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NOTE

# Experimental branch cooling increases foliar sugar and anthocyanin concentrations in sugar maple at the end of the growing season

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**Abstract:** Autumnal leaf anthocyanin expression is enhanced following exposure to a variety of environmental stresses and may represent an adaptive benefit of protecting leaves from those stresses, thereby allowing for prolonged sugar and nutrient resorption. Past work has shown that experimentally induced sugar accumulations following branch girdling triggers anthocyanin biosynthesis. We hypothesized that reduced phloem transport at low autumnal temperatures may increase leaf sugar concentrations that stimulate anthocyanin production, resulting in enhanced tree- and landscape-scale color change. We used refrigerant-filled tubing to cool individual branches in a mature sugar maple (*Acer saccharum* Marsh.) tree to test whether phloem cooling would trigger foliar sugar accumulations and enhance anthocyanin biosynthesis. Cooling increased foliar sucrose, glucose, and fructose concentrations 2- to nearly 10-fold (depending on the specific sugar and sampling date) relative to controls and increased anthocyanin concentrations by approximately the same amount. Correlation analyses indicated a strong and steady positive relationship between anthocyanin and sugar concentrations, which was consistent with a mechanistic link between cooling-induced changes in these constituents. Tested here at the branch level, we propose that low temperature induced reductions in phloem transport may be responsible for increases in foliar sugars that trigger anthocyanin displays at grander scales.

*Key words:* fall leaf color, low temperature, sucrose, glucose, fructose, *Acer saccharum*.

**Résumé :** L'apparition des anthocyanines dans les feuilles à l'automne est amplifiée par l'exposition à divers stress environnementaux et pourrait représenter un avantage adaptatif en protégeant les feuilles contre ces stress; ce qui permettrait par conséquent de prolonger la résorption des sucres et des nutriments. Des travaux antérieurs ont montré que l'accumulation expérimentalement induite des sucres après avoir annelé des branches déclenche la biosynthèse des anthocyanines. Nous avons émis l'hypothèse que le ralentissement du transport dans le phloème causé par les températures basses à l'automne pourrait augmenter la concentration des sucres; ce qui stimulerait la production des anthocyanines et entraînerait un changement de couleur plus intense à l'échelle de l'arbre et du paysage. Nous avons utilisé une tubulure contenant un fluide frigorigène pour refroidir des branches dans un érable à sucre (*Acer saccharum* Marsh.) mature afin de tester si le refroidissement du phloème déclencherait une accumulation des sucres dans les feuilles et augmenterait la biosynthèse des anthocyanines. Le refroidissement a augmenté la concentration de sucrose, de glucose et de fructose dans les feuilles de 2 à près de 10 fois (dépendemment de la nature du sucre et de la date d'échantillonnage) comparativement aux branches témoins et a aussi augmenté la concentration des anthocyanines dans environ la même proportion. Les analyses de corrélation montrent qu'il y a une relation positive étroite et stable entre les concentrations d'anthocyanines et de sucres qui correspond à un lien mécaniste entre les changements induits dans ces constituants par le refroidissement. Ayant testé l'hypothèse à l'échelle des branches, nous sommes d'avis que le ralentissement du transport dans le phloème causé par une température basse serait responsable de l'augmentation des sucres dans les feuilles qui déclencherait l'apparition plus marquée des anthocyanines. [Traduit par la Rédaction]

*Mots-clés :* coloration automnale des feuilles, température basse, sucrose, glucose, fructose, *Acer saccharum*.

## Introduction

Anthocyanins are water-soluble pigments that reside within cell vacuoles in a wide variety of plants (Archetti et al. 2009). They are responsible for the red to purple colors observed in flowers, fruits, stems, and roots (e.g., Chalker-Scott 1999; Gould et al. 2010; Neufeld et al. 2011). Anthocyanins have been identified in both deciduous and evergreen species growing in a variety of ecosystems (e.g., Chalker-Scott 1999; Hughes et al. 2005; Archetti et al. 2009). Their broad existence across a wide range of sites has led to considerable debate regarding the specific roles that anthocyanins play and the extent to which environmental triggers lead to their production. Research has linked the expression of anthocyanins to an assortment of environmental stresses, including drought (Zhang et al. 2007), exposure to ozone and ultraviolet radiation (Alexieva et al. 2001; Gravano et al. 2004), wounding (Jeannette et al. 2000; Gould et al. 2002), bacterial and insect attacks (Gonzáles et al. 2002; Edwards et al. 2008), and nutrient deficiencies (Schaberg et al. 2003). One of the most notable displays of foliar anthocyanin production occurs in autumn within deciduous temperate forests and coincides with the onset of low temperatures (Feild et al. 2001;

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Schaberg et al. 2003). Likewise, observations of anthocyanin production have been observed in the young foliage of some temperate species exposed to low temperatures in spring (Taulavuori et al. 2011). Among other temperature cues, freezing events have also been shown to trigger anthocyanin production (Hao and Arora 2009). Due to their consistent association with environmental stresses, anthocyanins are believed to impart a protective function within plant tissues. However, identifying a single specific protective role is currently the subject of much scientific debate, which has led to the development of numerous explanations regarding the ecological functions of anthocyanins (Archetti et al. 2009). Some of these include providing leaves with photoprotection (Neill and Gould 1999; Hughes et al. 2005), antioxidant capabilities (Gould et al. 2002; Neill and Gould 2003), prolonged periods of nutrient resorption (Hoch et al. 2003; Schaberg et al. 2008), serving as an "energy escape valve" (Hernández and Van Breusegem 2010), and providing signals to insects of a plant's defensive capabilities (Archetti et al. 2009).

In addition to anthocyanin production, a variety of environmental perturbations or stresses have also been shown to induce changes in sugar concentrations within plant tissues. For example, elevated carbon dioxide levels significantly increase soluble sugar concentrations in tree leaves (Liu et al. 2004), with a simultaneous up-regulation of the genes involved in anthocyanin expression (Tallis et al. 2010). Likewise, drought conditions (Gebre and Tschaplinski 2002), as well as pathogen infection (Bolouri Moghaddam and Van den Ende 2012), can also result in an accumulation of foliar soluble sugars and anthocyanins. In particular, it is well established that low-temperature exposure slows and can even halt phloem transport, thereby leading to foliar sugar buildups (e.g., Keskitalo et al. 2005; Thorpe et al. 2010). Mechanical disruption of the phloem can increase anthocyanin expression in a range of plant forms (e.g., Hughes et al. 2005), and previous research showed that girdling sugar maple (*Acer saccharum* Marsh.) branches just prior to the onset of autumnal low temperatures successfully trapped sugars within foliage and induced anthocyanin synthesis (Murakami et al. 2008). Foliar sugars and anthocyanin concentrations were up to five times greater in leaves from girdled branches than from control branches that were not girdled. Girdling stopped the flow of sugar transport, leading to an accumulation of sugars in leaves that triggered anthocyanin production as air temperatures decreased (Murakami et al. 2008). However, because both wounding (Jeannette et al. 2000; Gould et al. 2002) and low-temperature exposure (Feild et al. 2001) are associated with anthocyanin production, autumnal girdling experiments cannot distinguish between these possible triggers. In particular, what remains uncertain is whether or not the influence of low temperatures that slow phloem transport is analogous to the action of physical girdling in increasing sugar accumulation and stimulating anthocyanin biosynthesis.

We hypothesized that low temperatures in autumn act as the environmental cue to slow phloem transport, leading to a buildup of foliar sugars that triggers the biosynthesis of anthocyanins in sugar maple leaves. Here we present the results of a unique experiment in which sugar maple branches were not wounded but experimentally cooled to reduce phloem transport and then evaluated to determine if cooling increased both foliar sugar and anthocyanin concentrations. Results of this experiment highlight the independent influence of low-temperature exposure from wound-induced anthocyanin production — providing a physiological connection that is more directly relevant to tree-, stand-, and landscape-level displays of foliar reddening that accompany low air temperature exposure in autumn.

## Materials and methods

### Branch cooling and collection of plant material

A single open-grown sugar maple tree was chosen for this study at the USDA Forest Service Northern Research Station in South Burlington, Vermont, USA (elevation 92 m a.s.l.), based on its consistent display of yellow autumnal foliage from 1998 to 2008 (P.G. Schaberg, unpublished data). Using a tree that historically produced anthocyanins on a limited basis allowed for greater resolution of treatment-induced changes. This tree was approximately 10 m tall and 33 years old, and its leaves were green at the start of the experiment. Within this tree, two pairs of adjacent south-facing branches were selected, and within each pair, one branch was randomly assigned to be cooled and the other was exposed to ambient temperatures (the control). Thermocouples were inserted into pin-sized openings in the bark created with a stylus to the depth of the cambium to continuously monitor phloem temperature of each branch. Cooling of phloem tissues was achieved by circulating chilled liquid (ethylene glycol) from a cold-block system through plastic tubing encircling branches, similar to the system used by Johnsen et al. (2007). Branches assigned to the cooling treatment were wrapped with flexible plastic tubing to create a 30 cm long collar that was then covered with insulation. The average diameter of one cooled branch was 19 mm, with a total length of 2.33 m, and the average diameter of the other cooled branch was 18 mm, with a total length of 2.37 m. For these branches, a target temperature of ~3 °C was maintained by a feedback loop that periodically pumped chilled ethylene glycol from a freezer to branches.

One-half of the leaves from treatment and control branches were randomly and equally selected from along the length of each branch for harvest on 26 September 2008 (Julian Date (JD) 270), and the remainder were collected one week later on 3 October (JD 277) for sugar and pigment analyses. The total numbers of leaves collected per treatment were 23 for control branches and 24 for cooled branches. Leaves were placed in plastic bags and transported to the lab where they were immediately prepared for chemical analyses.

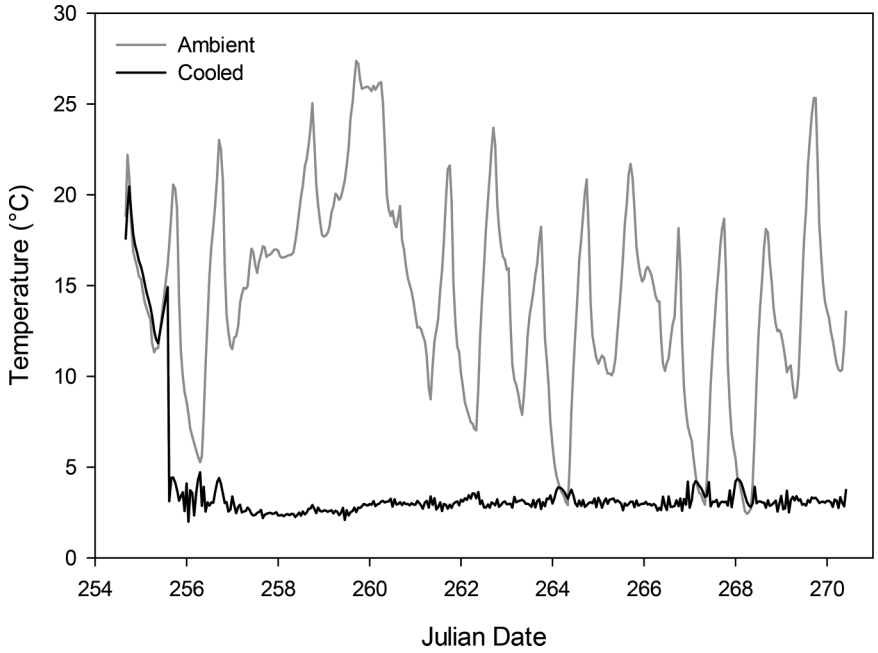
### Sugar analysis

Five 0.5 cm leaf disks from four randomly chosen leaves were pooled for a total of 20 disks per sample for analysis. Leaf punches were submerged in 5 mL of 80% ethanol and stored at –20 °C. Foliar glucose, fructose, and sucrose concentrations were determined from a series of ethanol extractions according to the methods of Hinesley et al. (1992). Chlorophyll was removed from soluble sugar extracts using a $C_{18}$ Sep-Pak Plus Cartridge (Waters Corporation, Milford, Massachusetts). In preparation for sugar analysis, 200 μL of the foliar sample extract was dried at 37 °C in a limited volume insert vial and then reconstituted in 200 μL 0.1 mmol·L$^{-1}$ Ca EDTA. Each sample was filtered through a 0.45 μm syringe filter and then analyzed using high-performance liquid chromatography (Waters Corporation) equipped with a Sugar-Pak column at 90 °C and using 0.1 mmol·L$^{-1}$ Ca EDTA as the eluent at a flow rate of 0.6 mL·min$^{-1}$. Empower software (Waters Corporation) was used to quantify sugar concentrations, expressed as mg·cm$^{-2}$ leaf area.

### Foliar pigment analysis

A separate group of 20 leaf disks comprised of five disks from each of the same four leaves used for sugar analysis was pooled per sample for pigment analysis. Leaf disks were shredded using a razor blade and placed in 6 mL of 80% acetone for chlorophyll extraction. Another set of 20 shredded leaf disks was placed in 6 mL of 3 mol·L$^{-1}$ HCl–$H_2O$–MeOH (1:3:16, by vol.) for extraction of anthocyanins using the methods of Gould et al. (2000). Absorbance of pigments was measured using a DU 800 UV/VIS spectrophotometer

**Fig. 1.** Mean hourly temperatures of phloem tissues for control (ambient) and treated (cooled) branches over the duration of the study. Cooling treatments began on day 256 (12 September) 2008. Julian Date is labeled at the midpoint of each day.




(Beckman Coulter, Fullerton, California). Chlorophyll *a* and *b* concentrations were determined from the acetone extracts using the equations provided by Lichtenthaler and Wellburn (1983) and were expressed as μg·cm$^{-2}$. Anthocyanin absorbance was measured at 530 nm from the extracts of acidified methanol, and concentrations were determined after adjusting for the overlap in chlorophyll absorbance ($A_{530} - 0.24A_{653}$) (Murray and Hackett 1991).

### Statistical analyses

To determine differences in soluble sugar and pigment concentrations between the foliage of cooled and control branches, *t* tests were used. Although disks from individual leaves were assessed, branch means were used for statistical analyses per date and for the two dates combined. Differences were considered significant if $P < 0.05$. Because variances were not equal in some instances, a Welch's test was used when necessary. Correlation analyses were conducted on pooled leaf samples and evaluated per treatment and date and for combined dates to evaluate relationships among sugar and pigment concentrations.

## Results

### Branch cooling verification

Figure 1 depicts the success of cooling efforts. Control and treated branches had temperatures that were indistinguishable prior to the start of the cooling treatment (before 12 September, JD 256). However, they showed marked differences after treatment induction, with cooled branches maintaining relatively stable temperatures of 2.82 ± 0.06 °C ($\bar{x}$ ± SE) and control branches showing diurnal temperature fluctuations that were generally well above treated comparisons (13.51 ± 0.82 °C) (Fig. 1).

### Soluble sugar concentrations

After 14 days of branch cooling, foliage collected on 26 September contained significantly greater concentrations of sucrose, glucose, and fructose ($P < 0.0001$) compared with foliage of control branches collected on the same day (Table 1). Indeed, cooling treatment resulted in foliar sugar concentrations that were approximately two to nine times greater than their control counterparts. Although foliage collected in October generally contained lower concentrations of soluble sugars than those measured in September, foliar sucrose concentrations from cooled branches were two times higher than levels measured in foliage from control branches ($P = 0.0015$). In contrast, fructose concentrations were marginally lower with cooling treatment ($P = 0.0933$), and there were no differences in foliar glucose concentrations. Foliar glucose concentrations from control branches changed little from September to October, but sucrose concentrations declined by more than half and fructose concentrations doubled ($P < 0.0001$). Patterns of sugar accumulation with treatment for both dates combined mirrored those for September, with two- to five-fold increases in sugar concentrations measured in leaves from the cooling treatment relative to controls (Table 1).

**Table 1.** Mean (±SE) foliar sugar concentrations measured in control and cooled branches of a sugar maple tree on 26 September (Julian Date (JD) 270), 3 October (JD 277), and both dates combined.

| | Sugar concentration (mg·cm$^{-2}$) | | |
|---|---|---|---|
| Sugar type per collection date | Control | Cooled | *P* |
| **Sucrose** | | | |
| 26 September | 0.063±0.008 | 0.139±0.012 | <0.0001 |
| 3 October | 0.016±0.003 | 0.032±0.004 | 0.0015 |
| Combined dates | 0.039±0.006 | 0.085±0.013 | 0.0025 |
| **Glucose** | | | |
| 26 September | 0.008±0.002 | 0.079±0.006 | <0.0001 |
| 3 October | 0.010±0.004 | 0.012±0.001 | 0.6573 |
| Combined dates | 0.009±0.002 | 0.046±0.008 | <0.0001 |
| **Fructose** | | | |
| 26 September | 0.017±0.004 | 0.145±0.010 | <0.0001 |
| 3 October | 0.037±0.009 | 0.019±0.002 | 0.0933 |
| Combined dates | 0.027±0.006 | 0.082±0.014 | 0.0010 |

**Note:** Differences were considered significantly different when $P \leq 0.05$. For all analyses, sample sizes were *n* = two branches per treatment.

### Chlorophyll and anthocyanin expression

Synchronous with changes in foliar sugars, branch cooling significantly altered chlorophyll and anthocyanin concentrations, with significant treatment differences apparent on both collec-

**Table 2.** Mean (±SE) foliar pigment concentrations measured in control and cooled branches of a sugar maple tree on 26 September (Julian Date (JD) 270), 3 October 3 (JD 277), and both dates combined.

| | Pigment concentration (mg·cm$^{-2}$) | | |
|---|---|---|---|
| Pigment per collection date | Control | Cooled | *P* |
| **Chlorophyll** | | | |
| 26 September | 20.615±1.122 | 6.667±1.559 | <0.0001 |
| 3 October | 2.274±0.132 | 0.254±0.054 | <0.0001 |
| Combined dates | 11.046±2.023 | 3.461±1.014 | 0.0021 |
| **Anthocyanin** | | | |
| 26 September | 0.089±0.006 | 0.372±0.037 | <0.0001 |
| 3 October | 0.093±0.006 | 0.152±0.012 | 0.0006 |
| Combined dates | 0.091±0.004 | 0.267±0.031 | <0.0001 |

**Note:** Differences were considered significantly different when $P \leq 0.05$. For all analyses, sample sizes were $n$ = two branches per treatment.

tion dates (Table 2). In September, leaves of cooled branches had over three times less chlorophyll than leaves of control branches ($P < 0.0001$), though green coloration was still evident. By October, chlorophyll concentrations in foliage of cooled branches were nearly nine times less than concentrations found in the foliage of control branches ($P < 0.0001$), and green coloration appeared faded. As expected, green color and foliar chlorophyll content decreased significantly ($P < 0.0001$) from September to October in control samples through natural senescence. Anthocyanin concentration also exhibited significant treatment differences on both collection dates (Table 2). Leaves harvested from cooled branches in September had distinct red hues and contained anthocyanin levels that were over four times greater ($P < 0.0001$) than those found in foliage of control branches. Similarly, in October, anthocyanin concentration was greatest ($P = 0.0006$) in leaves from cooled branches compared with those from control branches, though red coloration appeared somewhat faded relative to those from September. Surprisingly, anthocyanin concentrations changed little from September to October in leaves of control branches ($P = 0.6118$) but were significantly lower than concentrations in leaves of cooled branches ($P < 0.0001$), with the greatest concentration occurring in September (Table 2).

Correlation analyses confirmed a significant relationship between foliar sugar concentrations and anthocyanin expression during senescence-induced branch cooling. For leaves from cooled branches, pooling data from both collection dates revealed that for all foliar sugars (sucrose, $r = 0.59$, $P = 0.0028$; glucose, $r = 0.78$, $P < 0.0001$; fructose, $r = 0.87$, $P < 0.0001$; total sugars, $r = 0.78$, $P < 0.0001$), concentrations were significantly and positively correlated with anthocyanin levels. In leaves of control branches, only fructose ($r = 0.41$, $P = 0.0494$) and total sugars ($r = 0.41$, $P < 0.049$) were significantly correlated with anthocyanin levels. On individual dates, only foliage harvested from cooled branches revealed significant associations between sugar and anthocyanin concentrations; fructose ($r = 0.68$, $P = 0.0158$) in September and glucose ($r = 0.69$, $P = 0.0181$) and total sugars ($r = 0.65$, $P = 0.0307$) in October.

## Discussion

Overall, the hypothesis that branch cooling would slow phloem transport, lead to buildups of foliar sugars, and trigger anthocyanin biosynthesis in sugar maple leaves was supported by our data. Branch cooling increased foliar concentrations of sucrose, glucose, and fructose late in the growing season — a time when carbohydrate reserves generally move toward proximal winter storage sinks. These sugar increases were accompanied by two changes in leaf pigmentation: increased anthocyanin and decreased chlorophyll concentrations. Synchronous changes in foliar sugar and pigment concentrations with cooling suggest a mechanistic link between temperature triggers and physiological response. This link was supported by consistently strong correlations among the concentrations of various sugars and anthocyanins, as well as substantial experimental evidence from the literature.

Considerable evidence indicates that low temperatures slow phloem transport and increase foliar sugar concentrations (e.g., Keskitalo et al. 2005; Thorpe et al. 2010). Other studies have repeatedly linked sugar enhancement to anthocyanin production (Hiratsuka et al. 2001; Hara et al. 2003), including among woody species under field conditions. For example, Ishikura (1976) found an increase in total and reducing sugars followed by anthocyanin formation during autumnal senescence of leaves in three genera (*Acer*, *Rhus*, and *Euonymous*), whereas Chang et al. (1989) noted that increases in glucose and galactose were coupled with red color development in senescing quaking aspen (*Populus tremuloides*) leaves. Schaberg et al. (2003) also showed a significant and positive correlation between red coloration and sugar concentrations in senescing sugar maple leaves.

Girdling experiments have provided evidence of a more causative association between buildups of foliar sugars and anthocyanin expression. Jeannette et al. (2000) found that heat girdling at the base of maize (*Zea mays*) leaves caused an increase in foliar sucrose concentrations compared with controls and that the anthocyanin content of girdled leaves then increased considerably within 36 h. The mechanistic connection between sugar and anthocyanin concentrations has been bolstered by findings that sugars induce gene transcription required for anthocyanin biosynthesis via the shikimic acid pathway (Hara et al. 2003). Indeed, the expression of a range of anthocyanin biosynthetic genes (e.g., *CHS*, *CHI*, *C4H*, *F3H*, *DFR*, *ANS*, and *UFGT*) are enhanced by low-temperature exposure (Zhang et al. 2011; Zhang et al. 2012), with sugar signaling initiating the biochemical cascades that trigger this (e.g., Vitrac et al. 2000; Hara et al. 2003). In addition to their influence on anthocyanin biosynthesis, increases in sugar concentrations may play a significant role in triggering leaf senescence, especially the loss of chlorophyll in leaves (Wingler et al. 1998). We noted both anthocyanin increases and chlorophyll loss with branch cooling and associated sugar increases.

Biochemical feedbacks between sugar and anthocyanin accumulations could be of ecological benefit during senescence if they help coordinate changes in physiology that prolong leaf longevity and extend the resorption of soluble carbohydrates and nutrients that could be of competitive benefit during subsequent seasons. Anthocyanins may protect senescing leaves from the negative effects of photoinhibition and scavenge reactive oxygen species produced via photooxidation (Gould et al. 2002; Neill et al. 2002; Neill and Gould 2003). In addition, foliar anthocyanin expression may allow for a prolonged period of nutrient resorption especially in trees with a growing season that may be inherently short due to a combination of environmental stresses (Feild et al. 2001; Hoch et al. 2001, 2003). Hoch et al. (2003) proposed a "resorption protection hypothesis" in which anthocyanins of senescing foliage shade the photosynthetic system and prevent photoinhibition, allowing for a greater resorption of nutrients, particularly nitrogen. This could be particularly pertinent to plants that seasonally experienced low temperatures (Hoch et al. 2001). Similarly, Feild et al. (2001) emphasized the dual roles of anthocyanins in senescing foliage as scavengers of reactive oxygen species, as well as facilitators of nutrient recovery in red-osier dogwood (*Cornus stolonifera*). Schaberg et al. (2008) provided the first anatomical evidence that anthocyanins may support nutrient resorption in senescing leaves. They found that red sugar maple leaves were more firmly attached and had less abscission layer progression through petiolar vasculature compared with yellow leaves for which the abscission layer was complete — indicating a termination of phloem transport. Indeed, red leaves exhibited vascular connectivity more closely resembling green leaves (Schaberg et al. 2008). These results demonstrated a relationship between foliar

coloration and abscission zone formation and suggest that, at least during senescence, red expression in sugar maple may allow for an extended capacity for nutrient and sugar translocation compared with yellow leaf counterparts.

Our findings highlight the influence of increased foliar sugar concentrations on the autumnal expression of senescence and anthocyanin levels for cooled sugar maple shoots absent the influence of wounding. The basic physiological connections associated with experimental manipulation in our study are likely also pertinent to reports of red leaf coloration coincident with native stresses such as mechanical injury (Jeannette et al. 2000) and certain types of insect and fungal damage (Costa-Arbulú et al. 2001; Rostás et al. 2002) that alter phloem transport and sugar export. However, our low-temperature treatment more specifically mimicked a more pervasive, broad-scale trigger of anthocyanin production — reductions in autumnal air temperature. Low temperatures during autumn lead to reductions in phloem transport (Keskitalo et al. 2005). Here we show that experimental low-temperature exposure also increased foliar sugar concentrations and enhanced anthocyanin expression at the branch level. We propose that this same stimulus and response is pertinent to landscape-level foliar anthocyanin displays. An association between low-temperature exposure that triggers changes in carbohydrate relations that then alter pigment physiology could provide a mechanistic explanation for patterns of fall leaf coloration across time (e.g., between years of early versus late cold onset) and space (e.g., locations that experience cold early versus late in the season). Regardless of scale, we posit that increased anthocyanin expression can then enhance leaf protection and extend opportunities to retrieve and transport nutrients and soluble sugars to winter storage sites — a capacity that would be of adaptive benefit to deciduous species in cold environments with short growing seasons.

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