

Why some leaves are anthocyanic and why most anthocyanic leaves are red?

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Abstract

The adaptive significance of leaf reddening, as it occurs during specific developmental stages or after stress, has puzzled biologists for more than a century. Theoretically, the accumulation of a non-photosynthetic pigment competing with chlorophylls for photon capture would impose a photosynthetic cost, which should be paid off by the benefits afforded by anthocyanins under some circumstances. Hence, the proposed hypotheses presume protective functions against excess light, UV-B radiation, reactive oxygen species, water stress (osmoregulation) and herbivory. The existing arguments in favor of an anti-oxidant, anti-UV-B and osmoregulatory role are confounded by the co-occurrence in leaves of other compounds having the same properties, not absorbing visible light, attaining much higher concentrations and, in some cases, having a more appropriate location to fulfill the ascribed functions. Moreover, the excess light hypothesis should take into account that anthocyanins mainly absorb green photons, which are used photosynthetically in deeper cell layers needing less photoprotection. The more ecological, anti-herbivore hypotheses, consider red leaf color as a signal denoting high defensive commitment, as a camouflage obscuring the green reflectance indicative of a healthy leaf and/or as a device undermining the folivorous insects camouflage. The anti-herbivore hypotheses have not been thoroughly tested, yet they are compatible with the known optical preferences of insects and their underlying physiology. Overall, although a multiplicity of potential roles can be argued, the primary role may depend on the reference system, i.e. species, developmental stage or specific biotic and abiotic stressors.

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Introduction

The evolution of plants from the fresh water green Chlorophytes resulted in a land world where green dominated. Later on, the advent of Angiosperms and their co-evolution with pollinators added more chromatic variation, as a response to the need for flower

discrimination against the green background. Although the function of flower color as an optical advertisement facilitating flower localization was easily established (Faegri and van der Pijl, 1971), the sporadic appearance of red leaves still puzzles. Since the leaf is an organ optimized for photosynthesis, it is worth wondering why sometimes its color does not correspond to the spectral properties of photosynthetic pigments. Red leaf color is due to either anthocyanins or betalains, the

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latter being restricted to the suborder Chenopodineae within Caryophyllales (Clement and Mabry, 1996). Conversely, anthocyanins abound in almost every other member of the Angiospermae, yet the two kinds of pigments have not been found together in the same plant up to now (Stafford, 1994). In spite of their optical resemblance, the two groups of pigments differ greatly in their chemical structure. Betalains are nitrogen-containing alkaloids while anthocyanins are flavonoids with a C₆–C₃–C₆ skeleton and characteristic modifications in their structure that shift absorbance spectra within the visible range. There are also differences in color stability. Betalains are stable at physiological pH values, while the anthocyanic color fades at pH higher than 1, especially in the aglycon forms. Aglycons, however, are rarely found in plants while glycosylation, acylation with hydroxycinnamic acid and co-pigmentation with colorless flavones promote stability up to neutral pH values (Stintzing and Carle, 2004). The number of known anthocyanin aglycon structures are few: only 18 have been reported (Harborne and Williams, 2001) while six of them (pelargonidin, delphinidin, cyanidin, peonidin, petunidin, malvidin) have a wide distribution. Yet, glycosylation at various positions with various sugars and acylation/co-pigmentation with various phenolics result in a plethora of different anthocyanin structures which paint flowers and fruits with a biologically reasonable and aesthetically agreeable array of colors and tints. Interestingly, however, the major anthocyanin found in leaves is the red cyanidin-3-glycoside (Harborne, 1976). Hence, in seeking a *raison d'être* for the presence of leaf anthocyanins one should not only ask “why leaves are sometimes red” (Gould et al., 1995) but “why anthocyanic leaves are almost always red” as well.

In this short review, I shall try to re-address old questions and formulate new ones for the function of leaf anthocyanins on the basis of some recent findings and ideas. The subject is gaining attention and its fertility is reflected by the numerous hypotheses invented to describe the function of leaf anthocyanins and by the frequency of recently published relevant reviews (Chalker-Scott, 1999; Close and Beadle, 2003; Gould, 2004; Gould et al., 2002b; Hoch et al., 2001; Steyn et al., 2002). The possible functions of leaf betalains will not be discussed since corresponding investigations are scarce (but see Ibdah et al., 2002). However, spectral and antioxidative similarities between the two groups of pigments allow the reasonable assumption of functional similarities as well. Finally, it should be mentioned that anthocyanins are possibly ubiquitous in green leaves, yet at low quantities that cannot mask the chlorophyll color. In the present review, I shall consider only those cases where anthocyanin over-production is manifested as leaf redness.

When and how

There are very few plants possessing constantly red leaves, with redness covering either the whole leaf surface or appearing in patches, as in some tropical understorey plants of considerable importance as ornamentals. Transient redness, however, is much more common. Broadly, we can distinguish between developmentally and environmentally determined appearances of redness. In the first case, young leaves of some plants are red but gradually turn to green upon maturation; or, mature green leaves can become red during senescence. On the other hand, anthocyanin accumulation can be induced in mature green leaves by various biotic or abiotic agents like wounding (Bopp, 1959; Costa-Arbulú et al., 2001; Stone et al., 2001), pathogen attack (Hipskind et al., 1996), nutrient deficiency (Atkinson, 1973; Hodges and Nozzolillo, 1996; Kumar and Sharma, 1999), UV-B radiation (Lindoo and Caldwell, 1978; Mendez et al., 1999), transplant shock (Close et al., 2000) and high light in combination to cold temperatures (Christie et al., 1994; Close et al., 2001; Krol et al., 1995). The pattern of appearance of leaf anthocyanins in space and time (its “phenology” *sensu lato*) provided the first hypothesis for their function, that of photoprotection, which is most popular among physiologists.

Anthocyanins as sunscreens

It happens that the abiotic stresses inducing anthocyanin accumulation also predispose leaves to photoinhibition of photosynthesis (Long and Humphries, 1994). For example, under nutrient deficiency the levels of enzymes in the reductive pentose phosphate cycle may decrease (Long, 1985) and low temperatures decrease the activity of these enzymes. As a result, the electron sink capacity of the Calvin cycle is lessened and, when light is high, the co-ordination of electron flow and CO₂ assimilation is perturbed. Under these conditions, excitation energy from chlorophyll can lead to oxyradical production, increasing the risk of photoinhibitory damage (Smirnoff, 1993). Leaves afford an on-line array of biochemical and behavioral means to avoid the risk in the short term. First, the excess excitation energy can be harmlessly dissipated as heat directly in the pigment bed through the activation of the xanthophyll cycle (Demmig-Adams et al., 1996). Alternative electron sinks are also available, like the water–water cycle (Asada, 2000) and the C₂ photorespiratory cycle (Tolbert, 1997) which can act as electron valves whenever the sink capacity of the Calvin cycle is surpassed. In addition, light avoiding chloroplast (Williams et al., 2003) or leaf movements

(paraheliotropism; Pastenes et al., 2005) may work towards this goal. Under prolonged stress, acclimation may occur through enhancement of the cycles by elevating pool sizes of their components and enzymes (Hirotsu et al., 2004; Horton and Ruban, 2005; Tourneux and Peltier, 1995) while additional biochemical and/or behavioral avoiding reactions may come into play. Among these, adaptive decreases in chlorophyll content (Kyparissis et al., 1995) and leaf rolling (Kyparissis and Manetas, 1993) may be mentioned. Yet, some plants may not afford all extra preventive measures or may not afford them to an appropriate extent. As a result, the severity of stress may surpass their acclimative capabilities. Here comes the most popular (among physiologists) role of anthocyanins acting as sunscreens, attenuating the visible radiation penetrating into mesophyll and thus, reducing the excitation pressure (Gould et al., 2002b; Steyn et al., 2002). We may note at this point that such a photoprotective role can be fulfilled in two ways: either by simply screening visible radiation or/and by quenching oxy-radicals through the powerful anti-oxidative capacity of anthocyanins (Wang et al., 1997). Detailed evidence in favor of and against this hypothesis will be presented later. In the meantime, it can be said that the above arguments may also be valid for young and senescing leaves. In young leaves, the ability to absorb photons and evolve oxygen is developed before the full sufficiency of the CO₂ reduction system (Ireland et al., 1985; Miranda et al., 1981; Šesták et al., 1985) and this may explain their vulnerability to photoinhibition, in spite of the higher pools in the xanthophyll cycle components (Barker et al., 1997; Krause et al., 1995). A senescing leaf may encounter similar problems. Senescence is believed to be a programmed developmental stage where leaf metabolism is changed according to the needs of nutrient resorption (mainly nitrogen) towards persistent storage compartments like twigs and stems, facilitating rapid remobilization and use during the next growth season (Millard and Thomson, 1989). Thus, chlorophyll–protein complexes in the thylacoids of the senescing chloroplasts need to be dismantled before proteins of the light-harvesting antennae and reaction centers are decomposed, while at the same time enzymes of the Calvin cycle are also degraded (Matile et al., 1992). Interestingly, research on chlorophyll catabolism during senescence revealed that chlorophyll is in fact not so much degraded, but mainly “detoxified” into colorless catabolites stored in the vacuoles (Matile et al., 1999). Apparently, the removal of chlorophyll from its safe position in the chlorophyll–protein complexes, may render it photodynamically active, and this could be particularly damaging in the absence of photochemical and non-photochemical energy quenching. Hence a speed race to transform chlorophyll into colorless compounds is unfolded.

Under such conditions an anthocyanic umbrella would be welcome.

The photoprotective hypothesis is not new. Von Mohl (cited by Haberlandt, 1914) “drew attention to the frequent red coloration of young shoots and seedlings, the nascent chlorophyll of which is particularly liable to destruction by light”, thus relating anthocyanin presence with a kind of photoprotection. In addition, Kny (also cited by Haberlandt) performed experiments showing that chlorophyll bleaching by light in alcoholic solution is prevented by shading with an anthocyanic solution. The same authors, however, were skeptic, as Stahl (cited by Haberlandt) pointed out that a photoprotective function could not be applied to the red leaves of many understorey plants, especially when anthocyanins are located in the lower epidermis. As we shall discuss later, the photoprotective function is still open, more that a century after these initial thoughts, while more recently formulated ecologically oriented hypotheses are still at their infancy.

Although the physiological background of the sunscreen hypothesis seems convincing, experimental evidence is contradictory. In some cases, a correlation between presence of anthocyanins and tolerance to photoinhibition was found. Krol et al. (1995) reported that low-temperature acclimated pine seedlings accumulated anthocyanins and were less susceptible to photoinhibition. However, they also attained higher photosynthetic capacities compared to controls and, accordingly, the protection offered by the anthocyanin screen was confounded by an increased sink capacity of the reductive pentose phosphate cycle. Concerning senescing leaves of red-osier dogwood, photodamage of PSII by red light (which by-passes anthocyanins) was more severe in yellow compared to red leaves, yet red leaves were sampled from the canopy, whereas yellows from the shaded canopy interior (Feild et al., 2001). Pietrini et al. (2002) used anthocyanin-rich and anthocyanin-deficient corn genotypes with comparable CO₂ assimilation rates and light-adapted PSII photochemical efficiencies to show that the red genotype was more tolerant to photoinhibitory damage after high light treatment. Similar results were obtained when tolerance to photoinhibition was compared between young red and mature green leaves of *Rosa* sp. and *Ricinus communis* (Manetas et al., 2002). Although these results seem more straight-forward, it should be mentioned that the photoinhibitory treatments applied by Pietrini et al. (2002) and Manetas et al. (2002) were rather severe compared to the previous growth conditions of the plants. Finally, a more direct approach was that of Burger and Edwards (1996) using variegated leaves of *Coleus blumei* possessing patchy epidermises with red and colorless areas overlying an equally green mesophyll. In this case, no evidence of a higher tolerance to photoinhibition in the red patches could be shown.

All the above-cited investigations were performed in controlled environments, either during plant growth and/or photoinhibitory application treatments. Yet, the final criterion for the sunscreen hypothesis would be whether anthocyanin accumulation affords any protection in the field. Based on the in-door investigations just described, it is not surprising that field trials were equally contradictory. In an extensive study with four *Syzygium* species (two of them bearing green and two red young leaves), conducted in a naturally lit glass house, the “red” species had lower chlorophyll levels, lower photosynthetic capacities (measured as O₂ evolution at saturated CO₂) and suffered from greater chronic photoinhibition, assessed from the maximum PSII photochemical efficiency at pre-dawn (Dodd et al., 1998; Woodall et al., 1998). The authors concluded that an assessment for the role of anthocyanins in preventing photoinhibition could not be reached, due to the inherent differences in photosynthetic capacities between red and green species. Moreover, Lee et al. (2003) in an extensive survey with senescing leaves from 89 woody species found no evidence of photoprotection by anthocyanins, based on pre-dawn measurements of PSII photochemical efficiency.

The problem of species-specific differences was circumvented by taking advantage of the intraspecific variation in young leaf color displayed by *Quercus coccifera*. In this plant, one can distinguish individuals occupying the same habitat and having fully exposed red or green young leaves, while mature leaves are invariably green. Here, it was found that the dark-adapted PSII efficiency measured at pre-dawn was slightly (i.e. 5%) higher in the red morphs (Manetas et al., 2003). Both morphs had the same chlorophyll content. The authors concluded that anthocyanins may have a slight photoprotective role in this plant. A final difficulty in ascribing a light screen function for anthocyanins concerns the frequent finding of red leaves in many understorey plants. This puzzled Stahl (cited by Haberlandt, 1914) and made the early proponents of the photoprotective role very skeptic. However, shade leaves face the opposing needs of maximizing photon absorption and utilization on one hand, and reducing the potential risk of photoinhibition during sunflecks, on the other. In addition, the rates and magnitudes of change in light intensity in the understorey compared to canopy leaves are greater and, accordingly, the photoprotective demands more important (Chazdon, 1988). From this point of view, the spatial distribution of anthocyanins in the canopy tree *Quintinia serrata* are compatible with a photoprotective function especially targeted to shade leaves receiving sunflecks. Thus, an inverse correlation between annual irradiance and leaf anthocyanin content was established, that was only broken in the permanently shaded leaves, whose anthocyanins were as low as in the fully exposed

leaves (Gould et al., 2000). Although the sunscreen hypothesis seems to be strengthened by these results, one may argue that sunfleck receiving leaves need a more flexible photoprotective device (like a reinforced xanthophyll-cycle, Logan et al., 1997; Thiele et al., 1998) than a permanent red umbrella.

The optical properties of leaf anthocyanins may not be ideal for a sunscreen role

Ideally, the absorption spectrum of a sunscreen against a photodynamic action should match that of the potential photosensitizer. For example, phenolics important for protection against UV-B (280–320 nm) radiation damage of DNA and other crucial biomolecules absorb strongly within this spectral band (Caldwell et al., 2003). Yet, leaf anthocyanins being almost always red (Harborne, 1976) absorb maximally in the green band (at ca. 520–540 nm) i.e. at the spectral region where the probability of photon capture by chlorophylls is a minimum. Although some absorption occurs in the blue, the absorption coefficient is roughly 3-fold lower compared to the green maximum. Accordingly, the optical properties of anthocyanins may not be optimally designed for such a function, unless green light is photosynthetically harmful. There is no evidence to support such an idea. On the contrary, green light seems to be photosynthetically important deep within the leaf. Due to the sieve effect and the elongation of the light path within the non-homogeneous leaf structure (Fukshansky, 1981), green light is finally absorbed and used for CO₂ assimilation at deeper chloroplast layers (Sun et al., 1998). In fact, the green window in the chlorophyll absorption spectrum allows a leaf to be thick and differentially use the various spectral bands at the various cell layers (Nishio, 2000). Thus, at low photon flux, blue, red and, to a lesser extent, green light are absorbed efficiently at the first chloroplast layers below the epidermis and used with high photochemical efficiency without appreciable development of non-photochemical quenching (NPQ). Under high light, however, excess blue and red photons absorbed in these superficial chloroplast layers lead to the development of photoprotective NPQ, reducing the photochemical efficiency in the upper part of the leaf. Yet, penetrating green light can be used within the next layers with high efficiency, since self-shading by the overlying chloroplasts makes the engagement of NPQ needless (Sun et al., 1998). For example, the contribution of red and blue light to CO₂ fixation in a relatively thick (750 μm) spinach leaf is greater compared to green at the first 150 μm of depth. The situation is reversed after ca. 250 μm and up to the lower epidermis (Sun et al., 1998).

Therefore, what is the photosynthetic importance of closing the green window by anthocyanins? Apparently, there would be only a slight effect for photosynthesis or photoprotection in the upper part of the leaf, since mainly the blue and red bands are absorbed by chlorophyll in this part. One may argue that the blue anthocyanin absorbance, although low, could contribute to photoprotection. However, Karabourniotis et al. (1999), using microscopic light guides inserted in the leaves, found no differences in blue light penetration to the mesophyll when young green and red leaves of *Vitis vinifera* (having anthocyanins in the epidermis) were compared. In addition, Gould et al. (2002c) used chlorophyll fluorescence in transverse sections of green and red leaves of *Quintinia serrata* in order to assess penetration of blue, green and red monochromatic light. The effects of the anthocyanic screen on the penetration of blue and red light were negligible. Yet, green light, which in the green leaves penetrated deeply, was strongly absorbed in the uppermost palisade mesophyll of red leaves. Accordingly, the shade afforded by anthocyanins to the leaf mesophyll concerns deep cell layers which are already shaded by overlying chloroplasts and may not need photoprotection. However, their presence should negatively affect photosynthesis driven by green light in these deep layers. Indeed, the light-saturated photosynthetic rates were lower in red than in green leaves in some species (Burger and Edwards, 1996; Choinski and Johnson, 1993; Woodall et al., 1998). However, negligible differences have been reported for other species (Karageorgou and Manetas, 2006; Pietrini and Massacci, 1998) and even higher rates in red leaves of two understorey tropical species were observed (Gould et al., 1995). The reasons for these discrepancies are not known, yet one may mention that according to the results of Sun et al. (1998) the contribution of green light to photosynthesis should depend on leaf thickness and/or chlorophyll cross-section, being less in thin, low chlorophyll leaves. Hence, the attenuation of green light by anthocyanins in such leaves may not be photosynthetically important. Another variable could be the actual light attenuation by the anthocyanic screen. Although relative levels of extracted anthocyanins are given in most of the investigations, the actual light transmittance of an intact anthocyanic epidermis has not yet been reported. It is worth noting in this context that the so-called “package effect”, increases the probability of light absorption when a pigment is distributed in many small cell compartments like chlorophyll in chloroplasts (Niklas, 1997). Anthocyanins, however, are localized in vacuoles and the package effect is minimal. Finally, investigations correlating the levels of anthocyanins with the extent of photoinhibition of photosynthesis are based on the tacit assumption that the presence of anthocyanins is the only physiological difference between the compared leaves.

As we shall discuss later, this assumption may not be reasonable.

In conclusion, the contradictory experimental results and the non-ideal optical properties of anthocyanins do not allow a firm support for a direct photoprotective function. Leaf age and thickness, inherent ability to develop and use other photoprotective measures in various leaf depths and growth environment may be factors determining the photosynthetic importance of an anthocyanic screen.

Anthocyanins may not be an effective UV-B screen

Leaf anthocyanin levels often increase in response to UV-B radiation in laboratory trials (Alexieva et al., 2001; Brandt et al., 1995; Lindoo and Caldwell, 1978) and their presence can alleviate DNA damage (Kootstra, 1994; Li et al., 1993). Under field conditions, with balanced UV-A/visible radiation, mildly enhanced UV-B radiation may (Mendez et al., 1999) or may not (Dillenburg et al., 1995) result in increased anthocyanin levels. It has also been reported that red *Coleus* varieties are less damaged by both UV-B and UV-C radiation, when compared to green varieties (Burger and Edwards, 1996). Therefore, anthocyanins have been implicated in UV-B protection (Lee and Lowry, 1980).

Anthocyanins can protect against UV-B by attenuating UV-B radiation or through their oxy-radical scavenging capacity (Stintzing and Carle, 2004). However, in most of the above-mentioned studies, flavonoids and other phenolics, which act as both UV-B screens and antioxidants, were also induced. Accordingly, a causal relationship between anthocyanin increase and UV-B protection could not be easily established. In addition, a closer look at the anthocyanin spectral absorbing properties and their contribution to total leaf UV-B absorbing capacity renders their putative protection against UV-B radiation questionable.

Molar absorption coefficients for anthocyanins in the UV-B (280–320 nm) are ca. 3–5 times lower compared to corresponding values at their visible maxima (Giusti et al., 1999; Harborne, 1976). Glycosylation does not appreciably alter the spectral absorbance profile. However, acylation with phenolic acids increases UV-B absorptivity due to the superimposed absorbance of the phenolic ring, resulting in roughly equivalent absorption in both the visible and UV-B bands (Giusti et al., 1999; Harborne, 1976). This could lend support to an anthocyanin-based screening function against UV-B radiation, provided that the contribution of anthocyanins to the total UV-B absorbing capacity of a leaf is fairly sufficient. Yet, although molar absorptivities of acylated anthocyanins and the rest of simpler phenolics

and flavonoids in the UV-B band are comparable, the concentrations of anthocyanins are always a small fraction (ca. 1–1.5%) of the total phenolic pool (Grace et al., 1998; Jaakola et al., 2004; Woodall and Stewart, 1998). Accordingly, the contribution of anthocyanins to the total leaf UV-B absorbing capacity, especially under field conditions is negligible (Mendez et al., 1999; Woodall and Stewart, 1998). We may also add at this point that the UV-B protective hypothesis is further weakened for those forest understorey plants possessing anthocyanins in their lower leaf surface.

Anthocyanins as antioxidants

Apart from the direct, light screening function, anthocyanins may indirectly protect against excess light through their oxy-radical scavenging properties (Gould et al., 2002a; Steyn et al., 2002). Indeed, anthocyanins are powerful antioxidants *in vitro* and it may not be irrelevant that the strongest oxy-radical absorbing capacity is displayed by the red cyanidin-3-glycoside (Wang et al., 1997), i.e. the anthocyanin more often encountered in leaves (Harborne, 1976). However, direct experimental evidence for the *in vivo* engagement of anthocyanins in anti-oxidative defense of red leaves is lacking and indirect indications are sparse. Neil et al. (2002a) reported that leaf extracts from red morphs of *Elatostema rugosum* had higher anti-oxidant capacity compared to green morphs and anthocyanins contributed to that capacity more than the other low molecular weight antioxidants. This was not the case in *Quintinia serrata*, where both red and green leaf extracts displayed the same anti-oxidative capacities (Neil et al., 2002b). Gould et al. (2002a) applied mechanical wounding in paradermal sections of *Pseudowintera colorata* leaves containing red and green patches due to accumulation of anthocyanins in some palisade cells. Red cells were able to scavenge the produced H_2O_2 much more effectively compared to green cells. However, it cannot be excluded that differences in other anti-oxidative compounds between the two kinds of cells are more critical than anthocyanins in scavenging H_2O_2 .

A critical point to be considered in conjunction to an anti-oxidative hypothesis for anthocyanins concerns their localization. One could argue that effective *in vivo* anti-oxidants should reside as close as possible to the source of oxy-radical production. In leaves, the main source is the illuminated chloroplast in the mesophyll cells (Asada, 2000), while anthocyanins may be located in the upper and/or lower epidermis, in the palisade and/or spongy mesophyll or in any combination of those leaf cell layers (Lee and Collins, 2001). Moreover, their intracellular location is the vacuole, although colorless tautomers appear in the cytoplasm during the transit

time between their biosynthesis and transport to the vacuole (Hrazdina et al., 1978). To the best of my knowledge, there is no report for the presence of anthocyanins in chloroplasts. On the other hand, the superoxide radical produced in the Mehler reaction can not cross the tonoplast and, accordingly, it has to be detoxified either in the chloroplast or the cytoplasm (Takahashi and Asada, 1983). H_2O_2 can penetrate the tonoplast (Yamasaki et al., 1997) and may react with anthocyanins (Yamasaki and Grace, 1998; Yamasaki et al., 1996), provided that it could escape detoxification by other mechanisms or compounds in the chloroplast or the cytoplasm, including cytoplasmic colorless anthocyanins (Neil and Gould, 2003). Flavonoids and simple phenolics are also potential oxy-radical scavengers. Although slightly less effective than anthocyanins (Bors et al., 1994), their concentrations in leaves are at least an order of magnitude higher (Grace et al., 1998; Jaakola et al., 2004; Woodall and Stewart, 1998). They reside in the central vacuole (Hutzler et al., 1998), yet their presence in chloroplasts has also been documented (Saunders and McClure, 1976). Moreover, Takahama (1982) reported that the flavonols kaempferol and quercetin inhibited photobleaching of carotenoids in a concentration-dependent manner, while the extent of carotenoid loss was positively correlated to flavonoid oxidation. It comes from the above that it is difficult to ascribe the possibly higher anti-oxidative capacities of red leaves to anthocyanins alone. In fact, it is reasonable to assume that compounds with analogous oxy-radical scavenging properties, higher levels and more appropriate intracellular localization could serve this function. In addition, anthocyanins comprise the final steps of a biosynthetic route giving also flavonoids and simple phenolics (Saito and Yamasaki, 2002). Hence, high levels of all these constituents may co-occur as a result of activation of the pathway at an early stage (Close et al., 2001; Dominy and Lucas, 2004; Jaakola et al., 2004; Karageorgou and Manetas, 2006; Lee and Lowry, 1980; Neil et al., 2002a), although in some cases anthocyanins may accumulate preferentially, in the absence of measurable increases in the other constituents of the phenylpropanoid/flavonoid pathway (Gould et al., 2000).

An activation of the phenylpropanoid pathway leading to increased synthesis of phenolics and flavonoids is a common response to environmental stress (Dixon and Paiva, 1995). In fact, all environmental factors correlated with the accumulation of anthocyanins also correlate with increases in phenolics and the production of free radicals. Accordingly, a novel oxy-radical scavenging function for phenolics has been proposed, either complementary to the more traditional “anti-herbivore” role (Grace and Logan, 2000), or in partial opposition to that role (Close and McArthur, 2002). Grace and Logan (2000) went further to suggest

that phenylpropanoid biosynthesis may be an alternative sink for excess photochemical excitation energy under stress. The proposed scenario alleges that an excess of reducing power in the chloroplast could be consumed in the shikimate pathway producing phenylalanine, the precursor of phenolics. Phenylalanine could migrate to the cytoplasm and be directed to the phenylpropanoid/flavonoid pathway, consuming even more reducing power. We shall come back later on these biochemical competition models, discussing some more ecologically oriented hypotheses for anthocyanin accumulation and leaf redness. However, we may conclude at this point that the ascribed anti-oxidant and UV-B screen function for anthocyanins are difficult to be appreciated since they are obscured by the presence of phenolics and flavonoids which

- (a) co-accumulate with anthocyanins, yet at much higher levels,
- (b) have similar oxy-radical scavenging and UV-B absorbing capacities,
- (c) may be more optimally located within cells/tissues to fulfill these roles.

Anthocyanins may not substantially contribute to osmoregulation

Anthocyanin accumulation has been shown in many cases to be induced by environmental conditions which result, directly or indirectly, to water stress. Thus, drought, heat, cold and salinity have been correlated with high anthocyanin levels (see Chalker-Scott, 1999, 2002 and the literature there-in). This phenomenological co-occurrence led Chalker-Scott to propose that anthocyanin accumulation could reduce osmotic potential and allow leaves (or other organs) to maintain a low water potential and the ability to withstand or tolerate sub-optimal water levels. Moreover, the hypothesis is strengthened by the vacuolar localization of anthocyanins, their transient appearance as long as water stress is imposed in mature leaves and their frequent occurrence in juvenile leaves which need optimal turgor pressures for normal cell growth. Yet, in order to fulfill such a role, intracellular anthocyanin levels should be high enough comprising a substantial percentage of the total sap osmotic concentration. A well-watered mesophyte may have an osmotic concentration of 300 mmol kg^{-1} , while a non-stressed xerophyte can approach twice that value. After water stress and the accompanying osmoregulation, osmotic concentration can be 2–4 times higher, leaving an osmotic difference of $300\text{--}1800 \text{ mmol kg}^{-1}$ to be filled. Anthocyanins, however, even in bright red leaves, attain a concentration of $1\text{--}4 \text{ mmol kg}^{-1}$, which is very low to support an

osmoregulatory function (data compiled from Gould et al., 2000; Jaakola et al., 2004; Manetas et al., 2002; Mendez et al., 1999; Pietrini et al., 2002; Woodall and Stewart, 1998, assuming a 90% water content of leaves and cyanidin glycoside (molecular mass = 450) as the main leaf anthocyanin). Therefore, anthocyanins could have an osmotic role only when restricted into an organelle occupying a small fraction of the cell volume. This is not so, since anthocyanins are found in the vacuole. Conversely, total soluble leaf phenolics usually have concentrations one to two orders of magnitude higher, they are accumulated in vacuoles and, therefore, they could facilitate osmotic adjustment. In addition, phenolics stem from the same biosynthetic pathway and are induced by the same array of environmental stresses as anthocyanins. Thus, it is more plausible for soluble simple phenolics and flavonoids to have an osmotic role. To the best of my knowledge, such a role has not been proposed up to now.

Leaves in disguise: anthocyanins, leaf apparency and herbivory

Recently, the late W.D. Hamilton (Hamilton and Brown, 2001) hypothesized that bright colors in old senescing leaves may afford an optical warning signal, denoting a defensive commitment against insect consumers (handicap signal). At the same time, Lev-Yadun (2001) proposed a similar association for brightly colored thorns of some succulent plants. It seems that the idea originated from corresponding observations in animals (some observations date back to Charles Darwin and Alfred-Russel Wallace), where a bright coloration is correlated with unpalatability (see Lev-Yadun et al., 2002; Rowe and Guilford, 2000). The general arguments used by Hamilton and Brown (2001) were that autumn coloration is expensive and insects are damaging, together with observations that autumn colonizing aphids prefer yellow more than red senescing leaves of *Acer palmatum* (Furuta, 1986, 1990). The hypothesis triggered a series of opinion papers (Dominy et al., 2002; Holopainen and Peltonen, 2002; Wilkinson et al., 2002) either opposing or supporting the signal hypothesis.

Although the empirical evidence used by Hamilton and Brown (2001) to support the handicap hypothesis came from the special case of anthocyanic leaves, the general idea conveyed was that brightly colored trees (either red or yellow) are armed with a high defensive potential. For the latter case, the hypothesis was recently tested (and probably verified) for *Betula pubescens*, where leaf fluctuating asymmetry (an index of physiological stress) and insect damage in the following season was higher in dull green compared to

bright yellow individuals (Hagen et al., 2003). Moreover, a recent report by Numata et al. (2004) indicated a preferential damage by insects in species of the genus *Shorea* possessing young green leaves compared to species possessing young red leaves. Similar results were obtained by Karageorgou and Manetas (2006) with the Mediterranean tree *Quercus coccifera*, in which intraspecies differences in the expression of the anthocyanic character are found in young leaves of individuals occupying the same habitat. Although the above seem to support the “signal” status of leaf color in relation to herbivory, the underlying mechanisms may be different for bright red or yellow leaves.

In order to be effective against herbivory, an optical signal has to be perceivable by the consumers. Insect herbivores are interested in leaves either for direct consumption or for future use by their offspring (oviposition). Behavioral tests with artificial leaves have shown that folivorous insects from various orders, when searching for food or oviposition, are attracted by optical stimuli looking green or, more often, yellow to the human eye (Prokopy and Owens, 1983). Field trials similarly indicated a preference for young, yellowish leaves which thus suffer from higher herbivory pressures compared to mature dark-green leaves (Kelber et al., 2003). Although additional parameters, like the higher N/C ratios or less toughness of young leaves could have shaped this behavior, there is no doubt that folivorous insects should “see” and locate green/yellow objects. The preferences for specific optical stimuli are based on opponent interactions of specific photoreceptors. Most folivorous insects studied up to now seem to have three receptor types with sensitivity maxima at ca. 350, 440 and 540 nm (Kelber, 2001; Kelber et al., 2003). Accordingly, their maximum discriminating ability for reflected radiation lies mainly in the UV-A, blue and green spectral bands, with decreasing sensitivities at the two margins, up to 300 and 620 nm, respectively (Kelber et al., 2003). Accordingly, most insects may not see human red. Some butterflies possess a fourth receptor with peak sensitivity at 610 nm, extending the threshold of their effective color vision into red (Matic, 1983). It has been shown, however, that the stimulation of this red photoreceptor enhances an avoiding behavior against red objects in favor of greens for oviposition (Kelber, 1999).

A mature chlorophyllous leaf reflects in the green, while reflectance of a young or senescing leaf is shifted a bit to the yellow spectral band. Blue and red are both strongly absorbed by the photosynthetic pigments. Hence, it is not surprising that the selection of chlorophyll as the major photosynthetic pigment led the co-evolutionary race towards the selection of green and/or yellow sensitive photoreceptors in those organisms using leaves as major food. Yet, the superimposi-

tion of red (i.e. green absorbing) anthocyanins over chlorophyll closes the green window and levels off leaf reflectance along the whole range of folivorous insect’s effective visual range. Therefore, we may argue that a red leaf may not be easily distinguished by folivorous insects since it does not afford a useful optical cue. Redness cannot be considered a signal *sensu stricto*. Thus, although the handicap signal hypothesis seems to be strengthened by the observed low herbivory pressures on red leaves (Karageorgou and Manetas, 2006; Numata et al., 2004), the underlying insect physiology is more compatible with a function related to the masking of a perceivable spectral band. Considering the case of understory plants, red leaves could afford a real camouflage against the soil background, as proposed by Stone (1979), who further argued that red leaves would look more like dead leaves to the insect eye. However, one cannot exclude the possibility of the existence of red photoreceptors in insect species not examined up to now.

If red was a perceivable signal against herbivory (i.e. for those organisms “seeing” red), it could be either an honest or a seductive signal. In the first case, red leaves should display high investment in mechanical and/or chemical defenses. Concerning mechanical defense, toughness seems to defend from herbivory (Coley, 1983). A tough leaf needs extra energy and time to be exploited by a small consumer. In addition, leaf sclerification is brought about by the accumulation of indigestible structural material, which has a low nutritive value. However, there is no evidence that red leaves are tougher. On the contrary, young leaves which are often red are considerably softer than green mature leaves. A young leaf cannot be prematurely sclerified since this would restrict its growth. However, the situation may be different with chemical defense, especially that based on phenolics. Phenolics are synthesized through the phenylpropanoid biosynthetic pathway, some products of which feed the flavonoid pathway, with anthocyanins as end products (Winkel-Shirley, 2002). The regulation of the pathway is complex (Winkel-Shirley, 2002), but, from the point of view that anthocyanins are an honest signal against herbivory, the question is whether the accumulation of colored anthocyanins is accompanied by accumulation of other colorless phenolic classes as well. High levels of all constituents in the same organs have been reported in some studies (Close et al., 2001; Dominy and Lucas, 2004; Jaakola et al., 2004; Karageorgou and Manetas, 2006; Lee and Lowry, 1980). Hence, red color may be an honest signal of a high defensive commitment, provided that phenolic compounds are indeed defensive. In some other cases, however, high levels of anthocyanins are not accompanied by high levels of phenolics (Gould et al., 2000). In this case, a kind of a Batesian mimicry can be inferred.

In the ecological literature, phenolics are considered as deterrents against generalist consumers. Since they are not toxic, their antiherbivore action is related to their bitter taste and their capacity to precipitate protein in the digestive tract (Bennett and Wallsgrave, 1994; Feeny, 1970). Accordingly, they should decrease both palatability and digestibility in a concentration-dependent manner. In fact, the major theories of plant defense (see next section) were based to a large extent on the fluctuations of phenolics according to age, tissue type, differentiation stage, period of the year, habitat and stress. We may note, however, that not all phenolics (and, certainly, not anthocyanins) are bitter or astringent. It seems that tannins and possibly some simple phenolic acids do have negative effects on consumer's growth and health if taken at high doses (Bernays and Chapman, 2000; Chung-McCoubrey et al., 1997; Foley and Hume, 1987; Kause et al., 1999; Lempa et al., 2000). On the contrary, there is a vast recent literature (and public concern) on the health-promoting effects of dietary flavonoids, anthocyanins and some simple phenolics on animals (Stintzing and Carle, 2004). Thus, although leaf redness is often associated with high levels of phenolics, the health-promoting effects of some phenolic classes (including anthocyanins) may compensate for the growth inhibitory action of tannins. In order to discriminate between a potentially deterrent versus an attractive influence, the partial concentrations of individual compounds and their effects on insect physiology should be known.

The low rates of insect damage in the red leaves can also be explained by the alternative hypothesis recently coined by Lev-Yadun et al. (2004). According to this, a red color could undermine the insect camouflage. Most folivorous insects are greenish and, being on a green leaf, they probably escape predator attention. On a red leaf, however, they become more conspicuous, to the benefit of the plant. As the authors point out, this is a case of "the enemy of my enemy is my friend" and they consider this kind of plant defense as the visual parallel of the volatile chemical signals emitted by plants to attract predators (Schoonhoven et al., 1998). Hence, according to this hypothesis, red color is not a plant camouflage, but a plant signal addressed to insect predators. An experimental verification of this interesting hypothesis is still lacking.

In conclusion, there are interesting and inventive hypotheses ascribing an optical role in leaf anthocyanins against insect herbivory. Either as a warning signal addressed to consumers, an attractive signal addressed to consumer predators or a camouflage/masking device, anthocyanins may confine overconsumption. This could be particularly important for young, soft, developing leaves which are more vulnerable to herbivore attack. Accordingly, they should be heavily defended (McKey, 1979). Indeed, young leaves contain very high levels of

potentially harmful phenolics (Kause et al., 1999; Kouki and Manetas, 2002; Ossipov et al., 1997). The effectiveness of this chemical armament could be enhanced by an anthocyanic optical cue. Although confirming field evidence is still scarce (Furuta, 1986, 1990; Karageorgou and Manetas, 2006; Numata et al., 2004), it seems to be an attractive hypothesis.

Yet, the hypothesis cannot be extended to vertebrate herbivores. Many primates, for example, possess the ability to perceive red color (Bowmaker, 1998; Robinson, 1994). It has been reported that some folivorous primates do prefer young red leaves and this has led to the suggestion that their characteristic trichromatic vision evolved for the detection of such leaves as well (Dominy, 2002; Lucas et al., 1998).

Anthocyanins and the theories of plant defense

Plants have the ability to synthesize a vast array of organic compounds, mainly belonging to phenolics, terpenoids and alkaloids, which do not participate in primary metabolism. Up to the middle of the last century, these compounds were considered as useless metabolic products which, if not escaped to the atmosphere as volatiles or leaked to the soil by rain, were stored in the vacuole. However, Fraenkel, (1959) proposed that the so-called secondary compounds, may have primary roles in shaping the ecological plant–animal inter-relationships and, especially, the plant's defensive capability against herbivory. Since that time, evidence in favor of the idea has accumulated and it became generally accepted that these compounds (now mostly called natural products or, in a rather descriptive way, allelochemicals) do play decisive ecological roles. I shall briefly report on the hypotheses proposed to explain the variations observed in the levels of defensive compounds, not only because anthocyanins are natural products of phenolic origin, but also because their variations seem to parallel those of other phenolics in many cases. Accordingly, the hypotheses invented for defensive compounds may have heuristic value for anthocyanins as well.

The Optimal Defense theory (Feeny, 1976; McKey, 1979; Rhoades and Gates, 1976) is generally based on the economics of plant growth and development. It states that the most vulnerable and/or valuable plant parts should be heavily defended. Although vulnerability and value are rather abstract, not easily quantifiable parameters, one may predict that the more apparent plants or the more apparent plant parts may be more vulnerable to herbivores. For example, a fruit is valuable in terms of reproductive success and should not be eaten before maturation. If we consider the case of leaves, a developing leaf is vulnerable since it can not be

prematurely sclerified (Choong, 1996; Coley, 1983). In addition, it is valuable due to its future photosynthetic potential and the need to pay back its cost of production (Harper, 1989). Hence, young leaves contain high amounts of phenolics. Interestingly, many young leaves are red too. All kinds of phenolics (including anthocyanins) decrease with leaf maturation (Choinski et al., 2003; Kouki and Manetas, 2002; Ossipov et al., 1997) denoting a kind of replacement of chemical (or optical in the case of anthocyanins) defensive means with mechanical toughness. We may further speculate with the principle of replacement of defensive means in the case of anthocyanins. If a young leaf is adequately defended through its red color, then high levels of astringent phenolics may not be needed. In fact, the replacement of simple phenolics by anthocyanins may be a cost-effective strategy, since when both groups of phenolics are present in the same tissue, the levels of simple phenolics are usually at least an order of magnitude higher than anthocyanins.

The Growth–Differentiation Balance hypothesis (Herms and Mattson, 1992) also encompasses the Resource Availability Balance hypothesis (Coley et al., 1985) and the Carbon:Nutrient Balance hypothesis (Bryant et al., 1983). It was very successful in predicting environmentally induced variations in phenolic levels. In this hypothesis, “growth” refers to processes requiring cell division and/or elongation, while “differentiation” refers to processes that enhance the structure or function (maturation, specialization) of cells and tissues. A trade-off between growth and defense is also assumed with growth predominating over defense. Haukioja et al. (1998) and Jones and Hartley (1999) proposed a biochemical variant of the hypothesis stating that both phenylpropanoids (needed for defense/differentiation) and proteins (needed for growth) compete for a common precursor, i.e. phenylalanine. Accordingly, when growth is fast (either in inherently fast growing species or when there are ample environmental resources), there is no surplus carbon available for phenolic biosynthesis. When growth is slow, carbon can be diverted to phenolic biosynthesis. Similarly, when water, nutrients or temperature are limiting, growth is suppressed more than carbon acquisition through photosynthesis and the surplus carbon is allocated to phenolic accumulation. The predictions of the hypothesis have been verified in many cases (see Herms and Mattson, 1992; Stamp, 2003 and the literature therein). Interestingly, limiting nutrients, water stress and low temperatures induce the accumulation of anthocyanins as well. We may therefore conclude that the current plant defense hypotheses afford a useful base to predict leaf anthocyanin variations at various levels (according to tissue type and age, seasonal, intra- and inter-species). It remains to be seen whether the similarities in fluctuations of simple phenolics and anthocyanins are

merely based on their common biosynthetic pathway or if similar selection pressures have been exerted for chemical and optical defensive devices.

Why, then, are leaf anthocyanins (almost) always red?

Among the various hypotheses seeking an adaptive significance for leaf anthocyanins, only the camouflage/masking and the “undermined” camouflage hypotheses afford an answer which takes into account and is compatible with the spectral properties of major leaf anthocyanins. There is no need for an anti-oxidant or osmoregulator to be red, nor for a pigment protecting against UV-B radiation damage to also absorb in the visible band. Concerning the sunscreen hypothesis, a blue, yellow, red or neutrally absorbing compound should be much more effective in reducing excitation pressure on chlorophyll, than the green absorbing leaf anthocyanins. A green absorbing umbrella could only be addressed to deeply in the leaf-located chloroplasts, yet these are already self-shaded by overlying chlorophyll. Finally, the hypothesis of a warning signal against herbivory is not compatible with the fact that most folivorous insects examined up to now cannot perceive red light. On the contrary, folivorous insects land preferentially on green or yellow–green objects and possess appropriate photoreceptors in accordance to this behavior. Hence, leaf anthocyanins by closing the green reflectance window left by chlorophyll make the leaf less discernible to insect consumers (plant camouflage hypothesis). Alternatively (or in addition), the usually green folivorous insects, if found on a red leaf, are more easily recognized by their predators (undermining of insect camouflage by the plant).

Epilogue

Although some indirect evidence for antioxidant, anti-UV-B and osmoregulatory functions has been obtained, the conclusions are confounded by potentially similar functions of co-occurring compounds stemming from the same biosynthetic pathways and displaying much higher concentrations than anthocyanins in the corresponding leaves. A crucial point in these hypotheses is the intra- and inter-cellular location of anthocyanins in conjunction with the corresponding location of compounds having the same functions. Concerning the sunscreen function against photoinhibition by visible light, experimental evidence is contradictory, especially under field conditions. An additional problem is that the spectral properties of leaf anthocyanins are not ideal for such a function, unless their photoprotective services are

addressed to deeper chloroplast layers in thick leaves, where photosynthesis is mainly driven by green light. However, photoprotection in such depth may not be so important. We may therefore conclude that anthocyanin's contribution to these functions, if any, is probably weak and only complementary to other, more effective mechanisms. The neglected hypothesis of plant camouflage against herbivory and the recent opinion that leaf redness may undermine the green folivorous insect camouflage are theoretically more sound since they are compatible with folivorous insect vision physiology and also afford a reasonable explanation for the almost exclusive selection of red anthocyanins in leaves. Indeed, if the *raison d'être* of such compounds is to close the green window of leaf reflectance, only a red (i.e. green absorbing) anthocyanin could be selected. It should be admitted, however, that field observations and experiments supporting the camouflage hypotheses are scarce.

Seeking a suitable conclusion for this review, I can hardly find something better than that given by Haberlandt more than a century ago: "It must be admitted that, in spite of the numerous interesting detailed observations, the general physiological and ecological significance for the presence of anthocyanins in vegetative organs is still very obscure" (1914). Meanwhile, technical improvements, innovations, new observations and the accumulated knowledge added more hypotheses to the original "photoprotective" supposition, increasing obscurity.

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