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High contents of anthocyanins in young leaves are correlated with low pools of xanthophyll cycle components and low risk of photoinhibition

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Abstract

We checked the hypothesis that the transient presence of anthocyanins in young leaves serves a photoprotective function. For this purpose, *Rosa* sp. and *Ricinus communis* L., whose young leaves are red to become green upon maturation, were used. Thus, young leaves with high and mature leaves with low anthocyanin contents were analysed concerning their carotenoid (Car) composition and susceptibility to photoinhibition. Cars, including the components of the xanthophyll cycle, had similar contents in young and mature leaves, when expressed on a chlorophyll basis. Yet, when expressed on a leaf area basis or on the assumed photon absorptive capacity of leaves, Cars contents were considerably lower in anthocyanic young leaves. Although this may indicate a low photodissipative potential, red young leaves were considerably less susceptible to photoinhibitory damage. The results are compatible with a photoprotective function of anthocyanins, indicating also that their presence may compensate for a low capacity in the xanthophyll cycle-dependent harmless dissipation of excess excitation energy.

Additional key words: carotenoids; chlorophylls; fluorescence kinetics; leaf age; photosystem 2; *Ricinus communis*; *Rosa* sp.

Introduction

Photon energy drives photosynthesis, yet under some circumstances it may become inhibitory. Whenever the absorbed energy of photons exceeds that which can be used for CO_2 assimilation (*i.e.* under cold, drought, or mineral deficiencies), photo-oxidative conditions may be developed, unless the plant is capable of morphological and/or biochemical adjustments to avoid or dissipate the excess excitation energy (Smirnoff 1993, Long *et al.* 1994, Choudhury and Behera 2001). The xanthophyll cycle inter-conversions facilitate the harmless overflow of extra photons through the production of zeaxanthin and antheraxanthin. These compounds trap the surplus energy in the pigment bed of the light-harvesting complexes and transform it to heat before it reaches the reaction centres (Demmig-Adams *et al.* 1996). Accordingly, a plant with large pools of the xanthophyll cycle components is considered well equipped for adaptive short term down regulation of photosynthesis and may also withstand chronic photoinhibitory conditions (Long *et al.* 1994, Demmig-Adams *et al.* 1995).

An unbalance between photon absorption and utilisation may be inferred for young leaves, where the ability to absorb photons and evolve oxygen is developed before the full sufficiency of the CO_2 reduction system (Miranda *et al.* 1981, Ireland *et al.* 1985, Šesták *et al.* 1985). Indeed, the limited amount of data up to now has shown that young leaves are more prone to photoinhibition, although the pools of the xanthophyll cycle components are larger, compared to those of mature leaves (Krause *et al.* 1995, Barker *et al.* 1997).

The adverse effects of excess photons can be alleviated by the accumulation of non-photosynthetic, light screening pigments in the leaves. Anthocyanins, for example, are flavonoids with their absorbance maxima shifted within the visible part of the spectrum (Swain 1976). They occur in small amounts in almost every leaf. In rare cases their contents are so high that they mask the Chl greenness and give a red colour to the leaves. Red leaf anthocyanins have been correlated with resistance against biotic and abiotic agents such as fungi, herbivores, drought, cold, and radiation, both UV-B and visible (see reviews by Chalker-Scott 1999, Hoch *et al.* 2001, and the literature there-in). However, experimental tests for the hypothesis of a photoprotective role of antho-

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Abbreviations: Car, carotenoid; Chl, chlorophyll; PAR, photosynthetically active radiation; PS, photosystem; VAZ, violaxanthin + antheraxanthin + zeaxanthin.

cyanins in mature or senescent leaves gave conflicting results. Thus a correlation between tolerance to photoinhibition and anthocyanin accumulation was suggested in some studies (Krol *et al.* 1995, Mendez *et al.* 1999, Feild *et al.* 2001) but not in others (Burger and Edwards 1996, Dodd *et al.* 1998, Gould *et al.* 2000). In contrast, two studies performed with chlorophyllous and anthocyanic fruits presented evidence in favour of a photo-protective role (Smillie and Hetherington 1999, Merzlyak and Chivkunova 2000).

Much more common is the presence of red, young developing leaves that become green upon maturation (Harborne 1976). According to the Darwinian paradigm, the selection of this trait for transient investment in anthocyanin production in many species should indicate an adaptive significance for young leaves, but no benefit for mature leaves. Indeed, the attenuation of green and yellow radiation by anthocyanins (Neill and Gould 1999) may be protective under excess irradiance but does not allow the effective utilisation of the full visible solar spectrum by a mature chloroplast under low or moderate irradiance. However, in young, photoinhibition-sensitive leaves (Krause *et al.* 1995) the relative importance of photoprotection by anthocyanins may prevail. Such function has also been ascribed to the transient, highly reflective pubescence of young plane leaves (Bisba *et al.* 1997).

Based on the above, we sought experimental evidence for the photo-protective hypothesis by comparing the xanthophyll cycle-dependent photo-dissipative capacity and the sensitivity to photoinhibition in young (red) and mature (green) leaves of *Rosa* sp. and *Ricinus communis* L.

Materials and methods

Plant material and sampling: The experiments of this investigation were performed during the springs of 2000 and 2001 with *Rosa* sp. used as ornamental in the Patras University Campus and *Ricinus communis* L. growing wild in the vicinity. In both species new leaves burst during the spring (early March to late May) in a rather extended growth period. Therefore, by mid April one may find on the plants all leaf age classes from just bursting to maturity. Three individuals from each taxon were tagged and used throughout this study. On each sampling date, an equal number of young or mature leaves were harvested from each individual late in the afternoon, put in air-tight plastic bags containing moist filter paper, and left in the dark at room temperature all night, to be analysed the next morning. Care was taken to use leaves of comparable physiological age and irradiation history. Thus, criteria for leaf selection were full exposure and a similarity of their dimensions and Chl contents. Chl contents were assessed in the field non-destructively before harvest from the readings of a *Minolta SPAD-502* portable Chl meter. The credibility of this instrument in the measurements of Chl content of red leaves has been previously confirmed (Manetas *et al.* 1998). In general we used young leaves having attained 50 and 20 % of their final size (for *Rosa* sp. and *R. communis*, respectively) and 50 % of their mature Chl content.

Photosynthetic pigments: An appropriate leaf area (*ca.* 14.5 ± 2.3 and 19.7 ± 1.7 cm^2 for *Rosa* sp. and *R. communis*, respectively, *LI-3000* leaf area meter of *LI-COR*), taken from an equal number of young or mature leaves from each individual, was extracted. To facilitate extraction, the leaves were frozen in the mortar by adding a small volume of liquid nitrogen. Pigment extraction was performed in dim light by grinding the frozen samples in the mortar with 100 % acetone in the presence of a small amount of $CaCO_3$. The extract was centrifuged at 5 000×*g* for 10 min at 2 °C and the supernatant was further cleared by passing through a 0.45 μm filter. Chls were measured spectrophotometrically, using a *Shimadzu UV-160A* double beam spectrophotometer, and concentration was estimated according to the equations of Lichtenthaler and Wellburn (1983). Car separation was performed with a *Shimadzu LC-10 AD HPL* chromatograph, equipped with a non-endcapped *Zorbax ODS* (4.6×250 mm) column (*Rockland Technologies*, Chadds Ford, PA, USA) and calibrated against purified β-carotene (*Sigma Chemical*, St. Louis, MO, USA). Freshly prepared Cars were separated by thin-layer chromatography as described by Kyparissis *et al.* (1995). Development was performed isocratically at 16.7 $mm^3\ s^{-1}$ (20 min with acetonitrile : methanol, 85 : 15 v/v, and 20 min with methanol : ethyl acetate, 68 : 32 v/v), according to Thayer and Björkman (1990). Pigments were detected by measuring absorbance at 445 nm, using a *Shimadzu SPD-10A* UV-VIS detector. Peak areas were integrated by a *Shimadzu C-R6A Chromatopac.*

For anthocyanin determination, an aliquot of the acetone extract was acidified to 1 % HCl and absorbance was scanned from 400-700 nm. The peak anthocyanin absorbance was corrected for the contribution of chlorophyllous pigments at this wavelength (Mancinelli *et al.* 1975) and transformed to actual concentrations by using the mean molar absorption coefficient for anthocyanins according to Murray and Hackett (1991). The location of anthocyanins was examined in free-hand cross-sections of fresh leaves under a *Zeiss Axioplan* microscope.

Photoinhibitory treatment: Discs were cut from leaves kept in darkness overnight and Chl fluorescence transients were obtained with a commercial fluorimeter (*PEA*, Plant Efficiency Analyser, *Hansatech*, King's Lynn, UK). Chl was excited with red radiation of 1 500

μmol m^{-2} s^{-1} and the maximum photosystem 2 (PS2) photochemical efficiency (as F_v/F_m) was obtained. The discs were then placed on moistened filter paper in Petri dishes in a thermostated, double wall glass chamber (room temperature) and irradiated with a 400 W *Osram* quartz halogen bulb at 2 700 μmol m^{-2} s^{-1} PAR. In preliminary trials, the time required to obtain a sustained photoinhibitory reduction in F_v/F_m was established for each taxon. Thereafter, the discs were darkened for 25 min and F_v/F_m was measured again. Photosynthetically active radiation (PAR) at disc level was measured with a *Li-190* quantum sensor (*LI-Cor*, Lincoln, NE, USA). To avoid slight (±5 %) differences of PAR within the irradiation field, the dishes were rotated frequently.

Statistics: Significance of differences in the measured parameters between young and mature leaves was assessed through *ANOVA* tests by using the *SPSS 9.0* statistical package.

Results

Young red leaves of *Rosa* sp. and *R. communis* had *ca.* 50 % of the Chl content of green mature leaves while their anthocyanin contents were 27- and 21-fold higher, respectively (Table 1). Microscopical observations in leaf cross-sections revealed that anthocyanins were located in the epidermis of both the adaxial and abaxial surfaces (not shown). Concerning Cars, no significant differences were found between young (anthocyanic) and mature leaves, when their contents were expressed on a Chl basis (Fig. 1*A,C*). The only exception was lutein in *R. communis*, where young leaves had a *ca.* 40 % greater content. Since the expression of xanthophyll cycle-dependent photodissipative capacity on a Chl basis may be misleading (see Discussion), we present in Fig. 1*B,D*

Table 1. Chlorophyll (Chl) and anthocyanin contents [μmol m^{-2}] in young and mature leaves of *Rosa* sp. and *Ricinus communis*. Means ± SE from 12 (*Rosa* sp.) or 7 (*R. communis*) independent extractions. Peak absorbances for anthocyanins were at 528 nm for *Rosa* sp. and at 529 nm for *R. communis*.

	Rosa sp.		*R. communis*	
	young	mature	young	mature
Chl (*a*+*b*)	235 ± 19	505 ± 40	198 ± 22	402 ± 30
Anthocyanins	5 874 ± 563	215 ± 23	3 985 ± 1 005	193 ± 64

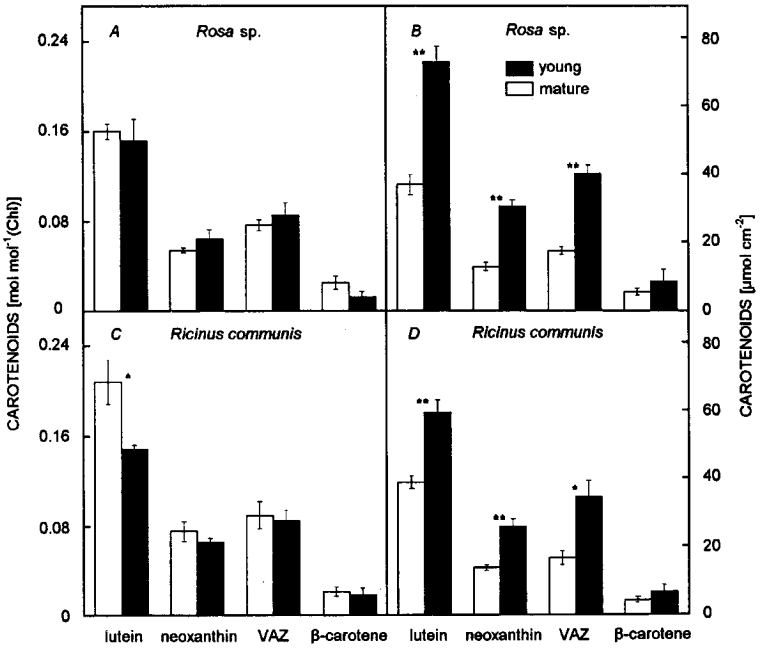

Fig. 1. Carotenoid contents of young and mature leaves of *Rosa* sp. and *Ricinus communis*, expressed on chlorophyll (*A, C*) and leaf area (*B, D*) basis. Means ± SE from 12 (*Rosa* sp.) and 7 (*R. communis*) independent extractions. *The asterisks* denote statistically significant differences at $p<0.01$ (**) and $0.01<p<0.05$ (*).

the results on a leaf area basis as well. In this case, contents of all Cars (with the exception of β-carotene) were significantly (about two-fold) greater in mature leaves. The same trend was observed in β-carotene, yet the differences were not statistically significant due to the high variation of the results.

Differences in the sensitivity to photoinhibition were evident between the two taxa, with *R. communis* being much more sensitive. Yet, in both cases, the young, red leaves exhibited considerable tolerance compared to green, mature leaves (Fig. 2*A,B*). Thus, after 2.5 h at high irradiance, the maximum PS2 photochemical efficiency in *R. communis* dropped to 66 and 34 % of the initial values for young and mature leaves, respectively. Corresponding values in *Rosa* sp. were 75 and 55 %, but after 8 h of photoinhibitory irradiation.

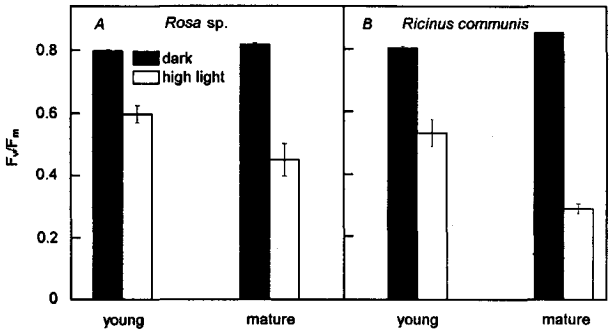

Fig. 2. Photosystem 2 photochemical efficiency (F_v/F_m) of predarkened young and mature leaves of *Rosa* sp. (*A*) and *Ricinus communis* (*B*) under photoinhibitory conditions (8.0 and 2.5 h at 2 700 μmol m^{-2} s^{-1} PAR, respectively). Means ± SE from 30 leaves (10 leaves per individual).

Discussion

Our results showed that young red and mature green leaves of both species had similar contents of almost all Cars, including the xanthophyll cycle components, when these contents were expressed on a Chl basis. This contrasts the results of Krause *et al.* (1995) comparing the same parameters in green young and mature leaves of three tropical forest trees. In that case, the molar ratio of VAZ to Chl was significantly higher in young leaves. In an additional study with the CAM plant *Cotyledon orbiculata* a similar result was found (Barker *et al.* 1997), although the young leaves were anthocyanic. However, in that case the photo-protection offered to young leaves by anthocyanins was gradually replaced during leaf development by a highly reflective wax layer, reducing radiation absorptance in mature leaves. Such a replacement of external photoprotective mechanisms was not evident in *Rosa* sp. and *R. communis*, as shown by absorptance measurements (see next paragraph). Therefore, we may conclude that the need for high, xanthophyll cycle-dependent, photodissipative capacity in young leaves (Krause *et al.* 1995) can be alleviated in anthocyanic leaves (present study) due to less Chl excitation pressure.

The expression of VAZ on a Chl basis follows the tacit assumption that the capacity of the xanthophyll pool should be correlated to the amount of potential targets for photosensitized action, *i.e.* Chl molecules (Adams and Demmig-Adams 1994). However, the amount of Chls may not necessarily reflect the actual potential of a leaf for photon capture. The complex internal architecture of a leaf results in the elongation of the radiation path, increasing the possibility of photon absorption even at low Chl content (Vogelmann 1993). For example, a *ca.* 50 % drop in Chl content results in a *ca.* 10 % reduction of leaf absorptance (Abadía *et al.* 1999). In our case, a 2-fold higher Chl content in mature leaves of both species corresponds to a 12 % increase in leaf absorptance at 680 nm (results not shown). At this wavelength, leaf optical properties are not influenced by the presence of anthocyanins (Neill and Gould 1999). Accordingly, the expression of the xanthophyll cycle pool on an absorbed photon base may be more appropriate (Bisba *et al.* 1997). However, this expression can be highly misleading in anthocyanic leaves, where considerable part of their absorptance in the green and yellow part of the spectrum is due to non-photosynthetic anthocyanins (Neill and Gould 1999). In order to by-pass this methodological difficulty, we may argue as follows: since the capacity for photon absorption of a leaf area is reduced by 10 % when Chl content is 50 % lower, a corresponding 10 % reduction in the area-based xanthophyll cycle pool size could be expected, if the photodissipative capacity of the leaf is to be maintained at the same level. However, our results show that young red leaves of both tested species had considerably lower area-based xanthophyll cycle pool sizes (57 % for *Rosa* sp. and 52 % for *R. communis*, Fig. 1*B,D*) and, accordingly, lower VAZ per absorbed photon compared to mature leaves. Recalculating the results for the non-anthocyanic plants studied by Krause *et al.* (1995) on a leaf area basis, it comes out that the xanthophyll cycle pools of young leaves were slightly (*ca.* 21 %) lower in two cases and considerably (48 %) lower in the third case, while corresponding values for Chls were 51, 55, and 42 %, respectively. We may finally conclude that young anthocyanic leaves may be deficient in the xanthophyll cycle-dependent photodissipative capacity when compared with their green counterparts.

Although the photodissipative potential of young anthocyanic leaves was probably low, these leaves were more tolerant to photoinhibitory conditions than mature, anthocyanin-less leaves. This is again in contrast to what has been found up to now with green young leaves of other species (Krause *et al.* 1995, Barker *et al.* 1997, Ishida *et al.* 2001). We may therefore correlate the presence of anthocyanins in young leaves with tolerance to

photoinhibition, apparently afforded through attenuation of visible radiation. This temporary anthocyanic screen may give the time needed for a young chloroplast to mature, avoiding the perturbations of excess radiation. We have to admit, however, that the PAR values used in our laboratory experiments were higher than mid-day rates under clear sky conditions. Work is needed in the field to examine if the risk of photoinhibition is indeed lower in young anthocyanic leaves under natural conditions, where, apart from high irradiance stress, other stresses may co-occur.

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