Mesophyll versus epidermal anthocyanins as potential in vivo antioxidants: evidence linking the putative antioxidant role to the proximity of oxy-radical source

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Abstract

The hypothesis that anthocyanins in red leaves may be potential in vivo antioxidants whose efficiency is linked to their proximity with the oxy-radical source was tested. Advantage was taken of intra-individual and intra-species variations in the anthocyanic trait and green and red leaves on the same individuals or leaves of green and red phenotypes were compared for the extent of PSII damage by reactive oxygen species generated by methyl viologen treatment in the light. Two species possessing anthocyanins in the mesophyll (Cistus creticus and Photinia fraseri) and two in the epidermis (Rosa sp. and Ricinus communis) were used, while red actinic light (which is not absorbed by anthocyanins) allowed discrimination between an indirect sunscreen and a direct antioxidant function. Red leaves whose anthocyanins were located in the mesophyll were more resistant to methyl viologen treatment than their green counterparts. In one of these species (Cistus creticus), where anthocyanins are induced in some individuals within the natural population after bright cool days in winter, both green and future-red morphs displayed the same sensitivity to methyl viologen before anthocyanin induction. Immediately after reddening, however, resistance to methyl viologen was considerably increased in the red morphs. By contrast, red leaves whose anthocyanins were restricted to epidermal cells were more sensitive to the herbicide. Total leaf phenolic levels in green/red pairs were similar. The results indicate that vacuolar anthocyanins may be an effective in vivo target for oxy-radicals, provided that the oxy-radical source and the anthocyanic detoxifying sink are in close vicinity.

Key words: Methyl viologen, phenolics, photoinhibition, reactive oxygen species, red leaves.

Introduction

The green colour of leaves can be transiently masked by the accumulation of red anthocyanins under some circumstances. A developmental and an environmental component for this change can be distinguished. In some plants, young leaves are red and turn to green upon maturation. In other cases, senescing leaves turn red before abscission. Finally, mature leaves may become temporarily red when stressed by UV-B radiation, low nutrients, wounding, or low temperatures accompanied by high light (Gould et al., 2002b; Steyn et al., 2002; Close and Beadle, 2003; Gould, 2004; Manetas, 2006).

However, the adaptive significance of a red leaf is obscure and this is reflected in the many roles ascribed to foliar anthocyanins. Thus, an anti-herbivore role has been championed by ecologists (Dominy et al., 2002; Levy-Yadun et al., 2004). Physiologists seem to prefer a photoprotective function which is apparently strengthened by the fact that the environmental factors inducing anthocyanin accumulation also make leaves more vulnerable to photoinhibition, since they perturb the fine tuning between chlorophyll excitation pressure and electron sink capacity of the reductive pentose phosphate pathway (Long and Humphries, 1994). The same hypothesis can be applied to juvenile and senescing leaves, which seem to have increased photoprotective needs (Ireland et al., 1985; Krause et al., 1995; Matile et al., 1999). Other proposed roles include osmoregulation and protection against UV-B radiation damage (Chalker-Scott, 1999).
Photoprotection by anthocyanins can be afforded by two means. Anthocyanins attenuate visible radiation thus reducing excitation load in the mesophyll, acting simply as sunscreens. Alternatively (or in addition), they may quench oxy-radicals produced under photoinhibitory conditions (Steyn et al., 2002; Gould et al., 2002a, c). Indeed, anthocyanins are powerful in vitro antioxidants (Wang et al., 1997) and this underlies the recent interest in these compounds for the human diet.

Concerning the antioxidant role, it has been shown that anthocyanins constitute a considerable part of the total oxy-radical scavenging capacity of red leaf extracts (Neill et al., 2002a) and they reduce H$_2$O$_2$ formed in vivo by wounding (Gould et al., 2002a). In addition, anthocyanins in the bathing medium of chloroplast suspensions alleviate photo-inhibition of photosynthesis induced by red light (Neill and Gould, 2003). However, evidence for a direct in vivo antioxidative function in intact leaves with normal compartmentation of anthocyanins is still lacking.

In leaves, the major source of oxy-radical production is the photosynthesizing chloroplast (Asada and Takahashi, 1987), which also contains all the known mechanisms for protection against excess excitation energy. Thus, the xanthophyll cycle (Demmig-Adams et al., 1996), the water–water cycle (Asada, 2000), and the C$_2$ cycle (Tolbert, 1987) function within the chloroplast or in close vicinity. It is argued that anthocyanins, in order to be competitive as potential oxy-radical scavengers in vivo (or to supplement the already existing mechanisms in case their capacity is surpassed), they should be located near the source of photosynthetic oxy-radical production. Existing evidence indicates no variation in their intracellular localization, with anthocyanins being exclusively in the vacuoles (Hrazdina et al., 1978). Yet, high inter-tissue and inter-species differences abound. Thus, anthocyanins may be found in the upper epidermis, lower epidermis, palisade mesophyll, spongy mesophyll, and trichomes, either in one cell type or in almost any combination of them (Lee and Collins, 2003).

The argument for an antioxidant role has been redefined by hypothesizing that the putative antioxidative function could be enhanced in the case of anthocyanins residing in the photosynthetic cells, and in vivo experiments have been designed for that purpose. Crucial determinants in the experimental design were the following: (i) plants possessing anthocyanins either in the epidermis or mesophyll were used; (ii) in addition to this variant, plants showing intra-species or intra-individual variation in the expression of the anthocyanic character were used, i.e. leaves of either green or red phenotypes were compared, or red young and mature green leaves from the same individuals were compared; (iii) oxy-radicals were induced by a combination of light and methyl viologen (MV). MV mediates the transfer of electrons from PSI to molecular O$_2$, generating reactive oxygen species (ROS) within chloroplasts (Iturbe-Ormaeche et al., 1998); and (iv) red light was used in order to by-pass anthocyanin absorbance and to discriminate between the antioxidant and light-screening alternatives. Under this scheme, the sensitivity of green and red leaves to ROS production was assessed by measuring PSII photochemical efficiency, through in vivo chlorophyll fluorescence.

### Materials and methods

#### Plant material

In preliminary trials, free-hand cross-sections of leaves were viewed under the microscope (Axioplan, Zeiss, Oberkochen, Germany) both under bright field and with an epifluorescence attachment. Bright field observations were used for anthocyanin localization and epifluorescence for the possible co-occurrence of chlorophylls in the anthocyanic cells. On the basis of these trials and the prerequisites detailed in the Introduction, four species were selected for further experimentation.

(i) **Cistus creticus** L. Mediterranean drought semi-deciduous shrub. Leaves of some individuals turn red during winter, while neighbouring individuals under the same conditions remain green. All shrubs assume a green colour after the shedding of old leaves in late spring (personal observations). Leaves of both morphs were sampled in the field.

(ii) **Rosa** sp. Common rose. Varieties producing either red or green young leaves during spring were used. All mature leaves are green. The plants were purchased from a local nursery.

(iii) **Photinia × fraseri** Dress. Ornamental shrub cultivated for its bright red young leaves, produced during spring and autumn under Mediterranean conditions. Mature leaves are green. The plants were purchased from a local nursery.

(iv) **Ricinus communis** L. Herbaceous ornamental naturalized in the Mediterranean. Red young leaves produced during spring and autumn turn green upon maturation. Leaves were sampled in the field.

All individual plants used in this study were fully exposed to solar radiation. South-facing (i.e. exposed) green and red leaves from each species were compared on the same experimental dates. In *P. × fraseri* and *R. communis* this was possible due to the extended period of leaf development, during which both young and mature leaves co-occur on the plants. The species used, the location of anthocyanins, and the comparisons performed are summarized in Table 1.

#### Experimental design

Leaves were sampled in the afternoon, put in air-tight plastic bags lined with moistured filter paper, and kept in the dark overnight at room temperature. During the next morning, discs from red or green leaves were cut under dim laboratory light (less than 1 μmol m$^{-2}$ s$^{-1}$ PAR) and infiltrated in water or water containing 1 mM MV for 10 min. This protocol was selected on the basis of preliminary experiments.

### Table 1. Characteristics of species used in the experiments

<table>
<thead>
<tr>
<th>Species</th>
<th>Location of anthocyanins</th>
<th>Comparison between</th>
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<tbody>
<tr>
<td><em>Ricinus communis</em></td>
<td>Epidermis</td>
<td>Young red versus mature green</td>
</tr>
<tr>
<td><em>Rosa</em> sp.</td>
<td>Epidermis</td>
<td>Young red versus young green</td>
</tr>
<tr>
<td><em>Cistus creticus</em></td>
<td>Mesophyll</td>
<td>Mature red versus mature green$^a$</td>
</tr>
<tr>
<td><em>Photinia × fraseri</em></td>
<td>Mesophyll</td>
<td>Young red versus mature green</td>
</tr>
</tbody>
</table>

$^a$ Leaves of the red phenotype turn red during mid-winter.
trials aiming at a modest, yet measurable MV effect and without extensive damage to the tissues. For example, no chlorophyll loss was observed during the course of this experiment. There was no need to buffer the MV solution. Dark-adapted maximum PSII efficiencies were measured on the upper disc side both before and after infiltration. The discs were then illuminated on the same side in Petri dishes, the bottom of which was covered by three layers of filter paper moistened with the infiltration liquid. Red light was provided by a quartz–halogen lamp filtered through a red film. The spectral transmittance of the film has been given elsewhere (Manetas et al., 2003) and this was selected because it matches the spectral transmittance of leaf anthocyanins. In all cases, \( \text{PAR} \) was kept at 400 μmol m\(^{-2}\) s\(^{-1}\). The light-adapted PSII photochemical efficiency was followed during illumination as well as in the subsequent dark recovery period. For each species the experiment was replicated 4–8 times with 5–10 discs per replication.

**Measurements**

PSII photochemical efficiency [as \( \Delta F/F_{\text{m}} = (F'_{\text{m}} - F)/F'_{\text{m}} \) according to Genty et al., 1989] was measured with a Pulse Amplitude Modulated Fluorimeter (MINI-PAM, Walz, Effeltrich, Germany). Since measurements had to be done without changing the incident \( \text{PAR} \) during application of the saturated beam, the optical fibre was attached to the so-called distance clip which was directly applied on the discs inside the Petri dish. Thus, the fibre received fluorescence signals at an angle of 30°, without appreciably impeding the incident actinic light.

\( \text{PAR} \) was measured with a LI-190 quantum sensor (Li-Cor, Lincoln, NE, USA).

Anthocyanins and total phenolics were extracted in a mortar and pestle with methanol/water/HCl (90:1:1 by vol.) and a small amount of washed sea sand. After clearing by centrifugation, spectral absorbance was read from 250–700 nm. The relative amount of anthocyanins was assessed from their peak absorbance (527–535 nm, depending on species), after correction for the contribution of pheophytins at this wavelength (Mancinelli et al., 1975). The relative amount of total phenolics was assessed from their mean absorbance between 250 and 400 nm. It has been shown that the absorbance at this band is linearly correlated with the actual amount of phenolics measured chemically with the Folin–Ciocalteu method (Levizou and Manetas, 2002).

**Statistics**

When needed, significance of differences between treatments, leaf types, etc. was computed by one-way ANOVA (SPSS 9.0 for Windows).

**Results**

Conventional (bright field) microscopy of leaf cross-sections indicated that anthocyanins reside in both the upper and lower epidermis of \( \text{Rosa} \) sp. and in the upper (exposed) epidermis of \( \text{R. communis} \). Epidermises of \( \text{C. creticus} \) and \( \text{P. fraseri} \) were devoid of anthocyanins, which were abundant in the palisade mesophyll of \( \text{C. creticus} \) and the whole mesophyll of \( \text{P. fraseri} \). Epifluorescence microscopy indicated the presence of chlorophyll in the anthocyanic mesophyll cells of these two species (Fig. 1). Thus, the test plants could be distinguished between those containing chlorophyll and anthocyanins in separate cells and those containing both pigments in the same cells. Table 2 presents the area-based levels of anthocyanins of the test plants and leaves. A trend for higher levels in leaves with epidermal anthocyanins may be noted.

Maximum, dark-adapted PSII photochemical efficiency of red leaves was slightly (i.e. 2–4%), yet significantly lower in red leaves when compared with green in \( \text{Rosa} \) sp. and \( \text{P. fraseri} \) (Table 3). The same, yet not significant trend was evident in the other two species. Note that the comparison in \( \text{R. communis} \) and \( \text{P. fraseri} \) refers to young red versus mature green leaves. In the other two species, the compared leaves had the same age. MV had no effect on \( F'_{\text{m}}/F_{\text{m}} \) in the dark up to 2 h after application. It may be

![Fig. 1. Freehand cross-sections of leaves, indicating the location of anthocyanins in the epidermis of Rosa sp. (A) and R. communis (B) and in the mesophyll of P. fraseri (C) and C. creticus (D). The bar corresponds to 250 μm.](https://academic.oup.com/jxb/article-abstract/57/10/2203/470351/1.10203473)
concluded that the initial green and red leaf material was equally healthy with respect to PSII yield and was appropriate for the photoinhibitory experiments described below.

Under constant illumination with red light at 400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) \( \text{PAR} \), the PSII effective yields of red leaves were lower in \( P.\times\text{fraseri} \) and \( \text{Rosa} \) sp., while the same was evident in \( \text{C. creticus} \) during the first 60 min of exposure. In \( \text{R. communis} \), the red leaves displayed higher PSII effective yields only after 3 h illumination (Fig. 2a). The extent of inhibition of PSII efficiency by MV in the light was species-specific, with \( \text{C. creticus} \) displaying slightly higher resistance to the herbicide, probably due to lower or slower uptake (Fig. 2b). Concerning differences between the two leaf kinds, red leaves suffered from significantly higher inhibition than green leaves in \( \text{Rosa} \) sp. (with the exception of the 2 h sampling) and \( \text{R. communis} \) (with the exception of the 1 h sampling). In \( P.\times\text{fraseri} \) the extent of inhibition by MV was the same in both leaf kinds (Fig. 2b). In \( \text{C. creticus} \), however, green leaves displayed higher values of inhibition at all sampling times.

In the dark period following illumination, PSII yield of control leaves gradually recovered, approaching the initial, dark-adapted values. The percentage recovery within the first 5 h of darkness ranged from 66% in \( P.\times\text{fraseri} \) to 96% in \( \text{Rosa} \) sp., with no differences between green and red leaves (results not shown). However, recovery in MV-treated leaves was less complete in most of the cases, indicating a permanent PSII damage. MV-induced inhibition of PSII dark recovery was the same between green and red leaves of three species, i.e. \( \text{Rosa} \) sp., \( \text{R. communis} \), and \( \text{C. creticus} \) (not shown). In \( P.\times\text{fraseri} \), however, MV-treated red leaves recovered to the same extent as control leaves, while green leaves suffered permanent PSII damage by MV (Table 4).

In a separate experiment, the effect of MV on PSII effective yield was followed in green and red individuals of \( \text{C. creticus} \) just before and after anthocyanin induction during winter. As shown in Fig. 3, percentage inhibition of PSII effective yield in both phenotypes was the same in the period between 13/01 to 18/01, i.e. before the future-red phenotypes turn red. Reddening was completed within a few days and in the following period ‘greens’ maintained roughly the same MV sensitivity, while percentage inhibition in ‘reds’ was considerably reduced (Fig. 3). Accordingly, the enhanced resistance of red leaves may be linked to the presence of anthocyanins.

In all tested species, high levels of anthocyanins were not accompanied by increased total phenolic concentrations (data not shown).

**Table 2.** Anthocyanin contents of green and red leaves of the indicated species

A cm\(^{-2}\) denotes the 1 cm light path absorbance at the peak for anthocyanins (\( \lambda \text{527–535 nm} \), depending on species), normalized for 1 cm\(^2\) of extract obtained from 1 cm\(^2\) leaf area. Values are means \( \pm \text{SE} \) from 8–12 independent measurements. Differences between green and red leaves within each species are statistically significant (\( P < 0.01 \)).

<table>
<thead>
<tr>
<th>Species</th>
<th>Leaf type</th>
<th>( A \text{ cm}^{-2} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Ricinus communis} )</td>
<td>Green</td>
<td>0.27 ( \pm ) 0.04</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>2.25 ( \pm ) 0.15</td>
</tr>
<tr>
<td>( \text{Rosa} ) sp.</td>
<td>Green</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>2.77 ( \pm ) 0.16</td>
</tr>
<tr>
<td>( \text{Cistus creticus} )</td>
<td>Green</td>
<td>0.48 ( \pm ) 0.02</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>1.36 ( \pm ) 0.10</td>
</tr>
<tr>
<td>( \text{Photinia}\times\text{fraseri} )</td>
<td>Green</td>
<td>0.29 ( \pm ) 0.05</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>1.67 ( \pm ) 0.35</td>
</tr>
</tbody>
</table>

**Table 3.** Dark-adapted PSII photochemical efficiency of green and red leaves in the indicated species

Values are means \( \pm \text{SE} \) from four (\( \text{R. communis} \), \( \text{Rosa} \) sp.), eight (\( \text{C. creticus} \)), and six (\( P.\times\text{fraseri} \)) independent experiments, with 5–10 leaf discs per experiment. The asterisks indicate statistically significant differences between red and green leaves for each species (\( P < 0.05 \)).

<table>
<thead>
<tr>
<th>Species</th>
<th>Type of leaf</th>
<th>( F_v/F_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{R. communis} )</td>
<td>Green</td>
<td>0.829 ( \pm ) 0.010</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>0.810 ( \pm ) 0.009</td>
</tr>
<tr>
<td>( \text{Rosa} ) sp.</td>
<td>Green</td>
<td>0.828 ( \pm ) 0.003*</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>0.789 ( \pm ) 0.005*</td>
</tr>
<tr>
<td>( \text{C. creticus} )</td>
<td>Green</td>
<td>0.773 ( \pm ) 0.007</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>0.764 ( \pm ) 0.006</td>
</tr>
<tr>
<td>( P.\times\text{fraseri} )</td>
<td>Green</td>
<td>0.817 ( \pm ) 0.003*</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>0.802 ( \pm ) 0.004*</td>
</tr>
</tbody>
</table>

Discussion

It is evident from the results of this investigation that MV-treated leaves of all plants tested suffered a reduction in light-adapted PSII photochemical efficiency, which persisted in the subsequent dark recovery period. MV is known to accept electrons from the reducing side of PSI (Fuji et al., 1990), acting competitively to NADP reduction. Accordingly, \( \text{CO}_2 \) fixation is inhibited (Preston, 1994), leading to a transient activation of the xanthophyll cycle and down-regulation of PSII efficiency (Thiele and Krause, 1994). Under short exposure to the herbicide, this dynamic photoinhibition is reversible within minutes in the dark. After longer exposure, however, permanent damage to PSII may occur, resulting in chronic photoinhibition. Hence, it was accepted that, under the conditions of these experiments, the transfer of electrons from MV to molecular \( \text{O}_2 \) and the concomitant production of the superoxide radical (\( \text{O}_2^- \)) and other highly reactive oxidative species resulted in a partial photo-oxidative damage of PSII (Iturbe-Ormaechea et al., 1998). Apparently, the intrinsic capacity of the chloroplast antioxidative enzyme complement to scavenge \( \text{O}_2^- \) (Asada and Takahashi, 1987) was surpassed in MV-treated leaf discs.

However, red leaves in two species (\( \text{C. creticus} \), \( P.\times\text{fraseri} \)) displayed higher resistance to photo-oxidative PSII damage, when compared with the corresponding green...
Fig. 2. Effects of MV on green and red leaves of the indicated species. (a) PSII photochemical efficiency versus time under constant red illumination (400 μmol m⁻² s⁻¹ PAR). (b) Percentage inhibition by MV. Values are means ±SE from four (R. communis, Rosa sp.), eight (C. creticus), and six (P. x fraseri) independent experiments with 5–10 leaf discs per experiment. Different letters within each data set indicate statistically significant differences (P <0.05). A data set consists of the values in the measured parameters obtained at the indicated time for red and green leaves of each species in the presence or absence of MV.
leaves. In these species, anthocyanins reside in the vacuoles of mesophyll cells. The higher resistance of red leaves in *C. creticus* was evident in the light, while in *P. xfraseri* it was evident in the dark recovery period. During the dark period in *C. creticus* and the light period in *P. xfraseri*, the extent of inhibition in PSII efficiency by MV was the same in both green and red leaves. The results do not allow any speculation concerning the above differences between *C. creticus* and *P. xfraseri*. Yet, although the timing for the expression of an assumed protective effect by anthocyanins against MV-induced ROS was different, the final results pointed towards the same direction. At this point it may be noted that ROS-induced PSII damage may extend well into the dark recovery period (Frankart *et al.*, 2003), probably as a result of autocatalytic membrane peroxidation (Halliwell, 1987). Also, the results cannot be explained by a reduction of the light intensity reaching the chloroplasts in red leaves (sunscreen hypothesis), since the red actinic light used is not absorbed by anthocyanins. However, it may be argued that the observed MV-resistance of red leaves could be associated with anthocyanins residing in mesophyll vacuoles and acting as competitive scavenging targets for reactive oxidative species. The argument is strengthened by the sharp reduction in the sensitivity of the red phenotype of *C. creticus* to MV, immediately after anthocyanin accumulation in the field (Fig. 3).

An alternative explanation, applied only in illuminated *C. creticus*, is that the greatest amelioration of the MV effects in red leaves is due to differences in MV uptake. Yet, such an interpretation is weakened by the fact that percentage inhibition in both the green and the red phenotype in the period immediately preceding anthocyanin accumulation was the same (Fig. 3) and there is no reason to assume a sudden decrease in penetration rate upon reddening.

A matter which should be discussed concerning this putative anthocyanin function is the nature of light- and MV-induced oxy-radicals and their capacity to reach the vacuole. O$_2^-$ is normally reduced in the chloroplast via the action of superoxide dismutase to H$_2$O$_2$, which, in turn, is transformed to H$_2$O plus O$_2$ by ascorbate peroxidase. If in excess or not properly handled, these oxidative agents can further produce hydroxyl radicals (HO$^-$) which attack proteins, lipids, and nucleic acids, initiating radical chain reactions. Although anthocyanins can *in vitro* detoxify all these active oxy-radical species, O$_2^-$ and HO$^-$ cannot penetrate the tonoplast and, accordingly, they have to be scavenged in the chloroplast or the cytoplasm (Takahashi and Asada, 1983). Yet, H$_2$O$_2$ can freely diffuse into the vacuole where anthocyanins abound (Yamazaki *et al.*, 1996). Moreover, it has been reported that the primary target of MV-induced photo-oxidative stress in leaves is ascorbate peroxidase, i.e. the enzyme responsible for H$_2$O$_2$ removal (Mano *et al.*, 2001). Since inactivation of ascorbate peroxidase precedes that of superoxide dismutase (Mano *et al.*, 2001), H$_2$O$_2$ transiently accumulates in the chloroplast stroma under photo-oxidative conditions (Nakano and Asada, 1981) and further diffuses to other cell compartments. Thus an anthocyanin-rich vacuole is a potential sink for excess H$_2$O$_2$ produced in the chloroplast, alleviating the photo-oxidative risk. The results with *C. creticus* and *P. xfraseri* are compatible with this possibility. A further argument is that this anthocyanin-based antioxidative potential is enhanced when anthocyanins are

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**Table 4.** PSII photochemical efficiency (as $F_v/F_m$) following recovery in the dark of pre-illuminated (3 h under red light of 400 μmol m$^{-2}$ s$^{-1}$ PAR) green and red leaves of *P. xfraseri*. (−) denotes control and (+) denotes MV treated leaves.

Values are means ±SE from six independent measurements with 5–10 leaf discs per experiment. Different letters within each row and for each leaf type (green or red) indicate statistically significant differences between control and treated leaves ($P < 0.05$).

<table>
<thead>
<tr>
<th></th>
<th>Green leaves ($F_v/F_m$)</th>
<th>Red leaves ($F_v/F_m$)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>30 min</td>
<td>5 h</td>
</tr>
<tr>
<td></td>
<td>0.530±0.026 a</td>
<td>0.433±0.012 b</td>
</tr>
<tr>
<td></td>
<td>0.588±0.047 a</td>
<td>0.388±0.066 b</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Percentage inhibition of PSII effective yield in the light (1 h, red light, 400 μmol m$^{-2}$ s$^{-1}$ PAR) by MV in green and red leaves of *C. creticus* sampled just before (13/01 to 18/01) and after (24/01 to 16/03) reddening of the corresponding phenotype in the field. The arrow indicates the first optical sign of colour change. Levels of anthocyanins in the corresponding leaves are shown in Table 2. Values are means ±SE from eight leaves per experiment and phenotype. The asterisks indicate statistically significant differences between phenotypes ($P < 0.05$).
located in the vicinity of H$_2$O$_2$ production, as happens in C. creticus and P.$\times$fraseri. Accordingly, one would expect the antioxidative function of anthocyanins to be weakened or absent as the distance between H$_2$O$_2$ source and anthocyanic sink is increased. In fact, in Rosa sp. and R. communis (with anthocyanins located in the epidermal vacuoles), red leaves appeared more sensitive to MV than green leaves. Although this result generally strengthens the initial hypothesis for an enhanced, anthocyanin-based, antioxidative capacity when anthocyanins are near the oxy-radical source, the reasons for the higher MV sensitivity of red leaves possessing anthocyanins in the epidermis cannot be determined from these experiments.

Alternatively, it may be argued that an explanation for these results could be sought on age- or morph- (green versus red) specific differences in enzyme and/or phenolic-based antioxidative potential. It has been shown with many plants that activities of superoxide dismutase, ascorbate peroxidase, and catalase increase with leaf age (Polle, 1997). Accordingly, the higher sensitivity of young red versus green mature leaves of R. communis to MV-induced PSII damage could be ascribed to the lower antioxidant enzyme pool of young red leaves. In young, red leaves of P.$\times$fraseri, however, the presence of anthocyanins within photosynthetic cells seems not only to compensate for the assumed lower antioxidant enzyme pools, but to render these leaves more resistant to MV-induced oxy-radical production than green mature leaves. Concerning pool differences in these enzymes between red and green leaves of the same age, Neill et al. (2002a) reported higher activities of superoxide dismutase, catalase, and ascorbate peroxidase in red leaves of the shade species Elatostema serrata (Neill et al., 2002b). At this point it may be noted that leaves used in the present study were taken from fully mature leaves, lacking the whole flavonoid biosynthetic pathway or just the final steps leading to anthocyanins may help to assess the relative importance of these compounds to antioxidative defence. It may also be noted here that the anthocyanin most often encountered in red leaves is cyanidin-3-glucoside (Harborne, 1976). Although the structure of anthocyanins in the test plants was not investigated, it can be noted that red anthocyanins have the highest antioxidative capacity which is further enhanced by glucosylation (Wang et al., 1997). Hence, an in vivo antioxidative function for anthocyanins seems plausible. These results not only confirm earlier reports indicating a possible antioxidative role for leaf anthocyanins, but extend this confirmation to an in vivo situation where the normal compartmentation of both anthocyanins and oxy-radical source is maintained.

**Conclusion**

It is concluded from the above that leaf vacuolar anthocyanins, although not optimally located in relation to the chloroplastic source of oxy-radical production, may afford a detoxifying sink for some reactive oxygen species when the chloroplastic, the first line of antioxidative defence, is surpassed. This putative function is more possible for anthocyanins located in mesophyll than in epidermal vacuoles.

**Acknowledgement**

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