Anthocyanins in photoprotection: knowing the actors in play to solve this complex ecophysiological issue

A response to Pena-Novas & Archetti (2020) ‘Biogeography and evidence for adaptive explanations of autumn colors’ and Renner & Zohner (2019) ‘The occurrence of red and yellow autumn leaves explained by regional differences in insolation and temperature’

Though there is compelling evidence for the suite of molecular events regulating the flavonoid branch pathway that leads to anthocyanin biosynthesis (Chen et al., 2019; Naing & Kim, 2018; Saigo et al., 2020), there is still no consensus about the functional significance of anthocyanins in plant–environment interactions (Steyn et al., 2002; Manetas, 2006; Hughes, 2011; Landi et al., 2015, 2021; Gould et al., 2018). This is also the case of colorless flavonoids, hereafter referred to as flavonoids, which accumulate in separate leaf tissues and subcellular organelles to anthocyanins (Pollastri & Tattini, 2011; Agati et al., 2012; Brunetti et al., 2018). Early studies performed on flavonoid-deficient mutants offered conclusive evidence of their ability to provide ultraviolet (UV)-B photoprotection (Li et al., 1993; Lois & Buchanan, 1994; Bieza & Lois, 2001). Their significance as reactive oxygen species (ROS) scavengers and modulators of various developmental processes mediated by ROS and phytohormone signaling (depending on light stress severity) has also been recently ascertained (Hernandez et al., 2009; Agati & Tattini, 2010; Watkins et al., 2017; Muhelemann et al., 2018; Chapman et al., 2019; Agati et al., 2020; Chapman & Muday, 2021). Anthocyanins, differently from flavonoids, have the peculiar capacity of absorbing wavelengths over a wider range, from UV-B to red, of the solar spectrum (Lopes da Silva et al., 2007; Skaar et al., 2014; Aguilar & Hernández-Brenes, 2015; Nichelmann & Bilger, 2017; Gould et al., 2018), and exclusively accumulate in the vacuoles of epidermal and/or subepidermal cells in red leaves (Hughes & Smith, 2007; Hughes et al., 2007; Hughes, 2011; Boldt et al., 2014; Tattini et al., 2017). They are optimally suited, therefore, to constitute an effective shield against supernumerary photons (thus contributing to the so-called avoidance mechanism), particularly over the visible portion of the solar spectrum, from reaching sensitive targets in the leaf. Inexplicably, after three decades of extensive research, colleagues world-wide are still in dispute about the effective photoprotection provided by anthocyanins in an in planta situation (Hughes, 2011; Landi et al., 2015, 2021; Gould et al., 2018).

Two articles, by Renner & Zohner (2019) and by Pena-Novas & Archetti (2020), have recently renewed the debate about the functional significance of anthocyanin biosynthesis in autumn (senescing) leaves. Renner & Zohner (2019) suggested that the primary function of anthocyanins is photoprotection: anthocyanins scavenge ROS and mitigate their formation through light attenuation while senescing leaves are dismantling their photosynthetic apparatus. In support of their hypothesis, Renner & Zohner (2019) reported that cyanic leaves occur more frequently in deciduous tree species inhabiting regions characterized by lower temperatures and higher solar irradiance during autumn/winter, which would render them more vulnerable to severe ‘light stress’ during leaf senescence. This is consistent with the notion that high light and cold stress are among the most effective environmental drivers for biosynthesis of anthocyanins (likely following stress-induced alteration in cellular redox homeostasis — see Page et al. (2012) and Viola et al. (2016) and references cited therein). Anthocyanin-induced photoprotection, in turn, allows senescing cyanic leaves to cope for longer against photooxidative stress and, consequently, to sustain greater nutrient resorption (the so-called photoprotection–resorption hypothesis; sensu Hoch et al., 2003) compared with the anthocyanin-deficient (yellow) counterparts. By contrast, Pena-Novas & Archetti (2020) suggested the ‘coevolution hypothesis’, which posits that the functional significance of autumn leaf color is to provide an honest warning signal to insects that lay eggs in trees during autumn, of high defense investment, and/or low leaf quality (Archetti & Leather, 2005; Archetti et al., 2009). In other words, pests prefer green leaves, simply because red leaves have greater chemical defense than green leaves and/or are lower in nutrient quality (Cooney et al., 2012; Menzies et al., 2016; Pena-Novas & Archetti, 2020).

Though we do not dispute the Pena-Novas & Archetti (2020) hypothesis for the functional/adaptive significance of leaf autumn color (Renner & Zohner (2020) have already replied to their argumentations), we strongly challenge the authors’ conclusions that there is not enough evidence in support of an effective photoprotective function of anthocyanins. In fact, their view is simply counterfactual — for recent research articles, see Tattini et al. (2014, 2017), Logan et al. (2015), Zhang et al. (2016, 2018), Zhu et al. (2016), Cooney et al. (2018), Gould et al. (2018), Zheng et al. (2019, 2021), Yu et al. (2019, 2021), Moustaka et al. (2020), and Landi et al. (2021) — and largely based upon superficial observations and reasoning proposed by Manetas (2006). Here, our discussion mostly concerns the anthocyanin’s ability to attenuate visible light, and thereby mitigate photooxidative stress.
Cyanic tissues have molar extinction coefficient maxima in the green, but effectively absorb over the blue and red portions of the solar spectrum

Although many common anthocyanins do indeed have molar extinction coefficient maxima ($\varepsilon_{\text{max}}$) in the green region of the solar spectrum, this does not imply anthocyanins are merely green-absorbing pigments, as erroneously reported in too many instances (for a recent review article, see Landi et al. (2021) and references therein). Anthocyanin derivatives of the most common aglycones detected in plants (cyanidin, delphinidin, malvidin, peonidin, pelargonidin, petunidin; Andersen & Jordheim, 2006) absorb effectively over the 450–500 nm (blue) waveband of the solar spectrum (Lopes da Silva et al., 2007; Merzlyak et al., 2008; Aguilar & Hernández-Brenes, 2015). In particular, derivatives of pelargonidin (a widely distributed class of anthocyanins) have $\varepsilon_{\text{max}}$ at c. 500 nm and absorb blue photons more efficiently than green photons (Rein et al., 2005; Aguilar & Hernández-Brenes, 2015). The blue-light-absorbing capacity may increase further when anthocyanins are acylated with aliphatic or aromatic acids, as reported in a wide range of species (Jordheim et al., 2016; Saha et al., 2020).

Therefore, the conclusion of a negligible contribution of anthocyanins in photoprotection, because ‘they absorb mainly green light’ (Manetas, 2006; Pena-Novas & Archetti, 2020), which is poorly absorbed by chlorophylls, is misleading. The capacity of anthocyanins to absorb over the visible portion of the solar spectrum requires the integration of $\varepsilon_c$ over the whole solar spectrum. Yet, the negligible blue light absorption capacity of anthocyanins reported in Manetas (2006) was based upon experiments comparing transmittance of monochromatic blue light—that is, 430 nm wavelength (Karabourniotis et al., 1999) and 450 nm (Gould et al., 2002)—through red vs green epidermis. Obviously, anthocyanins maximally absorb at longer blue wavelengths, which are also most effective in photodamage (Takahashi et al., 2010). The blue-light-absorbing capacities of anthocyanins have long been known (Merzlyack & Hendry, 1994; Feild et al., 2001; Pfundel et al., 2007) and may help to unveil, not only the significance of anthocyanins in photoprotection, but also the marked changes in morpho-anatomical traits displayed by cyanic and acyanic leaves (Tattini et al., 2014, 2017; Landi et al., 2021; see later for details).

We additionally note that the UV–visible spectral features of anthocyanins, as estimated by absorbance spectra of strongly acidic tissue extract solutions (usually at pH 1.5–2.0), are far from representing those of cyanic tissues (Agati et al., 2007; Ferrandino et al., 2017; Gould et al., 2018; Landi et al., 2021). First, vacuolar anthocyanins occur in an aqueous cellular milieu of c. pH 5.0, which shifts the absorbance maximum of the anthocyanin towards longer wavelengths, relative to the strongly acidic solutions often used for most anthocyanic extractions (Dangles & Fenger, 2018; Yun et al., 2019). Second, the UV–visible spectral features of cyanic tissues undergo additional hyperchromic and bathochromic shifts, since anthocyanins do not ‘live alone’ in the vacuole, but cohabit with a diverse community of phenylpropanoids, including flavones and flavonols. Copigmentation (i.e. the intermolecular complexes formed through noncovalent bonds between anthocyanins and both flavonoids and hydroxycinnamates) inevitably occurs in cyanic tissues and may shift their absorbance spectra up to 40 nm toward the red region of the solar spectrum (Trouillas et al., 2016).

Finally, in vitro ‘anthocyanin spectra’ may undergo additional bathochromic shift, since anthocyanins, as well as anthocyanin–flavone copigments, form stable complexes (or supramolecular complexes in the case of copigments) with a range of metal ions (e.g. magnesium, manganese, aluminium, iron; Sigurdson & Giusti, 2014; Tang & Giusti, 2020; Estévez et al., 2021), that may largely accumulate in the vacuoles of epidermal cells (Landi et al., 2015). In other words, cyanic tissues have the capacity to appreciably absorb red photons too (Agati et al., 2005, 2007; Ferrandino et al., 2017; Nichelmann & Bilger, 2021). In fact, anthocyanins in Plectranthus ciliates leaf hairs contain high concentrations of acylated anthocyanins (coumaric, caffeic, and malonyl derivatives) that effectively absorb blue and red photons (Jordheim et al., 2016). Gould et al. (2010) reported that red stems of Cornus stolonifera transmitted only 30%, 10%, and 50% of blue (450–500 nm), green–yellow (500–600 nm), and red (600–650 nm) wavelengths, respectively, compared with green stems. Markham et al. (2000) also showed appreciable absorption of blue and red light by vacuolar anthocyanin inclusions in red petals of Dianthus caryophyllus.

There is recent evidence that perception of low blue-light availability and relatively low red/far red (R/FR) may well explain the shade-type characteristics (Steyn et al., 2002; Manetas et al., 2003; Manetas, 2006; Hughes, 2011; Tattini et al., 2014) of cyanic leaves (Tattini et al., 2017; Landi et al., 2021). Low blue light largely affects the interaction between cryptochromes and phytochromes (Pedmale et al., 2016), resulting in increased activity of phytochrome interacting factors (Wang & Lin, 2020), which, in turn, promote shade avoidance responses (Castillón et al., 2009; Casal, 2012). Thus, anthocyanin-induced attenuation of blue light may lead to partial activation of photo2 and proper development of palisade cell formation (Kozuka et al., 2011), a phenomenon long known to occur in shaded leaves (i.e. experiencing low R/FR) (Aoyama et al., 1995; Franklin, 2008; Ciolfi et al., 2013).

By contrast, the green-light-absorbing capacity of anthocyanins is inconsistent with the shade-avoidant symptoms that they are often associated with – for a detailed analysis, see Landi et al. (2021). Shade avoidance responses follow perception of light highly enriched in green wavelengths (Dhingra et al., 2006; Wang & Folta, 2013), but these are the same wavelengths that are muted by anthocyanins. These contradictory observations have been ignored for decades by both plant ecologists and plant physiologists involved in anthocyanin research. We have recently offered compelling evidence that cryptochrome-regulated blue light signals (also involving phytochromes; Wang & Lin, 2020) are partially inactivated in cyanic leaves (Landi et al., 2021) and, therefore, responsible for their shade nature (Pedmale et al., 2016). As a corollary, this refutes previous ideas of anthocyanins being inherently unable to provide photoprotection to cyanic leaves. In the light of in vivo spectral features of cyanic leaves, the conclusion that ‘Anthocyanins thus appear to provide only limited protection to both flavonoids and hydroxycinnamates’ is inherently unable to provide photoprotection to cyanic leaves, whereas an effective sunscreen would be
expected to match the absorption spectrum of the target photo-dynamic chlorophyll molecules’ (Manetas, 2006) is definitively wrong, in turn leading to the wrong reasoning of Pena-Novas & Archetti (2020, 2021).

Do anthocyanins provide effective photoprotection? From lab to field studies, from plant physiology to plant ecology

Photoprotection is a qualitative parameter in its nature and, as such, difficult to quantify. It is indirectly estimated through the extent to which plants may avoid (limit) photoinhibition; that is, the light-induced depression in the maximum efficiency (usually estimated which plants may avoid (limit) photoinhibition; that is, the light-induced depression in the maximum efficiency (usually estimated by the chlorophyll fluorescence-derived parameter $F_v/F_m$) and/or the rate of photosynthesis ($A_N$, net CO$_2$ assimilation rate; Long et al., 1994; Baker, 1996).

Several studies have examined the photoprotective functions of anthocyanins by comparing permanently red vs green leaves acclimated to low light and then exposed to a sudden increase of radiant energy (therefore, ‘photoinhibitory’), usually followed by relief from photoinhibitory conditions (e.g. Hatier et al., 2013; Fondom et al., 2014; Hughes et al., 2014; Gould et al., 2018). In a few instances, light-induced declines in photosynthetic performance of either overaccumulating or anthocyanin-deficient mutants have been examined on a short-term (hours-to-days) basis (Hoch et al., 2003; Gould et al., 2018; Zheng et al., 2019). There are also examples of long-term (days-to-weeks) exposure to excessive light of red and green leaves grown under natural conditions (Tattini et al., 2014, 2017; Yu et al., 2021; Zheng et al., 2021). These studies are all consistent with effective photoprotection provided by anthocyanins: photosynthetic performance in cyanic leaves is less impaired and recovers faster upon relief from photoinhibitory light treatments, compared with the acyanic counterparts. There is consensus that anthocyanins operate mostly in absorbing supernumerary photons otherwise damaging the photosynthetic apparatus (avoidance mechanism), even though the ROS-scavenging ability of anthocyanins may increase in significance when light stress becomes particularly severe, as also hypothesized by Renner & Zohner (2019) in the case of senescing leaves. The biosynthesis of anthocyanins (and of other flavonoids) is part of the integrated and modular network of excess light-induced morpho-anatomical and biochemical adjustments, usually referred to as the flight strategy of sessile organisms (Potters et al., 2007), allowing plants to successfully counter the detrimental effects induced by a severe excess of light.

The issue becomes substantially more muddied when the photosynthetic performance of green and red individuals is measured in situ for review articles, see Hughes, 2011; Landi et al., 2015, 2021). This is in part due to the fact that plants usually face multiple environmental stressors in the field, such as the combined action of low temperature and high solar irradiance in autumn/winter. Although photoprotection sensu stricto is purely a physiological issue (as it merely concerns photoinhibition of photosynthesis), investigating the responses of transiently red vs permanently green leaves becomes a more complex ecophysiological issue. First, genetic or age-dependent color change unlikely involves just the biosynthesis of anthocyanins, but includes additionally a suite of morpho-anatomical and biochemical traits, all of which greatly affect the entry, interception, and management of supernumerary photons by the leaf (Hughes, 2011; Tattini et al., 2014, 2017; Cooney et al., 2018). Second, anthocyanins often accumulate in individuals that are more vulnerable to low-temperature photo-inhibition of photosynthesis (Pietrini et al., 2002; Hughes et al., 2005; Kytridis et al., 2008; Zeliou et al., 2009; Nikiforou & Manetas, 2010; Landi et al., 2015). These individuals suffer from more severe oxidative stress than the green counterparts do (particularly when growing in high sunlight), and the consequential drastic alteration in cellular redox homeostasis triggers the biosynthesis of anthocyanins (Pireyre & Burow, 2015; Viola et al., 2016; Plumb et al., 2018; Qu et al., 2018). The analysis of leaves (individuals) that become red during autumn is, therefore, not an ideal model to provide mechanistic insights on the photoprotective role of anthocyanins.

To overcome this issue, plant ecologists commonly use a classical top-down approach (Lucas et al., 2011), starting from the ecological drivers for the evolution of autumn leaf color, down to effects on color change at cellular through organ and even tissue levels. Though the identification of the ecological drivers responsible for anthocyanin biosynthesis is essentially observational, as is exactly the case of Renner & Zohner’s and Pena-Novas & Archetti’s hypotheses, disclosing their roles in photoprotection requires knowledge of their light-absorbing features (in turn influencing working hypotheses) and correct ‘measurements’ of photoprotection; this is mandatory, especially in field studies.

Since photoprotection is optimally estimated, by definition, when plants are exposed to photo-inhibitory (i.e. supersaturating) light levels, assessing the photoprotective role of any pigment requires the very same light condition, even in field studies. Unfortunately, this is not the case in most studies comparing red and green individuals, including those mentioned by Pena-Novas & Archetti (2020). For instance, Burger & Edwards (1996) explored the photosynthetic performance in partially shaded (acclimated to 480 µmol m$^{-2}$ s$^{-1}$) red and green leaves by means of oxygen evolution/photosynthetic photon flux density (PPFD) curves in the range 0–200 µmol m$^{-2}$ s$^{-1}$. Esteban et al. (2008) also examined the photosynthetic performance of variegated leaves of *Erytrimum dens-canis* L. plants growing in understory, and plants were subsequently exposed to a light treatment of only 300 µmol m$^{-2}$ s$^{-1}$ in the laboratory.

It also appears illogical that similar ‘predawn $F_v/F_m$’ displayed by cyanic and acyanic leaves has been taken as a proof of the negligible photoprotective roles of anthocyanins (Lee et al., 2003; Manetas et al., 2003; Kytridis et al., 2008; Manetas & Buschmann, 2011). Similar predawn $F_v/F_m$ indicates simply that green and red leaves recover to a similar extent (but likely with largely different recovery rates; Tattini et al., 2017) from previous, diurnal photoinhibitory conditions, as already hypothesized by Lee et al. (2003). In fact, green leaves sampled at predawn have both a higher concentration of violaxanthin cycle pigments (VAZ, relative to Chltot) and a higher de-epoxidation state of VAZ than corresponding red leaves (Kytridis et al., 2008; Hughes et al., 2012; Tattini et al., 2014). This indicates increased engagement of ‘sustained thermal
dissipation’ (mostly driven by nonphotochemical fluorescence quenching; sensu Demmig-Adams & Adams, 2006) in green compared with red leaves, representing an alternative energy-mitigation strategy for avoiding irreversible photodamage to photosystem II (PSII) during the most severe photoinhibitory light conditions. It is a matter of fact that excess energy on PSII (e.g. measured as 1 − gF, where gF is the photochemical quenching) is much lower in red than in green leaves, because anthocyanins absorb photons otherwise absorbed by chlorophylls and only in part used for CO2 assimilation (Tattini et al., 2014, 2017; Ospina Calvo & Lagorio, 2019). This offers further support to the idea of an efficient photoprotection afforded by anthocyanins, and the long-reported view of a constitutively lower ability of red leaves to dissipate thermally excess excitation energy on PSII (Manetas et al., 2003; Zeliou et al., 2009) is, consequently, erroneous.

In the same way, the lower photosynthetic rates (usually estimated at subsaturating light irradiance) displayed by cyanic compared with acyanic leaves in some studies (Kyparissis et al., 2007; Nikiforou et al., 2011) do not support the view of negligible photoprotection by anthocyanins. Net assimilation rate (AN) is lower in red leaves because the cyanic filter absorbs photons that are effective in photosynthesis, but instantaneous photosynthesis per se (as well as plant fitness, Mendez et al., 1999; Nikiforou et al., 2010) has nothing to do with photoprotection (Hughes et al., 2010; Landi et al., 2021). Instead, morning-to-midday declines in AN (or predawn-to-midday reductions in Fv/Fm)—that is, photoinhibition of photosynthesis—displayed by red and green leaves are best suited to quantify the actual photoprotection provided by anthocyanins. We have recently shown that AN was lower (−16%) in early morning (PPFD of 725 ± 63 μmol m−2 s−1), but substantially higher (+27%) at midday (PPFD of 1878 ± 168 μmol m−2 s−1) in purple than in green basil leaves (Tattini et al., 2017). Similarly, photosynthetic performance (estimated through measurements of both AN and a suite of chlorophyll fluorescence-derived parameters) was either lower or higher in red compared to green basil under partial shading, but higher under full sunlight (Tattini et al., 2014; Lo Piccolo et al., 2020). Although the data of these studies are both consistent with shade-type characteristics and reduced light availability imposed by the epidermal cyanic shield to red leaves, the issue needs extensive future research, examining species with different evolutionary history and adaptation.

We also note that previously reported (photosynthetic inferiority) (apparent carboxylation efficiency Vc,max(Ci)) of red compared with green leaves, calculated from AN/Ci (Ci, intercellular CO2 concentration) curves is equally erroneous (Kytridis et al., 2008; Nikiforou et al., 2011). There is compelling evidence that shade-acclimated leaves have lower mesophyll conductance to CO2 (gs) than sun-acclimated leaves do (Campany et al., 2016; Peguero-Pina et al., 2016). This is exactly the case of cyanic vs acyanic leaves growing in full sunlight (Tattini et al., 2017; Landi et al., 2021). The suite of morpho-anatomical (mesophyll areas exposed to intercellular air spaces, chloroplast accumulation to periclinial cell wall induced by low light availability) and biochemical changes (reduced synthesis and activity of carbonic anhydrase, induced by low blue light availability) imposed by the anthocyanin filter is likely responsible for the lower gs, displayed by cyanic leaves (Landi et al., 2021). Consequently, though Vc,max(Ci) may be lower (Nikiforou et al., 2011; Carpenter et al., 2014; Ranjan et al., 2014), actual carboxylation efficiency (calculated on the basis of chloroplastic CO2 concentration C) Vc,max(Ci) may even be higher in red than in green leaves (Tattini et al., 2017; Landi et al., 2021).

Finally, the chlorophyll fluorescence-derived parameter Fv/Fm is used as a proxy for photosynthesis of red and green leaves in the vast majority of ecophysiological studies. However, whereas AN measures photosynthesis at the whole-leaf level, Fv/Fm provides an estimation of the leaf photosynthetic potential only for a shallow layer of chloroplasts. Unfortunately, anthocyanins shield the first layers of chloroplasts from the measuring light as well (for both adaxial and abaxial surfaces), and so Fv/Fm may not always be a good proxy of AN in cyanic leaves. The discrepancy between AN and Fv/Fm in red leaves may become particularly relevant when performing chlorophyll fluorescence imaging, which often utilizes a blue measuring light beam, therefore resulting in signal interference by anthocyanins as well.

We suggest that measurements of photosynthesis, and the consequential estimation of the putative photoprotective role of anthocyanins conducted in most ecophysiological studies at just one point of the day, may provide misleading conclusions, unless taking into account the differential effects of environmental stimuli (stressors) on the suite of morpho-anatomical and biochemical features on red and green leaves (individuals).

Conclusions

Knowing the physicochemical features of anthocyanins is the sine qua non condition to reason about their functional roles. The inaccurate belief that anthocyanins absorb primarily green light, while negligibly absorbing blue and red photons, has resulted in a faulty working hypothesis and data interpretation. It is difficult to understand why plant scientists have not taken into proper account the contradiction between the green-light-absorbing features of anthocyanins and the old observation of the shade nature of cyanic leaves. The shade-type characteristics of cyanic leaves results from anthocyanins absorbing blue and red photons, and not on their ability to absorb green photons. This, in turn, makes anthocyanins well-suited to provide effective photoprotection, as conclusively reported in a series of studies conducted under controlled light conditions, on both short and long-term bases.

Apparently, contradictory conclusions emerge when individuals that produce red or green leaves on a seasonal basis — and hence upon changes in air temperature, soil water availability, and solar irradiance — have been analyzed. This usually occurs when scaling up from the cellular/organ level to the level of the whole plant interacting with multiple environmental stimuli. The analysis of these complex systems is usually performed using a classical top-down approach, starting from the knowledge of the environmental drivers of anthocyanin biosynthesis down to explore the photoprotection mechanisms, on the basis of photosynthetic performance and light-absorbing properties of cyanic vs acyanic leaves. This is exactly the case of both Renner & Zohner (2019) and Pena-Novas & Archetti (2020), although the two studies yielded
opposite conclusions. Renner & Zohner (2019) support their 'photoprotection hypothesis' using the mechanistic insights given by Gould et al. (2018), who used wild-type and anthocyanin-rich mutants of Arabidopsis exposed to excessive light energy. Pena-Novas & Archetti (2020) challenged Renner & Zohner’s conclusion for the evolution of autumn leaf color, not only posing concerns about the environmental drivers for anthocyanin biosynthesis (see also Pena-Novas & Archetti, 2021), but also basing their reasoning on the light-absorbing properties of anthocyanins reported by Manetas (2006). We have shown here that most of Manetas’s hypotheses are erroneous.

Additionally, we note that Pena-Novas & Archetti’s (2020, 2021) conclusion ‘… the photoprotection hypothesis posits that selection for reabsorbing nitrogen is the driving force behind the evolution of autumn colours’ is incorrect. In our opinion, the ‘driving force’ for the evolution of autumn leaf colors is to provide cold-sensitive individuals concomitantly exposed to high insolation (low temperature and high light trigger anthocyanin biosynthesis) with a flavonoid class primarily devoted to mitigating photooxidative stress. In turn, this allows for greater reabsorption of nitrogen. Resorption of nutrients is just an ‘ecological consequence’ (effect) of photoprotection. The coevolution hypothesis also relies on autumn red leaves having greater chemical defenses (or lower nitrogen), but with a flavonoid class primarily devoted to mitigating photooxidative stress. In turn, this allows for greater reabsorption of nutrients. Resorption of nutrients is just an ‘ecological consequence’ (effect) of photoprotection. The coevolution hypothesis also relies on autumn red leaves having greater chemical defenses (or lower nutritional quality) than the green leaves, both of which would correspond with greater sensitivity to environmental stress in red individuals. Higher activation of defense compound biosynthesis is usually associated with greater sensitivity to stress agents, as is exactly the case of light stress (Tattini et al., 2005).

The accumulation of anthocyanins in peripheral tissues may both protect leaves from supernumerary photons and serve as visual (warning) signal as well. However, whether ‘the production of color per se constituted the original function of anthocyanins’ is unclear (Fineblum & Rausher, 1997; Rausher, 2006). We speculate that anthocyanin biosynthesis did evolve, in both gymnosperms and angiosperms, to equip leaves with additional photoprotective pigments at specific leaf developmental stages, rather than protecting plants against their natural enemies. This may also be the cause for evolution of (colorless) flavonoid metabolism when plants moved from water to colonize land. Conclusive evidence shows that their beneficial action against natural enemies is not the driving force for their biosynthesis (Rausher, 2001; ultimate explanation), which is likely to be found upon the rise of oxidative stress signals that early plants faced when moving onto harsh terrestrial environment (Pollastri & Tattini, 2011). This conforms to the notion that oxidative stress signals modulate the expression of transcription factors regulating key genes of flavonoid biosynthesis, as probably occurs also for the biosynthesis of anthocyanins in senescing leaves (proximate explanation; Xu et al., 2017). There is also intriguing evidence that flavonoids may have served (and still serve) primary roles as modulators of redox signaling (both avoiding the generation and scavenging of ROS once they are formed) in early as well as present-day land plants facing multiple stress events associated with an ever-changing environment (Pollastri & Tattini, 2011; Brunetti et al., 2019; ultimate explanation). Though we are waiting for an unequivocal conclusion on this matter, the effective photoprotection provided by anthocyanins is strongly corroborated by a huge body of experimental data.

Acknowledgements

We are greatly indebted to Professor Nicole Hughes, at High Point University, for helpful comments on the manuscript. Our thanks go also to the reviewers of the manuscript for their constructive criticism.

Author contributions

GA focused on the spectral properties of anthocyanins; LG and ML discussed photoprotection and photoinhibition; MT conceived the article and wrote the MS draft.

ORCID

Giovanni Agati https://orcid.org/0000-0003-0855-9389
Lucia Guidi https://orcid.org/0000-0002-2472-720X
Marco Landi https://orcid.org/0000-0003-0121-0715
Massimiliano Tattini https://orcid.org/0000-0001-5434-8860

References

Agati G, Azzarello E, Pollastri S, Tattini M. 2012. Flavonoids as antioxidative in plants: location and functional significance. *Plant Science* 196: 67–76.
Agati G, Brunetti C, Fini A, Gori A, Guidi L, Landi M, Sebastiani F, Tattini M. 2020. Are flavonoids effective antioxidants in plants? Twenty years of our investigation. *Antioxidants* 9: 1698.
Agati G, Meyer S, Matteini P, Cerovic ZG. 2007. Assessment of anthocyanins in grape (*Vitis vinifera* L.) berries using a noninvasive chlorophyll fluorescence method. *Journal of Agricultural and Food Chemistry* 55: 1053–1061.
Agati G, Pinelli P, Cortes Ebner S, Romani A, Cartelat A, Cerovic ZG. 2005. Nondestructive evaluation of anthocyanins in olive (*Olea europaea*) fruits by *in situ* chlorophyll fluorescence spectroscopy. *Journal of Agricultural and Food Chemistry* 53: 1354–1363.
Agati G, Tattini M. 2010. Multiple functional roles of flavonoids in photoprotection. *New Phytologist* 186: 786–793.
Aguilar O, Hernández-Brenes C. 2015. Use of modified phenolic thyme extracts (*Thymus vulgaris*) with reduced polyphenol oxidase substrates as anthocyanin color and stability enhancing agents. *Molecules* 20: 22422–22434.
Andersen MO, Jordheim M. 2006. Anthocyanins. In: Anderson OM, Markham KR, eds. Flavonoids, chemistry biochemistry and application. Boca Raton, FL, USA: CRC Press. 471–530.

Aoyama T, Dong CH, Wu Y, Carabelli M, Sessa G, Ruberti I, Morelli G, Chua NH. 1995. Ecotypic expression of the Arabidopsis transcriptional activator Athb-1 alters leaf cell fate in tobacco. *Plant Cell*. 7:1773–1785.

Archetti M, Döring TF, Hagen SB, Hughes NM, Leather SR, Lee DW, Lev-Yadun S, Manetas Y, Ougham HJ, Schaberg PG et al. 2009. Unravelling the evolution of autumn colours: an interdisciplinary approach. *Trends in Ecology and Evolution* 24: 166–173.

Archetti M, Leather SR. 2005. A test of the coevolutionary theory of autumn colours: colour preference of *Rhabdamia padis* on *Prunus padus*. *Ethol* 110: 339–343.

Barker NR. 1996. Photoinhibition of photosynthesis. In: Jennings RC, Zuccelli G, Ghetti F, Colombetti G, eds. Light as an energy source and information carrier in plant physiology. New York, NY, USA: Plenum Press, 89–97.

Bieza K, Lois R. 2001. An Arabidopsis mutant tolerant to lethal ultraviolet-B levels shows constitutively elevated accumulation of flavonoids and other phenolics. *Plant Physiology* 126: 1105–1115.

Boldt J, Erwin JE, Meyer MH. 2014. Foliar anthocyanins: a horticultural review. *Horticultural Reviews* 42: 209–251.

Brunetti C, Fini A, Sebastiani F, Gori A, Tattini M. 2018. Modulation of photoreceptor signaling networks in plant responses to shade. *Horticultural Reviews* 296: 448–454.

Brunetti C, Sebastiani F, Tattini M. 2019. ABA, flavonols and the evolvability of land plants. *Plant Science* 280: 448–454.

Burger J, Edwards GE. 1996. Photosynthetic efficiency, and photodamage by UV and visible radiation, in red versus green leaf photosynthetic tissues. *Physiologia Plantarum* 116: 325–335.

Campany CE, Tjoelker MG, von Caemmerer S, Duursma MA. 2016. Coupled response of stomatal and mesophyll conductance to light enhances photosynthesis of shade leaves under sunflecks. *Plant Cell & Environment* 39: 2762–2773.

Carpenter KL, Keidel TS, Pilhl MC, Hughes NM. 2014. Support for a photosynthetic function of winter leaf reddening in nitrogen deficient individuals of *Lonicera japonica*. *Molecules* 19: 17810–17828.

Casal JJ. 2012. Photoreceptor signaling networks in plant responses to shade. *Annual Review of Plant Biology* 64: 403–427.

Castillon A, Shen H, Huq E. 2009. Blue light induces degradation of the negative regulator phytochrome interacting factor 1 to promote photomorphogenic development of Arabidopsis seedlings. *Genetics* 182: 161–171.

Chen L, Hu B, Qin Y, Hu G, Zhao J. 2019. Advance of the negative regulation of anthocyanin biosynthesis by MYB transcription factors. *Plant Physiology and Biochemistry* 136: 178–187.

Ciolfi A, Sessa G, Sassi M, Possenti M, Salvucci S, Carabelli M, Morelli G, Ruberti I. 2013. Dynamics of the shade-avoidance response in Arabidopsis. *Plant Physiology* 163: 331–353.

Coombs LJ, Logan BA, Walsh MJL, Ntanubeugo NB, Reblin JS, Gould KS. 2018. Photoprotection from anthocyanins and thermal energy dissipation in senescing red and green *Sambucus canadensis* peduncles. *Environmental and Experimental Botany* 148: 27–34.

Coombs LJ, van Kliew JW, Hughes NM, Perry NB, Scharfer HM, Menzies JJ, Gould KS. 2012. Red leaf margins indicate increased polygaloid content and function as visual signals to reduce herbivory in *Physodinaerina colorata*. *New Phytologist* 194: 488–497.

Dangles O, Fenger J-A. 2018. The chemical reactivity of anthocyanins and its consequences in food science and nutrition. *Molecules* 23: e1970.

Demming-Adams B, Adams WW III. 2006. Photoprotection in an ecological context: the remarkable complexity of thermal energy dissipation. *New Phytologist* 172: 11–21.

Dhingra A, Dies BH, Lehner KR, Folta KM. 2006. Green light adjusts the plastid transcriptome during early photomorphogenic development. *Plant Physiology* 142: 1256–1266.

Esteban R, Fernández-Marin B, Becerril JM, Garcia-Plazaola JI. 2008. Photoprotective implications of leaf variegation in *E. dens-canis* L. and *P. officinalis* L. *Journal of Plant Physiology* 165: 1255–1263.

Estévez L, Quezán M, Mosquera RA, Guidi L, Lo Piccolo E, Landi M. 2021. First characterization of the formation of anthocyanin–Ge and anthocyanin–B complexes through UV–vis spectroscopy and density functional theory quantum chemical calculations. *Journal of Agricultural and Food Chemistry* 69: 1272–1282.

Field TS, Lee DW, Holbrook NM. 2001. Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. *Plant Physiology* 127: 566–574.

Ferrandino A, Pagliarani C, Carlomagno A, Novello V, Schubert A, Agati G. 2017. Improved fluorescence-based evaluation of flavonoids in red and white winegrape cultivars. *Australian Journal of Grape and Wine Research* 23: 207–214.

Fineblum WL, Rauscher MD. 1997. Do floral pigmentation genes also influence resistance to enemies? The W locus in *Ipomea purpurea*. *Ecology* 78: 1646–1654.

Fondom NY, Castro-Nava S, Huerta AJ. 2014. Field assessment of sub-epidermal leaf anthocyanin, PSII photochemistry, and the xanthophyll-cycle as photoprotective mechanisms in two morphs of *Agyre stricta*. *Flora – Morphology, Distribution, Functional Ecology of Plants* 209: 131–141.

Franklin KA. 2008. Shade avoidance. *New Phytologist* 179: 930–944.

Gould KS, Duade DA, Neufeld HS. 2010. Why some stems are red: cauline anthocyanins shield photosystem II against high light stress. *Journal of Experimental Botany* 61: 2707–2717.

Gould KS, Jay-Allemand C, Logan BA, Baisac Y, Bidel LPR. 2018. When are foliar anthocyanins useful to plants? Re-evaluation of the photoprotection hypothesis using *Arabidopsis thaliana* mutants that differ in anthocyanin accumulation. *Environmental and Experimental Botany* 154: 11–22.

Gould KS, Vogelmann TC, Han T, Clearwater MJ. 2002. Profiles of photosynthesis within red and green leaves of *Quintinia serrata*. *Physiologia Plantarum* 116: 127–133.

Hatier J-HB, Clearwater MJ, Gould KS. 2013. The functional significance of black-pigmented leaves: photosynthesis, photoprotection and productivity in *Opipogon planiscapus* “Negrescos”. *PloS ONE* 8: e67850.

Hernández I, Alegré L, van Breugem F, Munné-Bosch S. 2009. How relevant are flavonoids as antioxidants in plants? *Trends in Plant Science* 14: 125–132.

Hoch WA, Singsas EL, McCown BH. 2003. Resorption protection. Anthocyanins facilitate nutrient recovery in autumn by shielding leaves from potentially damaging light levels. *Plant Physiology* 133: 1296–1305.

Hughes NM. 2011. Winter leaf reddening in ‘evergreen’ species. *New Phytologist* 190: 573–581.

Hughes NM, Burkey KO, Cavender-Bares J, Smith WK. 2012. Xanthophyll cycle pigment and antioxidant profiles of winter-red (anthocyanic) and winter-green (acyanic) angiosperm evergreen species. *Journal of Experimental Botany* 63: 1895–1905.

Hughes NM, Burkey KO, Neufeld HS. 2005. Functional role of anthocyanins in high-light winter leaves of the evergreen herb *Galax urceolata*. *New Phytologist* 168: 575–587.

Hughes NM, Carpenter KL, Keidel TS, Miller CN, Waters MN, Smith WK. 2014. Photosynthetic costs and benefits of abaxial versus adaxial anthocyanins in *Colocasia esculenta* ‘Mojito’. *Plant Science* 240: 971–981.

Hughes NM, Morley CB, Smith WK. 2007. Coordination of anthocyanin decline and photosynthetic maturation in juvenile leaves of three deciduous tree species. *New Phytologist* 175: 675–685.

Hughes NM, Reinhardt K, Field TS, Gerard AR, Smith WK. 2010. Association between winter anthocyanin production and drought stress in angiosperm evergreen species. *Journal of Experimental Botany* 61: 1699–1709.

Hughes NM, Smith WK. 2007. Attenuation of incident light in *Galax urceolata* (Diapensiaceae): concerted influence of adaxial and abaxial anthocyanin layers on photoprotection. *American Journal of Botany* 94: 784–790.

Jordheim M, Calcott K, Gould KS, Davies KM, Schwinn KE, Andersen ØM. 2014. High concentrations of aromatic acylated anthocyanins found in cauline hairs in *Plectranthus ciliatus*. *Phytochemistry* 128: 27–34.

Karabourniotis G, Borman JF, Liakoura V. 1999. Different leaf surface characteristics of three grape cultivars affect leaf optical properties as measured with fibre optics: possible implication in stress tolerance. *Functional Plant Biology* 26: 47–53.
Rauerh MD. 2006. The evolution of flavonoids and their genes. In: Grotewold E., ed. The science of flavonoids. New York, NY, USA: Springer, 175–211.

Rein MJ, Ollilainen V, Vahermo M, Yli-Kauhaluoma Y, Heinonen M. 2005. Identification of novel pyranoanthocyanins in berry juices. European Food Research and Technology 220: 239–244.

Renner SS, Zohner CM. 2019. The occurrence of red and yellow autumn leaves explained by regional differences in isolation and temperature. New Phytologist 224: 1466–1471.

Renner SS, Zohner CM. 2020. Further analysis of 1532 deciduous woody species from North America, Europe, and Asia supports continental-scale differences in red autumn colouration. New Phytologist 228: 814–815.

Saha S, Singh J, Paul A, Sarkar R, Khan Z. Banerjee K. 2020. Anthocyanin profiling using UV–vis spectroscopy and liquid chromatography mass spectrometry. Journal of AOAC International 103: 23–29.

Saigo K, Wang T, Watanabe M, Tohge T. 2020. Diversity of anthocyanin and proanthocyanin biosynthesis in land plants. Current Opinion in Plant Biology 55: 93–99.

Sigurdson GT, Giusti MM. 2014. Bathochromic and hyperchromic effects of aluminum salt complexation by anthocyanins from edible sources for blue color development. Journal of Agricultural and Food Chemistry 62: 6955–6965.

Skær I, Adaku C, Jordheim M, Byamukama R, Kiremire B, Andersen OM. 2014. Purple anthocyanin colouration on lower (abaxial) leaf surface of Hemigraphis colorata (Acanthaceae). Phytochemistry 105: 141–146.

Steyn WJ, Wand SJE, Holcroft DM, Jacobs G. 2002. Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. New Phytologist 155: 349–361.

Takahashi S, Milward SE, Yamori W, Evans JR, Hillier W, Badger MR. 2010. The solar action spectrum of photosystem II damage. Plant Physiology 153: 988–993.

Tang P, Giusti MM. 2020. Metal chelates of petunidin derivatives exhibit enhanced color and stability. Food 9: e1426.

Tattini M, Guidi L, Morassi-Bonzi L, Pinelli P, Remorini D, Degl’Innocenti E, Giordano C, Massai R, Agati G. 2005. On the role of flavonoids in the integrated mechanisms of response of Lignaria vulgaris and Phyliaea latifolia to high solar radiation. New Phytologist 167: 457–470.

Tattini M, Landi M, Brunetti C, Giordano C, Remorini D, Gould KS, Guidi L. 2014. Epidermal coumaroyl anthocyanins protect sweet basil against excess light stress: multiple consequences of light attenuation. Physiologia Plantarum 152: 585–598.

Tattini M, Sebastiani F, Brunetti C, Fini A, Torre S, Gori A, Centritto M, Ferrini F, Landi M, Guidi L. 2017. Dissecting molecular and physiological response mechanisms to high solar radiation in cyanic and acyanic leaves: a case study on red and green basil. Journal of Experimental Botany 68: 2425–2437.

Trouillas P, Sancho-Garcia JC, De Freitas V, Gierschner J, Otopyka M, Dangles O. 2016. Stabilizing and modulating color by co-pigmentation: insights from theory and experiment. Chemical Reviews 116: 4937–4982.

Viola I, Camoianaro A, Gonzalez DH. 2016. Redox-dependent modulation of anthocyanin biosynthesis by the TCP transcription factor TCP15 during exposure to high light intensity conditions in Arabidopsis. Plant Physiology 170: 74–85.

Wang Q, Lin C. 2020. Mechanisms of cryptochrome-mediated photoresponses in plants. Annual Review of Plant Biology 71: 103–129.

Wang Y, Folta KM. 2013. Contribution of green light to plant growth and development. America Journal of Botany 100: 70–78.

Watkins JM, Chapman JM, Muday GK. 2017. Abscisic acid-induced reactive oxygen species are modulated by flavonoids to control stomata aperture. Plant Physiology 175: 1807–1825.

Xu Z, Mahmood K, Rothstein SJ. 2017. ROS induces anthocyanin production via late biosynthetic genes and anthocyanin deficiency confers the hypersensitivity to ROS-generating stresses in Arabidopsis. Plant & Cell Physiology 58: 1364–1377.

Yu ZC, Lin W, Zheng X-T, Chow WS, Luo Y-N, Cai M-L, Peng C-L. 2021. The relationship between anthocyanin accumulation and photoprotection in young leaves of two dominant tree species in subtropical forests in different seasons. Photosynthesis Research 149: 41–55.

Yu ZC, Zhang Q, Zheng X, Huang X, Peng C. 2019. Anthocyanin accumulation in juvenile Schima superba leaves is a growth trade-off by consuming energy for adaptation to high light during summer. Journal of Plant Ecology 12: 507–518.

Yun D, Cai H, Liu Y, Xiao L, Song J, Liu J. 2019. Development of active and intelligent films based on cassava starch and Chinese bayberry (Myrica rubra Sieb. et Zucc.) anthocyanins. Royal Society of Chemistry Advances 9: e30905.

Zelion K, Manetas Y, Petropoulou Y. 2009. Transient winter leaf reddening in Cistus creticus characterizes weak (stress-sensitive) individuals, yet anthocyanins cannot alleviate the adverse effects on photosynthesis. Journal of Experimental Botany 60: 3031–3042.

Zhang T-J, Chow WS, Liu X-T, Zhang P, Liu N, Peng CL. 2016. A magic red coat on the surface of young leaves: anthocyanins distributed in trichome layer protect Catapnos fossa leaves from photo-inhibition. Tree Physiology 36: 1296–1306.

Zhang T-J, Zheng J, Yu Z-C, Gu X-Q, Tian X-S, Peng C-L, Chow WS. 2018. Variations in photoprotective potential along gradients of leaf development and plant succession in subtropical forests under contrasting irradiances. Environmental and Experimental Botany 154: 23–32.

Zheng X-T, Chen Y-H, Cai M-L, Yu Z-C, Peng C-L. 2019. ANS-deficient Arabidopsis is sensitive to high light due to impaired anthocyanin photoprotection. Functional Plant Biology 46: 756–765.

Zheng X-T, Yu Z-C, Tang J-W, Cai M-L, Chen Y-L, Yang C-W, Chow WS, Peng C-L. 2021. The major photoprotective role of anthocyanins in leaves of Arabidopsis thaliana under long-term high light treatment: antioxidant or light attenuators? Photosynthesis Research 149: 25–40.

Zhu H, Zhang T-J, Zhang P, Peng C-L. 2016. Pigment patterns and photoprotection of anthocyanins in the young leaves of four dominant subtropical forest tree species in two successional stages under contrasting light conditions. Tree Physiology 36: 1092–1104.

Key words: anthocyanins, autumn leaf color, coevolution, cold stress, light absorbance, light stress, photoinhibition, photoprotection.

Received, 10 April 2021; accepted, 24 June 2021.