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Association of red coloration with senescence of sugar maple leaves in autumn

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Abstract We evaluated the association of red coloration with senescence in sugar maple (*Acer saccharum* Marsh.) leaves by assessing differences in leaf retention strength and the progression of the abscission layer through the vascular bundle of green, yellow, and red leaves of 14 mature open-grown trees in October 2002. Computer image analysis confirmed visual categorization of leaves as predominantly green, yellow or red, and chemical quantification of leaf pigment concentrations verified that leaf color reflected underlying differences in leaf biochemistry. Significantly lower chlorophyll concentrations within red and yellow leaves indicated that senescence was more advanced in leaves from these color categories relative to green leaves. Among leaf types, only red leaves contained high concentrations of anthocyanins. There were significant differences in leaf retention capacity among color categories, with the petioles of green leaves being the most firmly attached to twigs, followed by red and then yellow leaves. Microscopic analysis indicated that yellow leaves had the most advanced extension of the abscission layer through the vasculature, with green and red leaves having significantly less abscission layer progression than yellow. A more limited progression of the abscission layer through vascular bundles may be evidence of delayed leaf senescence that could extend resorption of mobile leaf constituents. Together, results from this study suggest an association between leaf anthocyanin content and functional delays in senescence.

Keywords Anthocyanins · Fall color · *Acer saccharum* · Abscission zone · Leaf retention

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Introduction

The deciduous forests of the northeastern United States and neighboring Canadian provinces are world renowned for their brilliant and diverse foliar color displays in autumn. During this time, the mosaic of leaf colors across forested landscapes is primarily caused by variations in three plant pigments within and among tree species. Two of these classes of pigments, the chlorophylls that appear green and the carotenoids that appear yellow, are synthesized during the growing season to enable or protect photosynthetic light capture (Taiz and Zeiger 2002). In contrast, anthocyanin pigments that give leaves a red or purple hue are often synthesized toward the end of a leaf's lifespan (Matile 2000; Feild et al. 2001). Because anthocyanin production incurs a metabolic cost, it is assumed that this synthesis also has some compensatory benefit (Chalker-Scott 1999). But what could this benefit be for a leaf nearing senescence?

It has been suggested that anthocyanins are a nonfunctional by-product of leaf senescence (Archetti 2000; Matile 2000). However, exposure to an array of stressors, including UV-B radiation (Mendez et al. 1999), osmotic stress (Kaliamoorthy and Rao 1994), drought (Balakumar et al. 1993), low temperatures (Krol et al. 1995), nutrient

deficiencies (Rajendran et al. 1992), wounding (Ferreres et al. 1997), pathogen infection (Dixon et al. 1994), and ozone exposure (Foot et al. 1996) have been shown to elicit anthocyanin biosynthesis. This buildup of anthocyanins following stress exposure raises the possibility that anthocyanins may function, in part, to prevent or offset stress-induced damage. Indeed, anthocyanins have several qualities that could make their presence beneficial under times of stress. For example, anthocyanins can provide a "light screen" that decreases light capture by chloroplasts and may reduce the risk of photooxidative damage as leaves senesce (Feild et al. 2001). Anthocyanins are also powerful antioxidants that could prevent and repair damage caused by high light and other oxidative stresses (Neill et al. 2002). Furthermore, anthocyanins are osmotically active, so enhanced expression might benefit both cold hardiness and drought resistance through increased osmotic control (Chalker-Scott 1999).

Research on the adaptive benefit of anthocyanins to deciduous trees in autumn has provided evidence supporting their potential value in facilitating protracted nutrient recovery during leaf senescence. Feild et al. (2001) found that red-senescing leaves of red-osier dogwood (*Cornus sericea* L.) absorbed more light in the blue-green to orange wavelengths and had higher maximum photosystem II photon yields following high light stress than did yellow senescing leaves. They proposed that anthocyanins provide photoprotection during late-season dismantling of the photosynthetic apparatus, thereby reducing the risk of photooxidative damage and fostering more complete nutrient recovery from senescing leaves (Feild et al. 2001). Hoch et al. (2001) compared the anthocyanin production capacity of nine woody genera and observed that species at greatest risk of photoinhibition during leaf senescence (e.g., shade tolerant late successional species and plants native to cold environments) showed the highest capacities for anthocyanin production. They proposed that the primary functions of autumn anthocyanin production were to protect the photosynthetic apparatus from photooxidative damage, prolong carbon capture, and help supply the energy and osmotic control needed for phloem export of nutrients from senescing leaves (Hoch et al. 2001).

In a previous study of the factors influencing red color expression in the autumn foliage of sugar maple (*A. saccharum* Marsh.) trees, we found a link between leaf carbohydrate status, nutrient concentrations, and anthocyanin production. Trees with near-deficient foliar nitrogen (N) concentrations turned red earlier and more completely than trees with high foliar N concentrations (Schaberg et al. 2003). Low N trees also had higher foliar starch concentrations prior to red color development, and higher foliar glucose, fructose, sucrose and stachyose concentrations during peak red coloration than high-N trees. We hypothesized that N limitations provided the biochemical stimulus (low N and high sugar signals), energy, and carbohydrate building blocks needed for the facultative production of anthocyanins, which were preferentially expressed when N was limited and N recovery was most beneficial to the tree (Schaberg et al. 2003). In separate work, Hoch et al. (2003) showed that anthocyanin-deficient mutants of three woody species had lower N resorption efficiencies and proficiencies than wild-type counterparts following high light and low temperature stress.

Although progress has been made toward deciphering the potential physiological value and ecological role of autumnal anthocyanin production, numerous questions remain unanswered. For example, despite its reputed contribution toward prolonging leaf function during autumn, the association between anthocyanin production and the extent of leaf senescence has not been examined. The objective of this study was to test for potential differences in leaf retention strength and vascular bundle continuity among green, yellow, and red sugar maple leaves during autumn to assess if anthocyanin production was associated with delayed leaf senescence.

Methods

Plant material

Fourteen open-grown sugar maple trees at the USDA Forest Service Northern Research Station in South Burlington, Vermont, USA (elevation 92 m) that past work had documented could exhibit a full variety of fall leaf colors (Schaberg et al. 2003 and unpublished data from our lab) were sampled at peak color during the first three weeks of October 2002. Trees previously documented as having low N nutrition—a possible instigator of early red leaf coloration—(Schaberg et al. 2003), were excluded from this study. Trees were approximately 15 m tall and 40 years old at the time of sampling. Six sun-exposed branches approximately 30 cm long and 0.5 cm in diameter at their cut ends were collected from adjacent locations in the crown of each tree. Although red leaves can exist in the interior portions of tree crowns, shading can delay the synthesis of anthocyanins during autumn (Matile 2000). Branches were chosen so that two branches from each of three color classes were collected for every tree: two branches with leaves that were predominantly green, two branches with leaves that were mostly yellow, and two branches with red leaves. One set of branches from each color category was collected to conduct foliar color and abscission layer analyses ($n = 42$), and a separate collection was used to assess leaf retention strength ($n = 42$). All

tissue was immediately processed following harvest. Leaves from the three color categories had no visible differences in gross morphology, and had average leaf areas that were not significantly different from one another ($P > 0.3691$) based on ANOVA analysis of leaf areas generated as part of computer color analysis.

Foliar color analysis

Visual leaf color designations were quantified using digital image analysis. On the day of collection, leaves were scanned at 240 dpi on an Epson Perfection 1200U color scanner (Epson America, Inc., Torrance, CA) and saved in tagged image file format (TIFF). The scanner was calibrated using system software to ensure that the tone and contrast on the screen matched the original image. All image analyses were conducted using the public domain NIH Image program (developed at the US National Institute of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). Necrotic (brown) portions of leaves were graphically removed and omitted from color analysis. Each scanned leaf image was manipulated in NIH Image employing established methods (Schaberg et al. 2003; Murakami et al. 2005). By comparing the original scanned leaf image with the NIH-manipulated leaf image, regions of red, yellow, and green were identified and quantified on an area basis.

Although it is well established that the green and yellow colors of leaves are attributable to chlorophyll and carotenoid pigments, respectively (Taiz and Zeiger 2002), both carotenoid and anthocyanin pigments can contribute to the expression of red in leaves (Lee and Gould 2002). Previous testing determined that red leaf coloration measured using computer image analysis is highly correlated ($r = 0.877$, $P = 0.001$, $n = 22$) with the spectrophotometrically measured anthocyanin content of sugar maple leaves (Schaberg et al. 2003). These results support past findings that anthocyanins are major contributors to red leaf color during autumn among *Acer* species (Ishikura 1973), including sugar maple (Ji et al. 1992).

Leaf pigment quantification

For five leaves from each color category per tree, four fresh leaf disks were removed for pigment quantification. Disks were chopped using a razor blade and placed in either HCl:H_2O:MeOH (1:3:16, by vol.) for anthocyanin pigment extraction or acetone:H_2O (4:1, v:v) for extraction of chlorophyll and carotenoid pigments according to the methods of Gould et al. (2000). A Spectronic Genesys 8 spectrophotometer (Cheshire, UK) was used to quantify pigment absorbance. Anthocyanin absorbance was measured at A_{530} and was corrected for the overlap of chlorophylls by subtraction of $0.24 \times A_{653}$ (Murray and Hackett 1991). Absorbance of chlorophyll and carotenoid pigments was measured at A_{470}, A_{647} and A_{663} and pigment concentrations were determined using the equations provided by Lichtenthaler and Wellburn (1983).

Leaf retention strength

One twig with attached leaves per color class per tree was used to indirectly assess abscission layer progression by measuring the strength of leaf attachment to branches. Branches were securely suspended from a laboratory ring stand, and a nylon filament was attached to the petiole of one randomly selected leaf at the leaf blade interface. A 0.5 l, light-weight plastic collection container was attached to the other end of the filament, and standardized metallic micro-weights were gradually added until leaf separation from the stem occurred. Branches were oriented to standardize the angle of pull applied to leaves among the various samples. The force [mass (g) of micro-weights] required to induce leaf separation was recorded and used as an indicator of leaf retention strength.

Microscopic evaluation of abscission layer progression

The petiole and attached stem of one randomly selected leaf per color class per tree was embedded in EnviroTex Lite (ETI inc., Fields Landing, CA, USA), a resin polymer in 35×10 mm plastic petri dishes. Sequential 25 μm sections passing longitudinally through the abscission zone were cut from embedded samples with a sliding microtome. Sections from the approximate middle of the petiole-stem interface that included clear representations of the abscission layer, vascular bundles and axillary bud (for spatial reference) were examined microscopically at 40× magnification and digitally photographed. Digital images were analyzed using the public domain software NIH Image. The progression of the abscission layer through the vascular bundle from the petiole epidermis opposite the axillary bud toward the center of the petiole was measured to the nearest 0.01 μm, and represented as a proportion (%) of bundle width to provide an estimate of vascular continuity. Nuclear magnetic resonance spectroscopy imaging of the vascular continuity of *Prunus avium* (L.) L. petioles during leaf senescence indicated that abscission layer formation progresses towards the center of the petiole (Millard and Chudek 1993).

Statistical analyses

The non-parametric van der Waerden test was employed to test for differences in the relative leaf area spectrally identified as green, yellow or red among leaf color groups.

Analyses of variance were used to test for differences among other leaf parameter means associated with leaf color, and the Tukey–Kramer HSD test was used to detect specific differences among green, yellow and red leaves. Pigment data was transformed to meet statistical assumptions of equal variances among leaf color groups. For all analyses, differences were considered statistically significant if $P \leq 0.05$.

Results and discussion

Computer image analysis confirmed visual categorization of leaves as predominantly green, yellow or red (Fig. 1). Chemical quantification of leaf pigment concentrations verified that visual and computer-based differentiations in leaf color reflected underlying differences in leaf biochemistry. For example, green leaves contained significantly higher concentrations of total chlorophyll than red and yellow leaves (Fig. 2). Furthermore, red leaves contained significantly higher concentrations of anthocyanins than either green or yellow leaves, which contained very low and similar concentrations of this pigment (Fig. 2). In addition, green leaves contained the highest concentrations of carotenoid pigments, followed by red and then yellow leaves (Fig. 2). Although yellow leaves contained the lowest concentrations of carotenoids, they appeared yellow because they contained very low concentrations of chlorophyll or anthocyanin pigments that might otherwise mask yellow color expression (Matile 2000). Because chlorophyll catabolism is a defining characteristic of leaf senescence (Thomas et al. 2001), the low chlorophyll concentrations of red and yellow leaves provided initial evidence that these leaf types were more advanced in their senescence than green leaves.

Coincident with differences in leaf color and pigment concentrations, there were significant differences in leaf

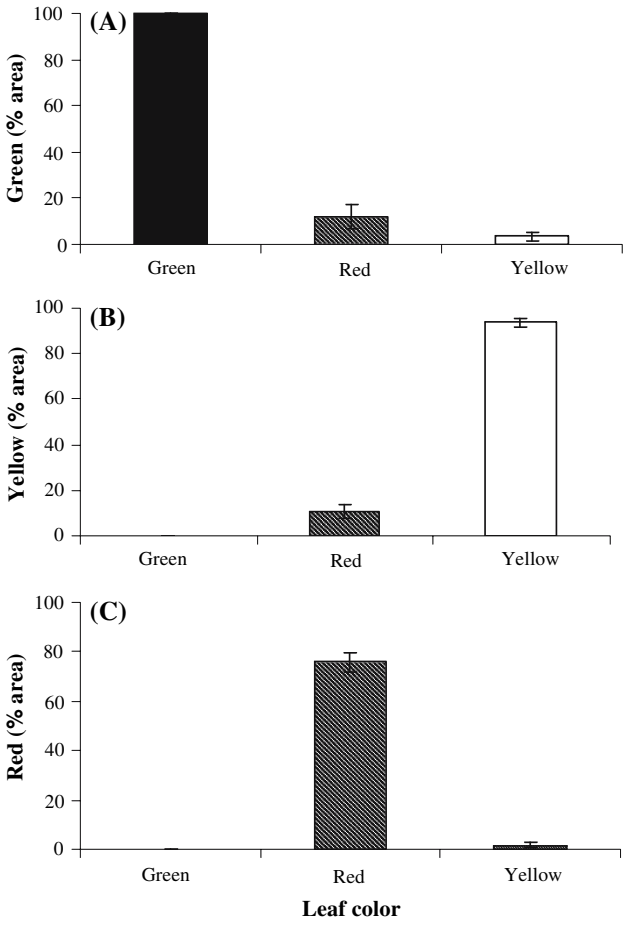

Fig. 1 Quantification of the relative leaf area (mean ± SE) spectrally identified as **a** green, **b** yellow or **c** red in leaves visually categorized as green, yellow or red. Leaves visually determined to be red and yellow included no areas measured as green using computer image analysis. In some cases, error bars are too small to be visible. For each leaf color type, leaf area means were significantly different ($P < 0.05$) based on results of the non-parametric van der Waerden test

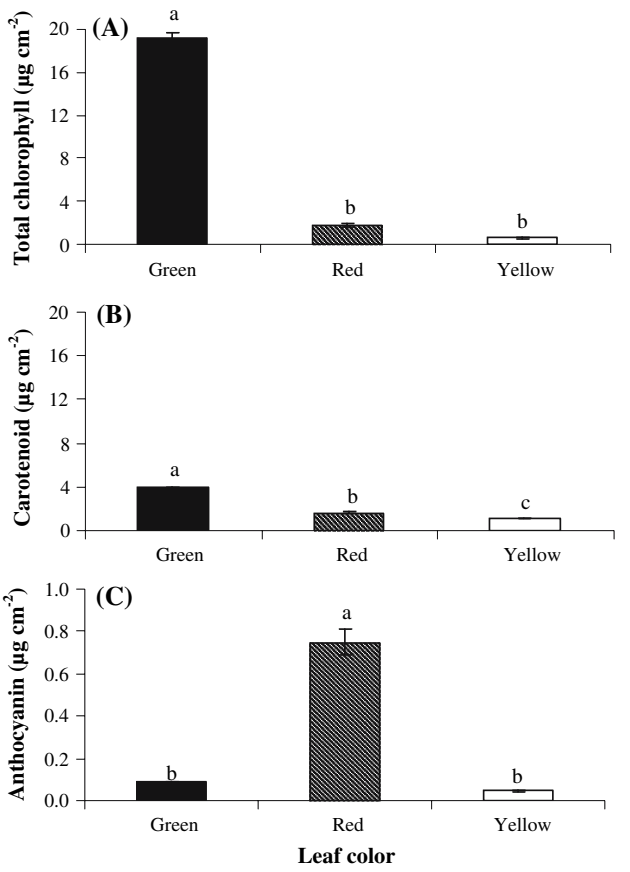

Fig. 2 Concentrations of **a** total chlorophyll, **b** carotenoid and **c** anthocyanin pigments (mean ± SE) for green, yellow or red leaves. Means with different letters are significantly different ($P < 0.05$) based on results of the Tukey–Kramer HSD test

retention capacities among leaf color types (Fig. 3). Both red and yellow leaves were more readily removed from stems than green leaves collected on the same date from the same tree. However, despite having comparable chlorophyll concentrations (Fig. 2; one indicator of senescence), red leaves had about double the retention strength of yellow leaves (Fig. 3). Although this provides an indication of delayed senescence for red leaves relative to yellow ones, a more specific analysis of the functional progression of senescence involved an evaluation of vascular bundle continuity as affected by abscission layer formation.

Microscopic analysis of the progression of abscission layer formation across vascular bundles indicated that green and red leaves had the least and statistically indistinguishable levels of abscission layer progression (Fig. 4). In contrast, yellow leaves had vascular bundles with more advanced abscission layer formation (Fig. 4). A more limited progression of the abscission layer through the vascular bundle provides additional evidence that red leaves appear to have delayed leaf senescence relative to yellow leaves. In addition, despite diminished retention strength relative to green leaves (Fig. 3), red leaves were indistinguishable from green leaves in the apparent progression of the abscission layer through the vascular trace (Fig. 4). The preservation of vascular bundle function is particularly important because this would allow for continued phloem transport, and sugar and nutrient recovery from senescing leaves.

Together with results from the leaf retention test (Fig. 3), measurements of abscission zone progression through vascular bundles suggest an association between anthocyanin content and a functional delay in senescence. It is possible that this association involves the purported role of anthocyanins in limiting oxidative stress or repairing oxidative damage, processes that may affect plant hormone systems and senescence. Anthocyanins accumulate in the vacuole (Winefield 2002)—a location that is suboptimal for scavenging reactive oxygen species (ROS)

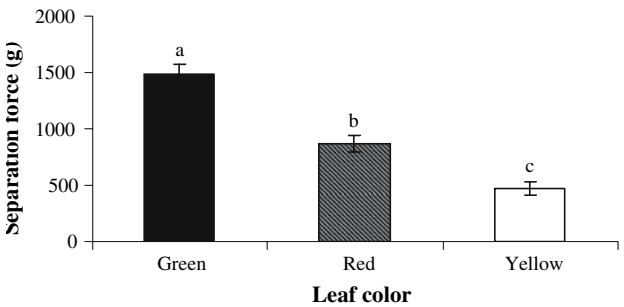

Fig. 3 Leaf retention strengths (mean ± SE) for green, red and yellow leaves as estimated by the mass (g) required to detach petioles from stems. Means with different letters are significantly different ($P < 0.05$) based on results of the Tukey–Kramer HSD test

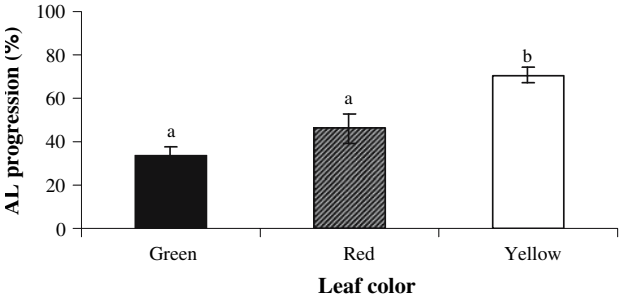

Fig. 4 Progression of the abscission layer (*AL*) through the vascular bundle, expressed as a percentage of the total vascular bundle width (mean ± SE) in the petioles of green, red and yellow sugar maple leaves. Means with different letters are significantly different ($P < 0.05$) based on results of the Tukey–Kramer HSD test

that are predominantly generated in organelles such as chloroplasts and mitochondria. However, anthocyanins are synthesized in the cytoplasm (Winefield 2002), where they may scavenge ROS before transport to the vacuole (Neill et al. 2002). Furthermore, because H_2O_2 can diffuse across the tonoplast, anthocyanins may contribute important antioxidant protection within vacuoles during periods of severe stress (Yamasaki 1997).

There is considerable evidence that ethylene and auxin coordinate the timing of leaf abscission; with ethylene accelerating and auxin retarding the process (see reviews by Roberts et al. 2000; 2002). Recent research suggests that oxidative stress influences both of these triggers of senescence. For example, Michaeli et al. (1999a) found that treatment with antioxidant agents such as butylated hydroxyanisole, *n*-propyl gallate, and vitamin E significantly reduced chilling-induced abscission, in part by decreasing tissue sensitivity to ethylene. Oxidative processes initiated by chilling (Michaeli et al. 1999b) or chilling combined with high light (Michaeli et al. 2001) also reduce the free auxin content in the abscission zone. Thus, through the combined influence of reduced auxin transport (Michaeli et al 1999b; 2001) and increased sensitivity to ethylene (Michaeli et al. 1999a) oxidative stress appears to hasten leaf senescence. Acting as antioxidants, anthocyanins could help sustain auxin concentrations, reduce tissue sensitivity to ethylene, and delay senescence.

Regardless of the mechanism(s) involved, if an accumulation of anthocyanins does help prolong leaf function, this would accommodate the extension of carbohydrate production, and sugar and nutrient resorption from leaves. The associations between anthocyanin expression and extended leaf retention and function that we report support the hypothesis that anthocyanins may benefit species like sugar maple by allowing for prolonged resorption of mobile leaf constituents. Experimental manipulations are needed to specifically assess the possibility that autumnal anthocyanin accumulation acts to delay leaf senescence

and is not simply a co-occurring phenomenon. Such experiments could use measurements of hydraulic conductance through the leaf-stem interface as an additional indicator of vascular continuity.

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