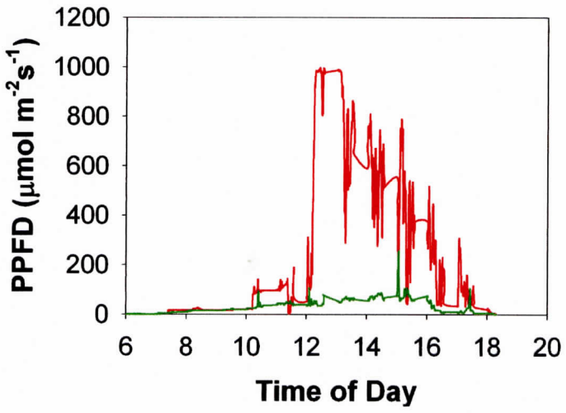

Figure 1. PPFDs of red and green leaves in a typical Galax plot (March 3).

High light leaves that had been sequentially shaded with neutral density cloth prior to reddening exhibited an anthocyanin gradient that increased linearly with light intensity ($p<0.0001$; $r^2= 0.76$ for potted plants [Figure 2A], and $r^2= 0.84$ for field plants [Figure 2B]). Leaves from the 0% shade treatment exhibited an average of 23-fold higher anthocyanin content than leaves in the 80% shade treatment. In both experiments, leaves in the 80% shade treatment were the only leaves that exhibited no visible reddening. Galax leaves exhibiting various shades of reddening are pictured in Figure 3.

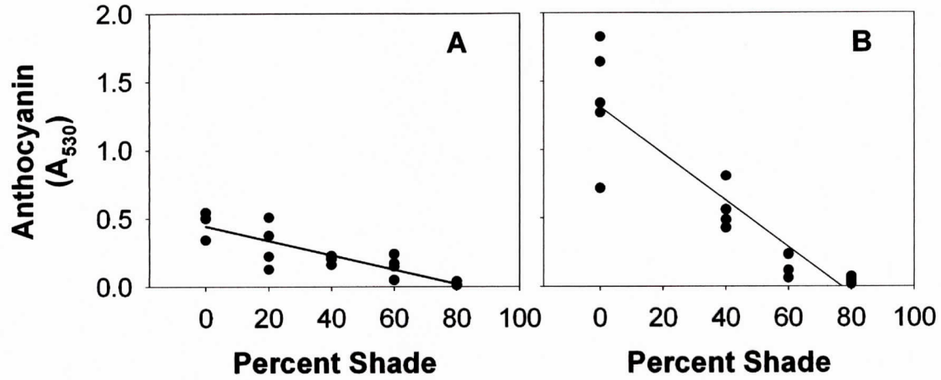

Figure 2. Percent shade effects on anthocyanin content in sequentially shaded potted plants, $r^2= 0.76$ (A) and field plants, $r^2= 0.84$ (B).

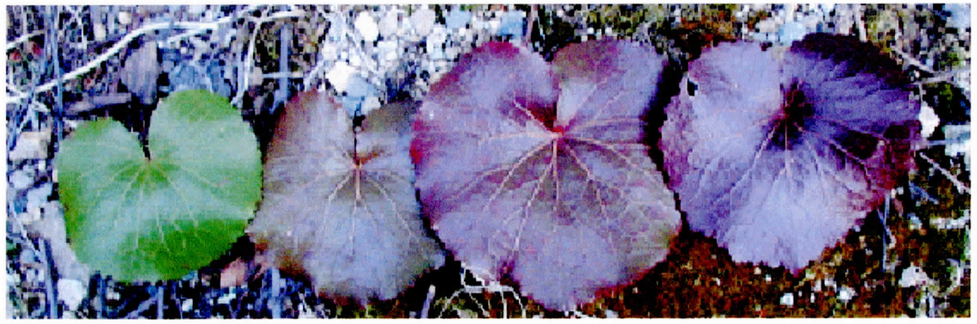

Figure 3. Galax leaves exhibiting increasing concentrations of anthocyanin.

Anthocyanins were observed to occur in the vacuoles (Figure 4A) of peripheral mesophyll cells closest to the epidermis (Figure 4B). Pigmentation was observed to occur on both abaxial and adaxial surfaces, depending on the orientation of the leaf relative to the light source. For those leaves oriented with adaxial surfaces facing the light source, with abaxial surfaces covered by litter or another leaf, the adaxial surface alone exhibited pigmentation. For those leaves oriented upside down, only the abaxial surface exhibited pigmentation. If the light was incident upon the adaxial surface, and the leaf's abaxial surface was uncovered by litter or other leaves, both the adaxial and abaxial surfaces were frequently observed to exhibit pigmentation (Figure 4B).

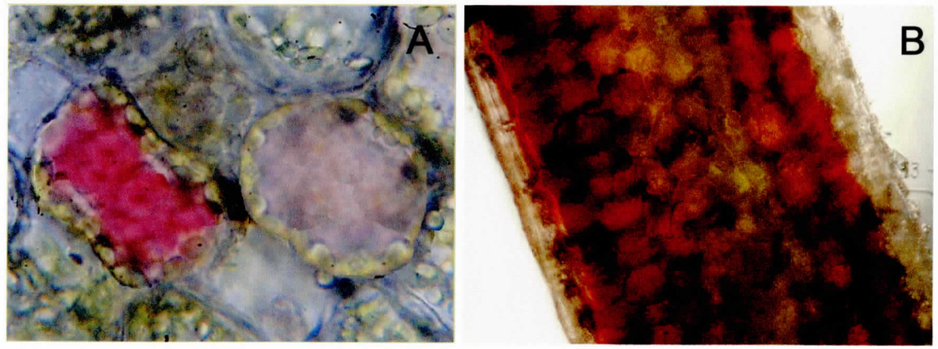

Figure 4. Galax cross sections. A: Vacuole containing anthocyanin (430X magnification). B: Cyanic mesophyll of both abaxial and adaxial surfaces (100X).

Levels of chlorophyll *a, b,* and the ratio of chlorophyll *a/b* did not significantly differ between sun and shade plants during the summer. During the winter, however, while chlorophyll *a* and *b* levels did not significantly differ between sun and shade plants, the ratio of chlorophyll *a/b* was significantly lower in sun plants compared to shade ($p = 0.005$), and was found to significantly decrease with light intensity ($p < 0.005$; $r^2 = 0.36$

for potted plants [Figure 5A]; $r^2 = 0.42$ for field plants [Figure 5B]). This decline appeared to be primarily due to decreases in chlorophyll *a,* as sun leaves exhibited an average of 10% less chlorophyll *a* than shade leaves, but only 2% less chlorophyll *b* (see Table 1).

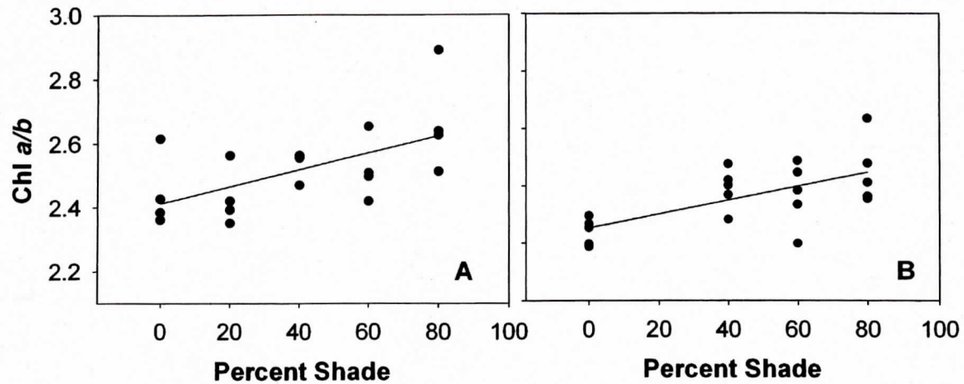

Figure 5. Chlorophyll *a/b* ratios in sequentially shaded potted plants, $r^2 = 0.36$ (A) and field plants, $r^2 = 0.42$ (B).

The effects of shade on TNCs yielded less consistent results between field and potted plant groups. Shade appeared to have no effect on starch or soluble sugar content in field plants ($p = 0.95$ and 0.65 respectively), but a very significant effect in potted plants ($p < 0.01$, $r^2 = 0.386$, and $p < 0.01$, $r^2 = 0.329$), with starches and soluble sugars both increasing with light intensity (Figure 6). Potted plants also generally exhibited higher levels of carbohydrates than field plants at most shade levels.

UV light did not appear to be necessary to induce anthocyanin synthesis, as green leaves were able to synthesize anthocyanins in both the presence and absence of UV (data not shown). This was observed in plants in the natural habitat (under UV-screens), plants

that had been potted (under UV screens), and leaves that had been cut at the petiole and placed in water (exposed to light through a UV filter).

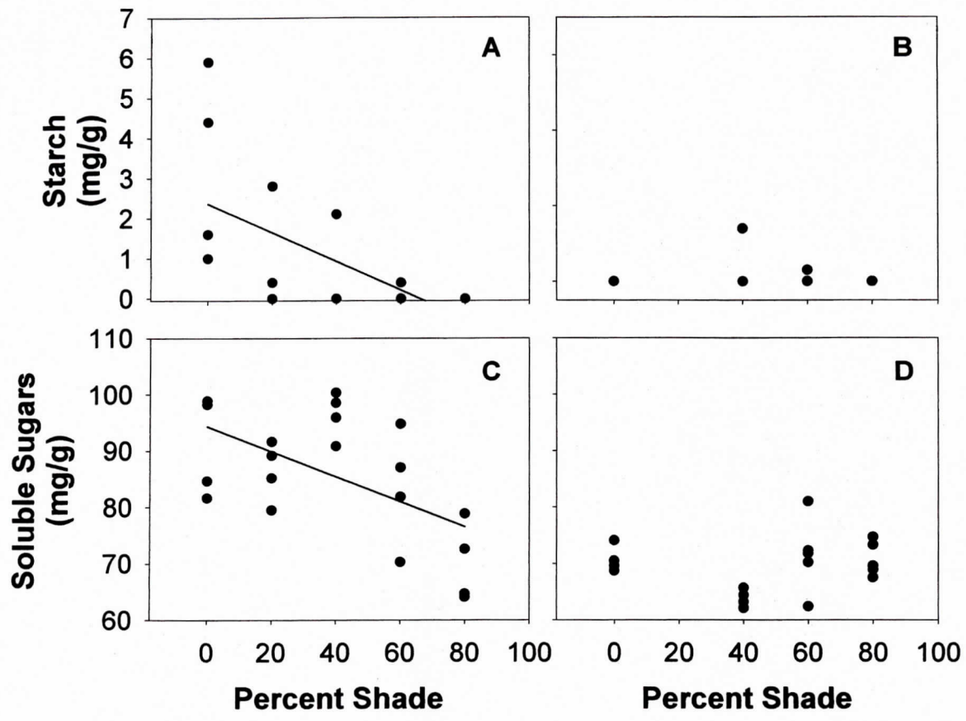

Figure 6. Effects of percent shade on starch content in sequentially shaded potted plants, $r^2 = 0.386$ (A) and field plants (B). Percent shade vs. soluble sugars for potted plants, $r^2 = 0.329$ (C) and field plants (D).

Seasonal Variation in Biomolecules

A comparison of biomolecule levels from sun and shade leaves sampled in summer and winter is illustrated in Table 1. Both chlorophyll *a* and *b* levels were significantly lower in winter leaves compared to summer leaves ($p= 0.002$ and $p= 0.058$), though the

ratio of chlorophyll *a/b* did not significantly differ between seasons. Seasonal comparison of TNCs show that starch was significantly higher in summer sun leaves than winter sun leaves ($p< 0.0001$), but levels in summer and winter shade plants did not significantly differ. Both sun and shade winter leaves exhibited significantly higher soluble sugar content than summer leaves, as winter leaves exhibited a nearly 2-fold increase in soluble sugars ($p< 0.0001$). Total sugars did not significantly differ between summer and winter sun plants, though total sugars were significantly (2-fold) higher in shade winter leaves compared to shade summer leaves ($p<0.0001$). Anthocyanins were never visually observed in summer plants, and only present in low concentrations in shade winter plants, though they were consistently observed in winter sun plants.

Table 1. Summer and winter biomolecules for high light and low light leaves in the field. Winter sun leaves represent red leaves, winter shade represent green. Values are means from 5 samples ± SE.

	Anthocyanin (A_{530})	Chl *a* (μg/cm²)	Chl *b* (μg/cm²)	Total Chl	Chl *a/b*	Starch (mg/g)	Soluble Sugars (mg/g)	Total Sugars (mg/g)
Summer sun	0.0201 (0.0010)	34.4 (2.1)	12.9 (1.2)	47.3 (3.2)	2.70 (0.11)	25.3 (3.1)	45.7 (1.2)	71.0 (3.6)
Summer shade	0.0229 (0.0012)	36.4 (2.4)	15.6 (2.1)	52.0 (4.3)	2.47 (0.19)	0.00 (0.0)	32.7 (1.2)	32.7 (1.2)
Winter sun	1.36 (0.17)	24.8 (1.2)	11.1 (0.52)	35.9 (1.7)	2.24 (0.019)	0.300 (0.30)	74.6 (0.8)	74.9 (1.0)
Winter shade	0.0500 (0.010)	27.6 (0.69)	11.3 (0.46)	38.9 (1.1)	2.45 (0.046)	0.100 (0.10)	71.8 (3.8)	71.9 (3.8)

Ecophysiological Profiles of Red and Green Leaves

Red leaves exhibited significantly and consistently lower dark-adapted maximum light-capture efficiency values (F_v/F_m) than green leaves in the field ($p < 0.0001$). This difference corresponded with level of irradiance incident upon the leaves, as F_v/F_m decreased with light intensity ($r^2 = 0.87$, Figure 7).

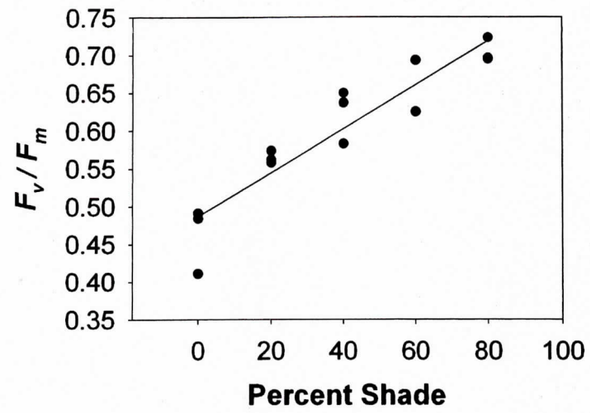

Figure 7. F_v/F_m as a function of light intensity, $r^2 = 0.87$. Data derived from sequentially shaded field plants.

The difference in F_v/F_m of red and green leaves within a given plot in the field typically ranged from approximately 0.1 to 0.3 F_v/F_m units, depending on the difference in light dynamics within each plot. Generally, plots with a less steep light gradient exhibited smaller differences in F_v/F_m between red and green leaves (Figure 8A), relative to plots where the difference in light environment between red and green leaves was more extreme (8B).

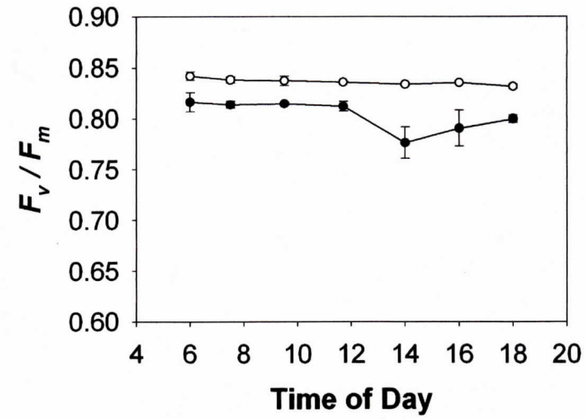

Figure 11: Diurnal F_v/F_m for sun (black) and shade (white) leaves within a single plot on Sept. 13 (min: 11°C; max: 20°C). Points represent means (±SD) for 3 replicates.

Light-response curves derived using the PAM fluorometer showed that green leaves possessed lower photosynthetic electron transport capacity than red leaves when irradiances exceeded approximately 200 μmol m^{-2} s^{-1} (Figure 12). No saturation was observed in red leaves, even at highest the PPFD. Green leaves exhibited a light saturation point of approximately 400 μmol m^{-2} s^{-1}.

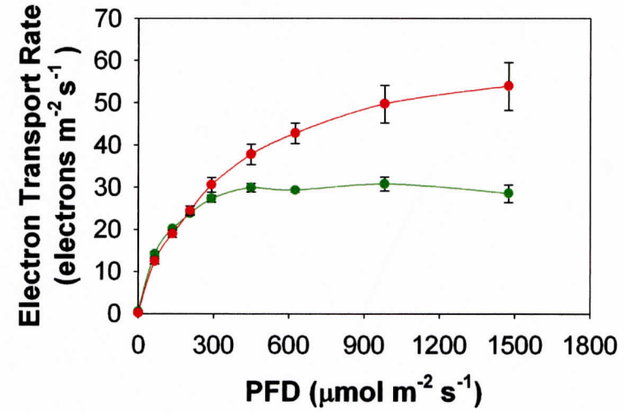

Figure 12. Light response curves of red and green leaves derived from the field with similar starting F_v/F_m values. Points represent means ±SE for 5 replicates.

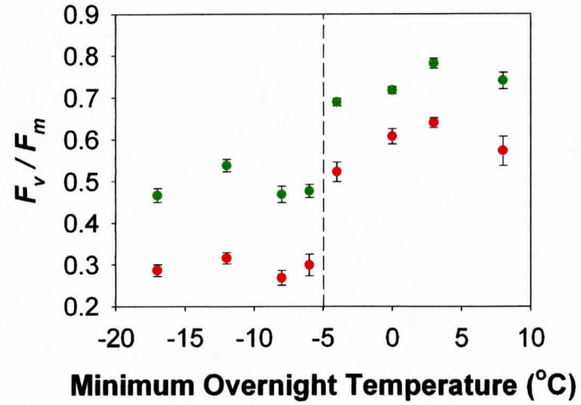

Figure 9. Pre-dawn F_v/F_m of red and green leaves in the field as a function of minimum overnight temperatures. Points represent means ±SE for 6-9 replicates.

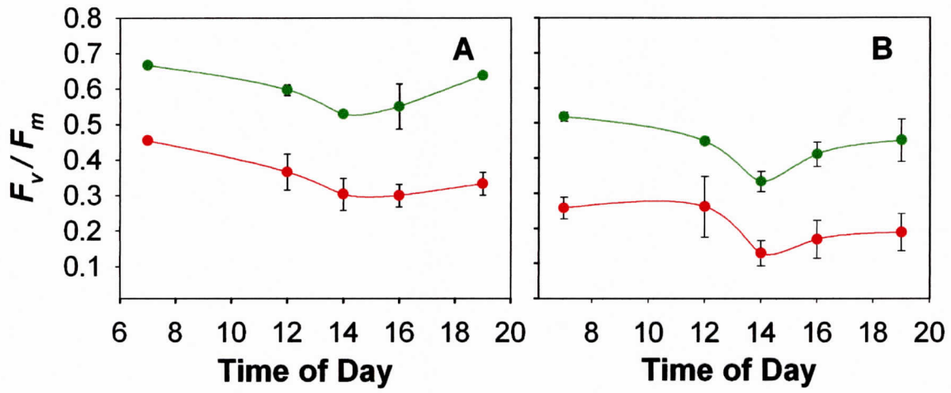

Figure 10. Diurnal F_v/F_m of red and green leaves in the field occurring within a single plot on Dec. 15 (max: 0.5ºC; min -4ºC) (A) and the same leaves on Jan. 23 (max: 4ºC, min: -13.5ºC) (B). Points represent means ± SE of 3 replicates.

Winter values of F_v/F_m were consistently lower than F_v/F_m values observed during the summer for both shade and sun leaves ($p < 0.0001$). During the summer, F_v/F_m seldom dropped below 0.75 (Figure 11). However, light levels within Galax plots were also substantially lower during summer than during the winter (data not shown).

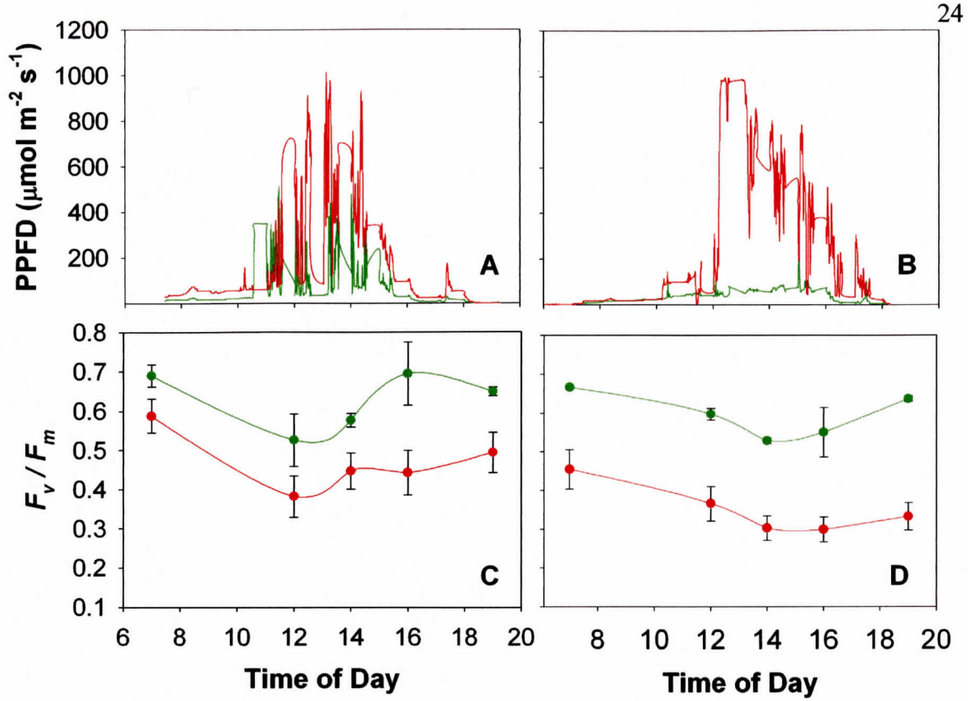

Figure 8. PPFD and F_v/F_m of red and green leaves within a field plot exhibiting a narrow light gradient (A) and a steep light gradient (B). Measurements taken on Dec.15 (min temp: -4 ºC, max temp:0 ºC). Points represent means ±SE of 3 replicates.

Minimum overnight temperatures were also found to affect pre-dawn F_v/F_m values in the field. When night time temperatures fell below -5 ºC, pre-dawn F_v/F_m values for both red and green leaves were significantly lower than when minimum temperatures were above this temperature ($p<0.0001$, Figure 9). Consequently, diurnal F_v/F_m values for both red and green leaves were substantially lower on days with extreme sub-freezing temperatures than days that were at or above freezing (Figures 10A and 10B). However, the mean difference in F_v/F_m between red and green leaves within individual plots remained relatively constant, averaging approximately 0.17.

Leaf Temperature

Leaf temperatures of red and green leaves did not differ significantly when placed under equal irradiances (data not shown). This trend was consistent across all three trials.

Leaf Optics

Red leaves absorbed significantly more light energy compared to green, most notably in the green (500-600 nm) wavelengths (Figure 13). When percent absorbance was converted to energy equivalents, red leaves were found to absorb 11% more light energy in green wavelengths than green leaves, a difference which was highly significant ($p < 0.0001$).

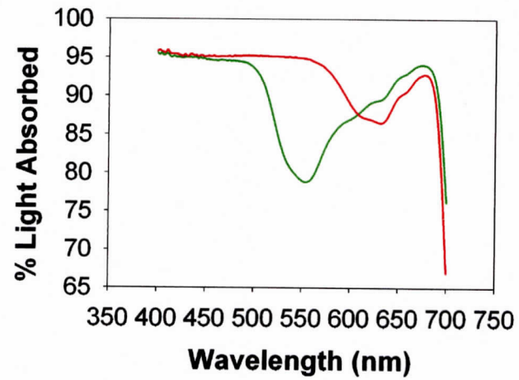

Figure 13: Absorbance spectra for red and green leaves. Lines represent means of 5 leaves.

High-Stress Recovery

When adaxial surfaces of red and green leaves were exposed to the high white-light stress treatment following pre-treatment to equalize starting F_v/F_m values, mean green leaf F_v/F_m was observed to decline by 86%, while F_v/F_m of red leaves only declined by 55%. Subsequently, red leaves recovered to near-starting F_v/F_m values after 1 day, while green leaves required 5 days (Figure 14A). In leaves that were exposed to the high stress treatment immediately following removal from the field (without a pre-treatment period to equalize F_v/F_m), very similar trends were observed. Green leaves exhibited declines in F_v/F_m of approximately 78%, while red leaves exhibited declines of only 52% (Figure 14B). Additionally, red leaves recovered to starting F_v/F_m after 1 day, while green leaves required 4 days.

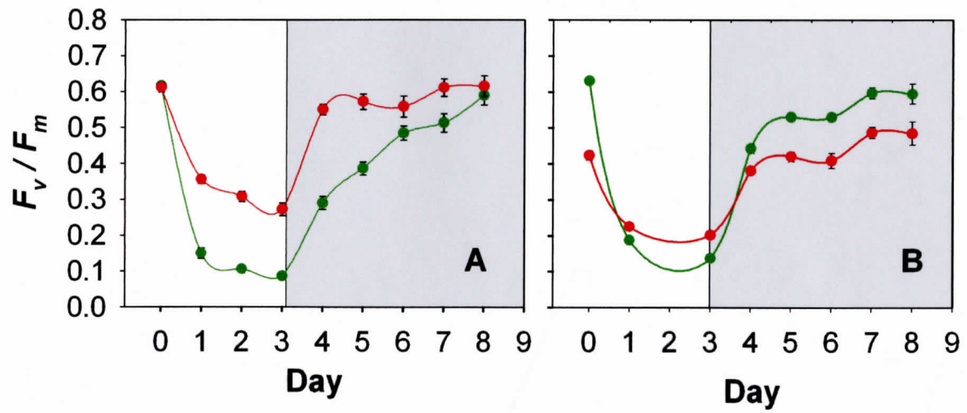

Figure 14. F_v/F_m recovery of pre-treated (A) and non pre-treated (B) red and green leaves (adaxial surfaces). White area represents high stress treatment, gray represents recovery period. Points depicted are means ±SE of 5 replicates.

This experiment was repeated (with pre-treatment) on the green abaxial surfaces of red and green leaves to compare F_v/F_m recovery rates without interference by anthocyanins (Figure 15). Decline and recovery of the green undersides of both red and green leaves were nearly identical, with a decrease in F_v/F_m of approximately 88% during the high stress period. Recovery rates of both groups of leaves were similar to those exhibited by the green abaxial surfaces (Figure 14).

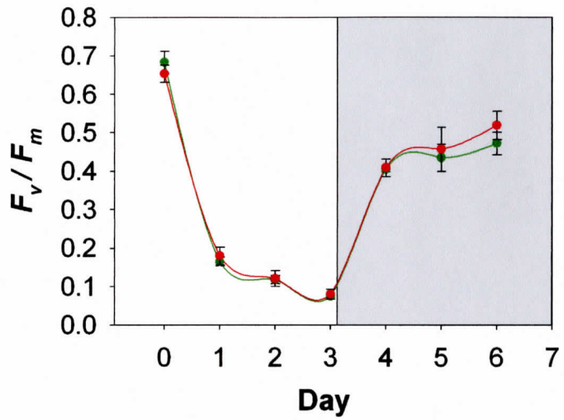

Figure 15. F_v/F_m recovery of pre-treated red and green leaves (acyanic abaxial surfaces). White area represents high stress treatment, gray represents low stress treatment. Points depicted are means ±SE of 5 replicates.

In response to green light (Figure 16A), red leaves exhibited significantly less of a decline in maximum PSII efficiency compared to green leaves, as red leaf F_v/F_m declined by 19% in response to the high light treatment, while green leaf F_v/F_m declined by 72%. Red leaves required 2 days to recover to starting F_v/F_m values, while green leaves required 3 days. Upon exposure to red light (Figure 16B), however, declines in maximum PSII efficiency were nearly identical between groups, with both red and green leaf groups

exhibiting a 63% decline in F_v/F_m; all leaves in this treatment recovered to near starting F_v/F_m values after 1 day of recovery.

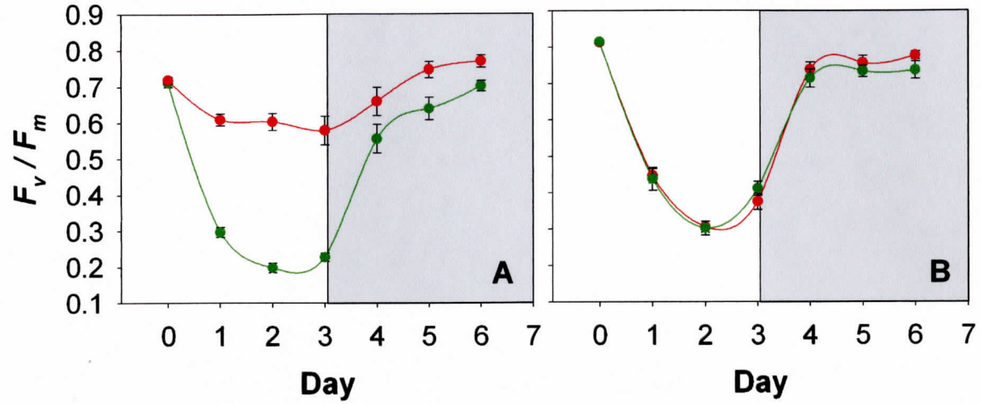

Figure 16. F_v/F_m responses of pre-treated red and green leaves to high stress period consisting of green light (A) and red light (B). White area represents high stress period, gray area represents recovery period. Points depicted are means ±SE of 5 replicates.

Antioxidants

Summer sun leaves exhibited antioxidant activity that was marginally higher than that of summer shade leaves ($p=0.066$), as evidenced by lower IC_{50} values in Figure 17. Antioxidant activities of winter sun (i.e. red) leaves were significantly higher than those of sun and shade leaves harvested during the summer ($p < 0.0001$; Figure 17). However, antioxidant activities of red leaves did not significantly differ from those of winter green leaves, and anthocyanin concentration did not appear related to antioxidant activity (Figure 18). Levels of reduced ascorbic acid also did not significantly differ between red and green leaves (data not shown).

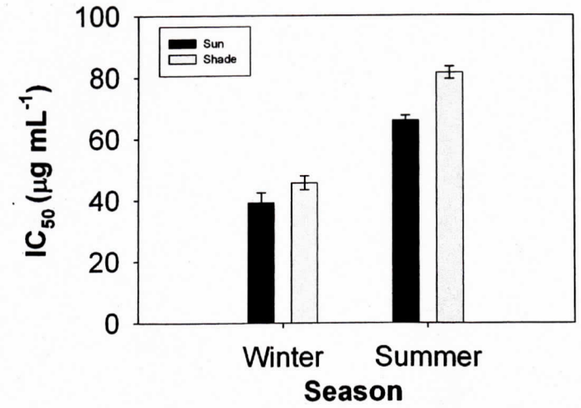

Figure 17. Mean IC_{50} by light environment and season. Bars represent means ±SE for 5-15 replicates.

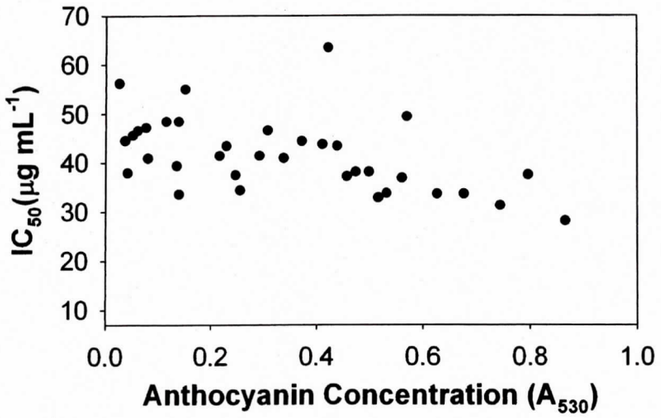

Figure 18. Relationship between anthocyanin concentration and IC_{50}.

Vein Severing

Severing of major veins resulted in significant increases in starch (p= 0.0003), soluble sugars (p= 0.0002), and hence total sugars (p= 0.0002) relative to the half of the leaf where veins had not been severed (Figure 19A). Furthermore, the halves of leaves with

severed veins produced significantly and visibly more anthocyanins (p= 0.0002) than un-severed halves (Figure 19B) . However, no consistent linear relationships between anthocyanins and starches, sugars, or total sugars were evident in this experiment.

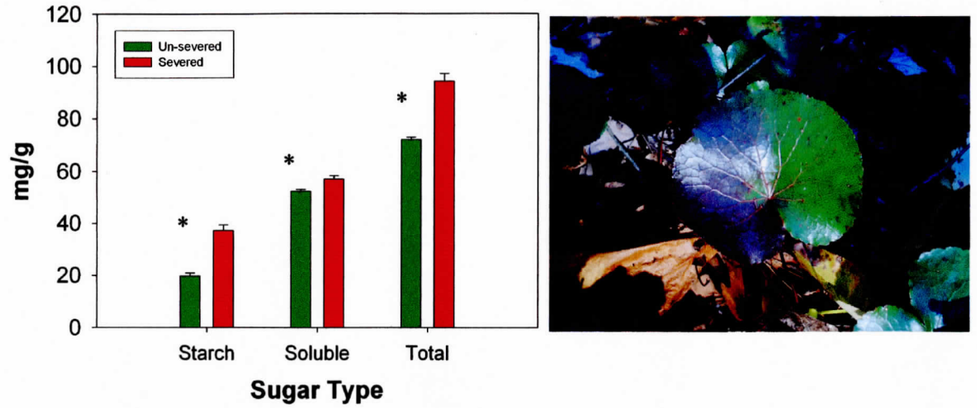

Figure 19. Left panel (A): Effects of vein severing on starch, soluble sugar, and total sugar concentrations. Bars represent means ± SE of 5 replicates. Right panel (B): Effects of vein severing on anthocyanin synthesis. In the leaf pictured, veins on the left side of the leaf were severed, while veins on the right side were not.

DISCUSSION

The fundamental assumption that anthocyanins occur in leaves most susceptible to light stress was clearly illustrated in this study. As light intensity increased, susceptibility of Galax plants to photooxidative damage increased proportionately, as illustrated by decreases in maximal light capture efficiency (F_v/F_m) observed in plants grown across a light gradient (Figure 7), and by plants grown in shaded versus unshaded habitats (Figure 8). This decline in F_v/F_m in response to increasing irradiance has been documented in several species, and has been attributed to the an up-regulation of PSII-specific photoprotective mechanisms implemented as a response to increased photostress. These photoprotective responses include increases in xanthophyll pool size, increased conversion of violaxanthin to zeaxanthin, and selective degradation and/or sustained-phosphorylation of D1/D2 protein and whole PSII cores (Adams *et al.* 1994; Adams *et al.* 2001b; Ebbert *et al.* 2001; Verhoeven *et al.* 1996). These mechanisms decrease light capture efficiency of the photosynthetic apparatus either by absorbing light energy from chlorophyll molecules and dissipating it away as heat (in the case of zeaxanthin), or by retarding the water-splitting process to decrease introduction of new electrons and divert electron flow cyclically around PSII (degradation or dissociation of D1 and PSII cores) (Adams *et al.* 1994; Adams *et al.* 2002; Critchley and Russell 1994). Although these photoprotective parameters were not directly measured in this study, patterns of

chlorophyll fluorescence that have been associated with their implementation were observed, and used to infer the degree of photooxidative stress exhibited by leaves under various environmental conditions, as described below.

Diurnal fluctuations in F_v/F_m mirrored changes in environmental conditions which could potentially exacerbate photooxidative stress. Specifically, declines in F_v/F_m were observed to be the greatest on very cold days (Figures 9 and 10) and in leaves exposed to the highest levels of irradiance (Figures 7 and 8). In the field, this translated into the consistently lower values of F_v/F_m for red leaves compared to green leaves, with differences in F_v/F_m being the greatest when the light gradient between red and green leaves was very steep (Figure 8). This is presumably due to the greater need of high light leaves to implement photoprotective mechanisms to reinstate the balance between light capture and photosynthesis during such conditions, a problem less realized by deeply shaded leaves (Demmig-Adams 1998). Pre-dawn F_v/F_m values were also shown to be significantly affected by overnight temperatures, with maximum light capture efficiencies being the lowest when overnight temperatures were several degrees below freezing (Figure 9). Several studies have related this pattern to the sustained retention of zeaxanthin and antheraxanthin and sustained PSII protein phosphorylation through the night, proposing that retention of these molecules in their primed energy-dissipating states may function to prepare the leaf for exposure to sunlight the subsequent day (Adams *et al.* 2002; Adams *et al.* 2001a; Adams and Demmig-Adams 1995). The delayed rise in F_v/F_m below freezing temperatures may also be attributed to the slow turnover of D1 protein, a process which has been shown to be severely retarded by very low

temperatures (Leitsch *et al.* 1994; Schnettger *et al.*1994; Thiele *et al.* 1996).

The above observations confirm the initial assumption that Galax leaves under high light, cold temperature conditions are indeed more susceptible to photooxidative damage than shaded leaves, as evidenced by their need to implement these photoprotective mechanisms. The observation that anthocyanin synthesis coincides with increases in light intensity (Figure 2), and hence susceptibility to photooxidative stress, thus strengthens the prospect that anthocyanins are produced in response to increasing light stress, and thus may serve some type of photoprotective function.

The first hypothesis described in this study purported that anthocyanins protect underlying mesophyll cells in high light leaves by absorbing blue-green light and dissipating it away as heat. In contrast to the heat dissipating process of the xanthophyll pigments, which are located in the antennae complex and accept energy directly from excited chlorophyll molecules, anthocyanins are exclusively vacuolar (Figure 4), and therefore intercept and dissipate light energy yet unabsorbed by other pigments. The light attenuation hypothesis predicts that the presence of anthocyanins should cause cyanic leaves to exhibit less photooxidative damage than acyanic leaves (which would be evidenced by higher F_v/F_m) when exposed to equally high levels of irradiance perpendicularly to the cyanic surface, assuming starting levels of other photoprotective mechanisms to be roughly equal. However, if the light is shone on any acyanic regions of red leaves, such as the green abaxial surface, those tissues should exhibit declines in F_v/F_m comparable to green leaves. Both of these predictions gained experimental support in this study. In Figure 14, leaves with red and green adaxial surfaces with equal starting

F_v/F_m values exhibited drastically different fluorescence responses when exposed to high PPFDs, as the decline in F_v/F_m of green leaves was 29% greater than the decline observed in red leaves. Furthermore, green leaves required 4 more days than red leaves to recover to starting maximum PSII efficiency, suggesting that red leaves had experienced a lesser degree of stress than green leaves during the high light period. When the light was shone from the acyanic abaxial surfaces, however, maximum light capture efficiency of red and green leaves both declined by 93% (Figure 15), and exhibited recovery rates similar to those of adaxial green surfaces. These results suggest that the cyanic layer does indeed convey some degree of photoprotection in high light leaves.

In order to more specifically attribute the protective mechanism employed by red leaves to anthocyanins, adaxial surfaces of red and green leaves were again exposed to high levels of irradiance, but at wavelengths that were either strongly or poorly absorbed by anthocyanins. Upon exposure to green light, which anthocyanins strongly absorb, green leaves exhibited a significantly greater degree of photooxidative stress than red leaves; however, when leaves were exposed to red light, which anthocyanins poorly absorb, red and green leaves exhibited equal declines in F_v/F_m (Figure16). These findings indicate that red leaves are indeed less susceptible to photooxidative damage induced by green wavelengths than green leaves, but that they are equally susceptible to damage induced by red wavelengths. Since these absorbance qualities match those of anthocyanin, the hypothesis that anthocyanins function to protect high light leaves from excess irradiance by absorbing green wavelengths gains strong support.

One question that should be addressed at this point is- why green light? If

photosynthesis is primarily a function of red and blue light, it seems that natural selection would favor expression of a light attenuator that absorbs strongly in these highly absorbed wavelengths, rather than the weakly absorbed green wavelengths. One possible answer to this question, as previously described, is based on the observation that anthocyanin's absorption range (approximately 450-575 nm) overlaps with that of chlorophyll *b* (400-500 nm), with the major overlap occurring in the blue-green wavelengths. Although both chlorophyll *a* and *b* do absorb most strongly in red and blue wavelengths, previous studies on spinach have shown that the majority of these wavelengths are attenuated in the adaxial palisade mesophyll (93% in blue wavelengths, 75% in red, but only 65% in green), resulting in proportionately more green wavelengths being transmitted to the lower mesophyll relative to blue and red (Vogelmann and Evans 2002). The ability of chlorophyll *b* to absorb green wavelengths more efficiently than chlorophyll *a* may then explain the general decrease in chlorophyll *a/b* ratios which occur as leaf depth increases (Cui *et al.* 1991). These observations may also be used to explain the findings of Sun *et al.* (1998), who showed that carbon fixation was highest in the palisade mesophyll in response to blue and red light, but highest in the lower mesophyll in response to green light. Given the significant role green light plays in the lower mesophyll cells, then, it becomes apparent that decreasing the transmittance of these wavelengths could be advantageous in the case of high light stressed plants.

A second possible advantage of absorbing green light rather than red or blue pertains to the potential interference a red or blue light-absorbing molecule would pose towards the function of plant photoreceptors, such as phytochrome, cryptochrome, and

phototropins (McClure 1975). These molecules have been shown to be sensitive to red and blue light, using changes in their intensity and duration to regulate cellular processes including stomatal opening, shade detection, phototropism, photoperiodism, and chloroplast development (Hopkins 1999; Lin 2000; Lin 2002). By avoiding absorption of these wavelengths, anthocyanins may effectively curtail photooxidative stress without interfering with red and blue light-mediated cell function.

Anthocyanin's absorbance of blue-green light may also be responsible for the changes in chlorophyll ratios observed in anthocyanic leaves during the winter. As shown in Figure 5, chlorophyll *a/b* ratios were observed to significantly decrease as light intensity (and hence, anthocyanin concentration) increases. This trend is unusual in that pigment analyses of evergreens in previous studies have shown *increases* in chlorophyll *a/b* in response to increasing irradiance, rather than decreases, presumably corresponding with higher ratios of core to light-harvesting complexes, indicative of a physiological shift away from light-capture and towards carbon fixation (Cui *et al.* 1991; Demmig-Adams 1998; Grace and Logan 1996). It is very interesting to note, however, that chlorophyll ratios of other plant species that produce anthocyanins (either transiently or genetically) also exhibit lower chlorophyll *a/b* ratios in red leaves compared to green, even when there is no difference in the light environment in which they are grown. Such species include the tropical understory plants *Begonia pavonina* and *Triolena hirsuta,* and the evergreen herb *Mahonia repens* (Grace and Logan 1996; Gould *et al.* 1995). The propensity for red leaves to consistently exhibit lower chlorophyll *a/b* ratios than green conspecifics would lead one to suspect that increases in anthocyanin content may, in some way, be the cause

for this change. One possible explanation for this could be that anthocyanins are so efficient at shielding underlying mesophyll from light that these subjascent cells are shifting photosynthetic emphasis back towards light capture, and away from photosynthesis, since shade leaves typically exhibit lower chlorophyll *a/b* ratios than sun leaves (Burkey and Wells 1996; Cui *et al.* 1991). However, this supposition is incongruous with the stream of observations previously described which indicate that high light (i.e. red) Galax leaves exhibit F_v/F_m patterns characteristic of plants primed for photoprotection. Further studies should be conducted to elucidate this relationship.

The hypothesis that anthocyanins function to increase leaf temperature was not supported in this study, as there was no significant difference in leaf temperature between red and green leaves. This is most likely because the increase in absorbed energy between 400-700 nm exhibited by red leaves relative to green leaves (2-3%) was not great enough to subsidize heat lost through convective cooling and re-radiation.

Antioxidant analyses also yielded results that were inconsistent with initial hypotheses. Although winter leaves, in general, exhibited significantly greater antioxidant activity than summer leaves (as evidenced by lower IC_{50} values in Figure 17), winter sun (i.e. red) leaves did not differ significantly with regards to LMWA activity when compared to shaded (green) leaves. In fact, many leaves with very low anthocyanin concentration exhibited antioxidant activities equal to or greater than those of some leaves with higher anthocyanin concentrations (Figure 18). Subsequent assays on reduced ascorbic acid, known to be a significant contributer to the antioxidant pool, also showed no differences in levels of this antioxidant between red and green leaves (data not shown).

These results differ from those of previous studies on acyanic cold-acclimated broad-leaved evergreen species, in which an upregulation in antioxidant activity in response to increasing irradiance was typical (Grace and Logan 1996). Our results are also incongruent with those of studies on some understory plants with red and green morphs, such as the tropical understory herb *Elatostema rugosum* (Neill *et al.* 2002a). In *E. rugosum,* red leaves were shown to exhibit greater antioxidant activities than green leaves, with anthocyanins contributing significantly to the overall antioxidant pool. However, our results do resemble those derived from the anthocyanic sun-tolerant tropical canopy tree species, *Quintinia serrata* (Neill *et al.* 2002b). In *Q. serrata,* as with Galax, there was no apparent difference in antioxidant activities between red and green morphs, and some green leaves exhibited IC_{50} values equal to or below those of red leaves (Figure 18). Although Galax is not closely related to *Q. serrata*, and red morphs of *Q. serrata* arise due to genetically, rather than environmentally, controlled polymorphisms, our results may be similar due to the fact that the green leaves used in both studies were susceptible to periodic exposure to potentially damaging levels of irradiance. With *Q. serrata,* red and green morphs were derived from the natural forest canopy, and probably experienced equal susceptibility to irradiance either directly or via sunflecks. With Galax, although winter green leaves are masked by shade throughout most of the day, sunflecks exceeding 500 μmol $m^{-2}s^{-1}$ were observed to occur in some plots (Figure 8A), including those sampled in this study. A well known cost of keeping photosystems primed for energy capture in the deep shade is an increased vulnerability to photooxidative damage due to sunflecks when they occur (Demmig-Adams and Adams

1992; Watling *et al.* 1997). It is conceivable, then, that a shade plant which experiences frequent sunflecks may maintain a steady pool of antioxidants as a protective measure throughout the day, since the intensity of sunflecks may result in high light stress (and hence increased ROI production), but their brevity would not allow plants to effectively engage xanthophyll-related photoprotective mechanisms. The fact that the leaves used in this study were sampled from their natural environment, where sunflecks readily occur, is therefore a factor which should be taken into account when comparing these results to those of studies where plants were grown in homogenous light environments, such as the those in Grace and Logan's (1996) study on broad-leaved evergreens, or under conditions where temperature-exacerbated photooxidative damage is not as threatening (Neill *et al.*'s 2002a study on tropical understory herbs). Regardless of why green leaves exhibited such high antioxidant activities, though, the implication of these results still remains- that anthocyanin content does not appear to significantly affect the total leaf antioxidant pool in Galax, and therefore, anthocyanins are most likely not being formed as antioxidants in this specie.

Analyses of leaf carbohydrates yielded results which suggest that anthocyanins are most likely not produced as a result of source:sink imbalance in high light leaves during the winter. The major assumptions of the carbon overflow hypothesis were (1) that high light (red) leaves fix a greater amount of carbon than green leaves, and (2) that carbon sinks are limited during the winter due to cessation of growth, inhibition of translocation due to soil freezing, and/or reduced metabolic rates; these two factors in combination would then result in overflow of carbon into an alternative sink, the phenylpropanoid

pathway, and production of anthocyanins. Regarding the first assumption, our results do suggest that high light leaves fix more carbon than their shaded counterparts. This was supported by the observations that TNCs in potted plants increased across a light gradient (Figure 6), and also that high light (red) leaves appeared to be engaged in higher rates of photosynthesis (up to 3x greater) on warm winter days than their shaded counterparts in the field (data not shown). One would expect, then, that if sinks were limiting in field plants, that plants grown under increasing irradiances would exhibit increasing levels of soluble sugars and/or starch as well, and that red leaves would possess higher levels of TNCs than green leaves. Our results did not support this assumption, as TNC analyses showed no significant differences in starch, soluble sugar, or total sugars between field leaves grown across a light gradient, and no significant differences in TNCs between red and green field leaves (Figures 6 and Table 1). Furthermore, if anthocyanins were acting as a carbon sink, one would expect to see anthocyanins formed in shade leaves in the field as well, since carbohydrate levels were equally as high and even exceeded levels found in sun plants in some cases during the winter (Figure 6). What these data seem to suggest instead is that high light Galax leaves *are* fixing more carbon during the winter, but sink capacities are adequate enough to maintain consistent concentrations of carbohydrates in leaf tissues- levels which are comparable to those observed in shade plants. Possible reasons why potted plants may have accumulated TNCs could be the fact that rhizomes in potted plants were significantly shorter than naturally occurring rhizomes, representing only a fraction of the normal root system, and/or the possibility that the potting process inhibited root growth, translocation, or metabolic processes in some way.

Although red and green leaves did not differ with regards to TNCs relative to each other, it should not escape notice that all winter leaves exhibited a 2-fold increase in soluble sugar content relative to the summer (Table 1). Previous studies have shown that plants often up-regulate soluble sugars as a mechanism to prevent tissue freezing during the winter (Sasaki *et al.* 1996; O'Neill 1983). This cryoprotectant function would certainly be advantageous for evergreen plants, such as Galax, which are susceptible to temperatures that plunge well below freezing at night, and could therefore be a legitimate ultimate cause for their synthesis and retention. However, results from the vein severing experiment described below suggest that this increase in soluble sugar content may also be serving an additional function- perhaps as a cue necessary to initiate anthocyanin synthesis in the presence of high light.

As shown in Figure 19, Galax could be induced to synthesize anthocyanins by severing veins of a leaf in warm, moderate to high light conditions. Under no other conditions were Galax leaves observed to synthesize anthocyanins in this study without the presence of cold temperatures. Figure 19 shows that starch and sugar concentration significantly increased in the side of the leaf where veins had been severed, and that anthocyanins were also only produced on that side of the leaf, despite roughly equal PPFDs on both cut and un-cut sides. In a separate experiment (data not shown), placement of an opaque object over a portion of the severed half would cause that area to remain green, illustrating the necessity of light for reddening to occur. What is similar between this experiment and the natural reddening observed in Galax during the winter are the carbohydrate and light dynamics involved. In both scenarios, only leaves (or

portions of leaves) that have high soluble sugar content and are exposed to moderate to high light produce anthocyanins. As described previously, several studies have observed anthocyanin synthesis to be initiated in response to increases in sugar-feeding and girdling (Jeanette *et al.* 2000; Onslow 1925), and the necessity of light for anthocyanin synthesis has been very well documented (for review see Chalker-Scott 1999). Perhaps the increase in soluble sugars that occurs during the winter in Galax serves a dual function of acting as a cryoprotectant, as well as a biochemical cue for initiating anthocyanin synthesis in the presence of high light. This explanation seems to be the most parsimonious, as it would explain both why high light summer leaves fail to produce anthocyanins (as most sugars are stored as starch), and why shaded winter leaves fail to produce anthocyanins (Table 1).

In summary, these results lend strong support for a light-attenuating function of anthocyanins in high light leaves of *Galax urceolata*, namely through their ability to protect high light leaves from excess blue-green light. However, anthocyanins were not found to affect leaf temperature or antioxidant activity, since no significant differences between red and green leaves were evidenced in either experiment. Finally, anthocyanins are probably not produced as a benign byproduct of excess carbon flow through the phenylpropanoid pathway, though carbohydrates probably do play a role in initiation of their synthesis during the winter.

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After graduating in 2004, Nicole plans on sharing her passion for teaching and biology as an educator. She is currently a member of Sigma Xi research society, Beta Beta Beta biological honors society, and the Association of Southeastern Biologists. Her permanent address is 5104 W. Longfellow Ave. Tampa, FL 33629.