

*Letters*

## 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; Muheleemann *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 ( $\epsilon_{\max}$ ) 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  $\epsilon_{\max}$  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  $\epsilon_s$  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 epidermises. 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 (Merzlyak & 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 vivo* ‘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, 2017; Landi *et al.*, 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 (Castillon *et al.*, 2009; Casal, 2012). Thus, anthocyanin-induced attenuation of blue light may lead to partial activation of phot2 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 confutes 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 against photoinhibition, 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 by the chlorophyll fluorescence-derived parameter  $F_v/F_m$ ) and/or the rate of photosynthesis ( $A_N$ , net CO<sub>2</sub> 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 muddled 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 photoinhibition 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 photoinhibitory (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  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) red and green leaves by means of oxygen evolution/photosynthetic photon flux density (PPFD) curves in the range 0–200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Esteban *et al.* (2008) also examined the photosynthetic performance of variegated leaves of *Erytronium dens-canis* L. plants growing in understory, and plants were subsequently exposed to a light treatment of only 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  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  $\text{Chl}_{\text{tot}}$ ) 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  $1 - q_p$ , where  $q_p$  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 CO<sub>2</sub> 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 ( $A_N$ ) 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  $A_N$  (or predawn-to-midday reductions in  $F_v/F_m$ ) – 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  $A_N$  was lower (–16%) in early morning (PPFD of  $725 \pm 63 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), but substantially higher (+27%) at midday (PPFD of  $1878 \pm 168 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) in purple than in green basil leaves (Tattini *et al.*, 2017). Similarly, photosynthetic performance (estimated through measurements of both  $A_N$  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  $V_{c,\max}(C_i)$ ) of red compared with green leaves, calculated from  $A_N/C_i$  ( $C_i$ , intercellular CO<sub>2</sub> 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 CO<sub>2</sub> ( $g_m$ ) 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 periclinal 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  $g_m$  displayed by cyanic leaves (Landi

*et al.*, 2021). Consequently, though  $V_{c,\max}(C_i)$  may be lower (Nikiforou *et al.*, 2011; Carpenter *et al.*, 2014; Ranjan *et al.*, 2014), actual carboxylation efficiency (calculated on the basis of chloroplast CO<sub>2</sub> concentration  $C_c$ )  $V_{c,\max}(C_c)$  may even be higher in red than in green leaves (Tattini *et al.*, 2017; Landi *et al.*, 2021).

Finally, the chlorophyll fluorescence-derived parameter  $F_v/F_m$  is used as a proxy for photosynthesis of red and green leaves in the vast majority of ecophysiological studies. However, whereas  $A_N$  measures photosynthesis at the whole-leaf level,  $F_v/F_m$  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  $F_v/F_m$  may not always be a good proxy of  $A_N$  in cyanic leaves. The discrepancy between  $A_N$  and  $F_v/F_m$  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 sequential 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 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.


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## 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.

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

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