DOI: 10.1007/s11099-011-0061-8 PHOTOSYNTHETICA 49 (4): 515-522, 2011

The interplay of anthocyanin biosynthesis and chlorophyll catabolism in senescing leaves and the question of photosystem II photoprotection

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Abstract

Fully exposed, senescing leaves of *Cornus sanguinea* and *Parthenocissus quinquefolia* display during autumn considerable variation in both anthocyanin and chlorophyll (Chl) concentrations. They were used in this study to test the hypothesis that anthocyanins may have a photoprotective function against photosystem II (PSII) photoinhibitory damage. The hypothesis could not be confirmed with field sampled leaves since maximum photochemical efficiency (F_v/F_m) of PSII was negatively correlated to anthocyanin concentration and the possible effects of anthocyanins were also confounded by a decrease in F_v/F_m with Chl loss. However, after short-term laboratory photoinhibitory trials, the percent decrease of F_v/F_m was independent of Chl concentration. In this case, a slight alleviation of PSII damage with increasing anthocyanins was observed in *P. quinquefolia*, while a similar trend in *C. sanguinea* was not statistically significant. It is inferred that the assumed photoprotection, if addressed to PSII, may be of limited advantage and only under adverse environmental conditions.

Additional keywords: anthocyanins; chlorophyll fluorescence; *Cornus sanguinea; Parthenocissus quinquefolia;* photoinhibition; photoprotection; senescence.

Introduction

Spectacular colour changes accompany specific developmental phases of leaves (Lee 2007). Hence, juvenile or senescing leaves in some plants may appear red due to anthocyanin accumulation. Also, mature leaves may turn red when stressed by various environmental factors. Since anthocyanins absorb part of incident radiation without being photosynthetic, a function has been sought to compromise for the cost of lost photons. Recent reviews emphasize on two major functional hypotheses, *i.e.* protection against high light and protection against excessive herbivory (Hoch *et al*. 2001, Gould 2004, Lev-Yadun and Gould 2007, Manetas 2006, Archetti *et al*. 2009). Empirical tests for the photoprotective hypothesis, however, produced contradictory results. Hence, the predictions of the hypothesis were confirmed in many cases (Feild *et al*. 2001, Manetas *et al*. 2002, Hoch *et al*. 2003, Nagata *et al*. 2003, Hughes *et al*. 2005, Kytridis and Manetas 2006, Hughes *et al*. 2007, Hughes and Smith 2007). Yet, in a considerable number of studies the hypothesis could not be confirmed (Burger and Edwards 1996, Lee *et al*. 2003, Karageorgou and Manetas 2006, Kyparissis *et al*. 2007, Esteban *et al*. 2008, van den Berg *et al*. 2009). In some other cases, the red leaf trait was found to characterize phenotypes which were inherently sensitive to photoinhibition (Zeliou *et al*. 2009, Nikiforou *et al*. 2010, Nikiforou and Manetas 2010).

In the particular case of senescing leaves, one may ask whether a protective function against photoinhibition of photosynthesis is reasonable in a leaf which is going to be soon shed. However, efficient resorption of nutrients, especially nitrogen, from senescing leaves to overwintering plant parts requires the dismantling of thylakoids and the transformation of chloroplasts to gerontoplasts (Matile 2000). In the meantime, Chl is dissociated from the chloroplast membranes and enzymatically degraded to colourless catabolites in order to avert possible photodynamic production of reactive oxygen species (Hortensteiner 2004, Ougham *et al*. 2005). Accordingly, a function for anthocyanins as a light screen and/or as antioxidant (Steyn *et al*. 2002) may be critical to keep the

Received 10 May 2011, *accepted* 20 October 2011.
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Abbreviations: ARI – anthocyanin reflectance index; Chl – chlorophyll; CI – chlorophyll index; F_0 – minimal fluorescence yield of dark-adapted state; F_m – maximum fluorescence yield of dark-adapted state; F_v – variable fluorescence; F_v/F_m – maximum photochemical efficiency of PSII; KIT – Karlsruhe Institute of Technology; LED – light-emitting diodes; NIR – near infrared reflectance; PSII – photosystem II; R – reflectance.

remaining PSII reaction centers functional. This may be additionally important, since nutrient resorption is an energetic process requiring energy inputs from photosynthesis, at least up to a certain stage of leaf senescence (Keskitalo *et al*. 2005).

However, in those studies dealing specifically with senescing leaves, results were again contradictory. Feild *et al*. (2001) found in laboratory trials that red exposed leaves of *Cornus stolonifera* suffered less photoinhibitory damage (as judged by measurements of F_v/F_m) when compared to yellow, shaded leaves of the same individual. Similar results were found by Hoch *et al*. (2003) in three deciduous trees (including *Cornus sericea*), when red-senescing wild types were compared to corresponding anthocyanin-deficient mutants in both laboratory and field trials. However, Lee *et al*. (2003), in an extensive survey of 89 tree species (including *Cornus alternifolia*) found no evidence for less photoinhibitory risk in red-senescing leaves in the field.

Apparently, the question may be more directly approached by using an intra-species system. In this study, initial observations in the field indicated that in *C. sanguinea* and *P. quinquefolia*, apart from an apparent variation in leaf redness between fully exposed and shaded leaves (the latter being less red), there was also considerable variation in fully exposed, south-facing leaves. In addition, senescence in these plants was asynchronous, as revealed by variations in Chl content at each sampling date. Accordingly, the opportunity is given to compare photoinhibitory risk in leaves of different colour but similar exposure and on the same tree. In this way, confounding effects of different light history (Feild *et al*. 2001) or the possibility of pleiotropic effects of specific mutations (Hoch *et al*. 2003) are alleviated. We also argued that the assumed photoprotective potential of anthocyanis would depend on their concen-tration, a question which, to the best of our knowledge, has not been tested up to now. However, vulnerability of PSII to photoinhibition in senescing leaves increases with the progress of senescence, which is reflected in Chl concentration (Hoch *et al*. 2003, Lu *et al*. 2003, Keskitalo *et al*. 2005). Based on the considerations mentioned above, we sought for correlations between redness, greenness and photoinhibitory risk in fully exposed, senescing leaves of *C. sanguinea* and *P. quinquefolia*. It was expected that, if anthocyanins are indeed photoprotective, slightly red leaves would suffer greater photoinhibitory risk, when compared to fully red leaves of similar Chl concentrations, *i.e.* of similar senescence stage.

Materials and methods

Preliminary field observations in the vicinity of the Karlsruhe Institute of Technology (KIT) during early October 2009, indicated that *C. sanguinea* L. and *P. quinquefolia* L. display considerable variation both in Chl and anthocyanin levels of canopy leaves. Two healthy plants were selected for further experiments, based on roughly similar plant size and means and ranges in the parameters to be measured, *i.e.* Chl and anthocyanin concentration and maximum PSII photochemical efficiency. At the indicated sampling dates, a number of leaves were harvested from the two plants in late afternoon. Only south-facing, fully exposed canopy leaves were chosen to assure similar light acclimation. Care was taken to sample leaves with varying strengths of redness and greenness. Since anthocyanins in these two plants are accumulated in palisade cells (Mertzlyak *et al*. 2008), a visual inspection of the adaxial and the abaxial leaf surface guaranteed considerable red and green colour variation in the sample. The leaves were put in air-tight, plastic envelopes lined internally with moist filter paper and dark-adapted all night in a black container kept at ambient temperature in the field.

During next early morning the leaves were inserted in leaf clips for Chl fluorescence measurements. Subsequently, leaf spectral reflectance was measured on the same spot by attaching an optical fiber directly on the leaf through the open window of the leaf clip. The diameter of the optical fiber (3 mm) was slightly smaller than that of the leaf clip.

In some experiments performed during a cold mid-October event, discs from the measured leaf area were cut and subjected to a photoinhibitory treatment (1 h at 1,700 μmol m^{-2} s^{-1} white light from an overhead projector) at room temperature. The photon fluence rate used was 50% higher than that measured at midday of clear days at leaf level in the field. After the treatment, Chl fluorescence was measured after dark adaptation for 30 min. Percent photoinhibition was calculated as % reduction in F_v/F_m after the photoinhibitory treatment.

For fast Chl fluorescence rise measurements, a fluorometer (*Handy-PEA*, *Hansatech Instruments Ltd.*, King's Lynn, Norfolk, UK) with continuous excitation was used. Saturated light at 3,000 μmol m^{-2} s^{-1} was given for 1 s by a bank of 3 red LEDs. Maximum PSII photochemical efficiency was computed as $F_v/F_m = (F_m - F_0)/F_m$, where F_0 is the fluorescence yield when all PSII reaction centers are open (*i.e.* at 20 μs after onset of illumination) and F_m is the maximum fluorescence yield obtained with all PSII reaction centers closed, typically after 300–600 ms of illumination.

Spectral reflectance was measured with a spectroradiometer (*Unispec, PP Systems*, Haverhill, USA) equipped with an internal white light source and a bifurcated optical fiber directly and perpendicularly attached on the leaf. A spectralon standard (95% reflectance for the whole 400–1,100 nm effective spectral band) was used to calibrate the instrument. Anthocyanin and Chl concentrations were estimated by using the

spectral indices and formulas recently developed by Gitelson *et al*. 2009. The authors used four species, one of which is used in the present study, to derive species-independent equations for nondestructive estimation of leaf pigments. In brief, the anthocyanin reflectance index (ARI) was computed as:

$$ARI = (R^{-1}_{green} - R^{-1}_{red\ edge})$$

and the Chl index

$$CI = (R_{NIR}/R_{red\ edge}) - 1,$$

where R_{green}, $R_{red\ edge}$, and R_{NIR} are mean reflectances in the green (λ = 540–560 nm), red edge (λ = 690–710 nm) and near infrared (λ = 770–800 nm) bands, respectively. ARI versus actual anthocyanin concentration is described by a nonlinear function with reduced accuracy above 50 nmol cm^{-2}, while the CI versus actual Chl concentration is given by a straight line (Gitelson *et al*. 2009).

Photosynthetically active radiation in the field and the laboratory was measured with a *LI–COR* quantum sensor. Climatic data for the area were kindly given by the KIT Meteorological station. Best fit lines were computed with the *SPSS 9.0* statistical package.

Results

Climatic data: As shown in Fig. 1, the climatic conditions on the test site were characterized by comparatively mild temperatures during the first ten days of October 2009 followed by a gradual decrease in temperatures displaying minimum values roughly at the middle of the month. Subsequently, temperatures increased gradually after October 20. Sampling was performed before, during and after the mid-October cold event. Fig. 1 also indicates that the first cold days were accompanied by high global radiation.

C. sanguinea: Leaf anthocyanin concentrations were negatively correlated to Chl concentrations at the first two sampling dates (6 and 18/10), but not at the third sampling date (22/10) (Fig. 2*A*). Maximum PSII photochemical efficiency (F_v/F_m) was significantly decreasing with anthocyanin concentration at the first sampling date, yet this effect was abolished during the second and third sampling dates (Fig. 2*B*). We may also note a general decrease of F_v/F_m with the progress of season. PSII photochemical efficiency was also diminishing with the progress of leaf senescence, as evidenced by the negative correlation between Chl concentration and F_v/F_m observed at the first, but not at the second and third sampling dates. (Fig. 3). In separate laboratory experiments performed during the cold event, the percent loss of PSII maximum efficiency after a photoinhibitory treatment was independent of Chl concentration (not shown). However, a marginally significant (p=0.083) trend for decreasing PSII damage with increasing anthocyanin concentrations (Fig. 4) was evident in the laboratory photoinhibitory trials.

P. quinquefolia: As already shown for *C. sanguinea*, the more red the leaves of *P. quinquefolia* were, the less Chl they contained (Fig. 5*A*). One may also note that the Chl *vs.* anthocyanin regression line becomes steeper with the progress of season, in contrast to an opposite trend observed with *C. sanguinea* (Fig. 2*A*). Maximum PSII photochemical efficiency was negatively correlated to anthocyanin concentration at all sampling dates and a gradual increase in the steepness of the F_v/F_m *vs.* anthocyanin curve was evident at mid and further at late October (Fig. 5*B*). This contrasts with what we have observed with *C. sanguinea* (*see* Fig. 2*B*). The Chl *vs.* PSII photochemical efficiency correlation in *P. quinquefolia* was characterized by a decrease in F_v/F_m with the progress of leaf senescence, *i.e.* with decreasing Chl (Fig. 6*A*). In addition, the correlation becomes steeper with the progress of season.

In the laboratory photoinhibitory trials, the extent of PSII damage was independent of Chl at pigment concentrations ranging between *ca.* 60–20 nmol cm^{-2}. At lower concentrations, photoinhibition of PSII increased sharply with Chl drop (Fig. 6*B*). Accordingly, we present in Fig. 7 the percent decrease in F_v/F_m *vs*. anthocyanin concentrations in two separate data sets, *i.e.* for leaves containing 60–20 nmol cm^{-2} or <20 nmol cm^{-2} Chl, respectively. As shown, a slight and marginally significant

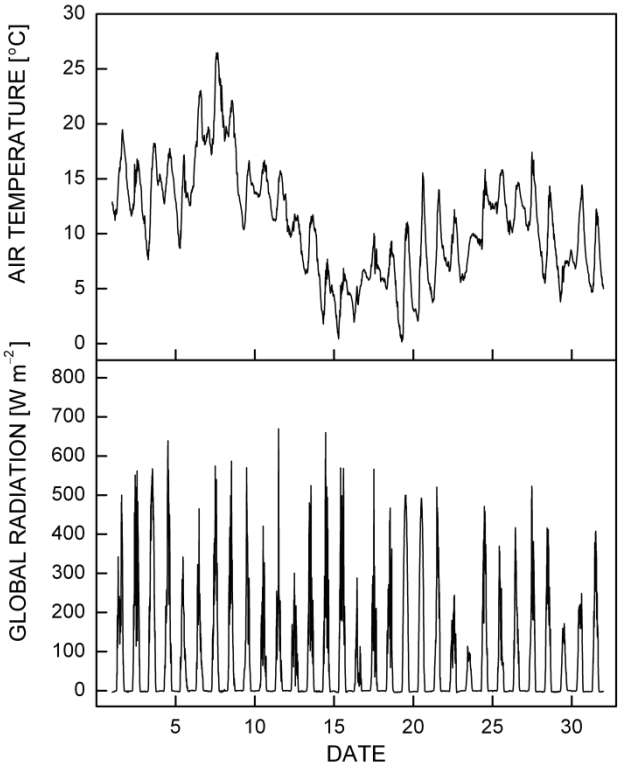

Fig. 1. Air temperature and global radiation at the experimental site during the sampling period (October 2009).

decrease in photoinhibition with increasing anthocyanins is evident in leaves with high to medium Chl levels. For low Chl levels, the percent photoinhibition is apparently higher, while the slightly negative correlation between photoinhibition and anthocyanin concentration is, again, marginally significant (Fig. 7).

Discussion

According to the literature, considerable species-specific variation exists in the pattern of autumnal leaf reddening. Thus, in some trees the whole foliage becomes red while in others only well exposed, canopy leaves accumulate anthocyanins (Lee 2007). It seems that variation also appears in the onset of reddening which, apart from being species-specific, occurs earlier if autumn is cold. There is also a general agreement that anthocyanin accumulation starts rather suddenly when the Chl concentration (reflecting the progress of senescence), drops below a threshold level (Sanger 1971, Hoch *et al*. 2003, Keskitalo *et al*. 2005). Other investigators, however, were able to harvest from other trees leaves with more or less Chl and varying anthocyanin levels (Gitelson *et al*. 2009), indicating that the dependence of autumnal reddening on the extent of Chl catabolism may not be so strict. The test plants used for this study were selected from this second category, as shown by the linear regression describing the inverse relationship between Chl and anthocyanin concentrations (Figs. 2*A*, 5*A*; first sampling date). Hence, the between-leaf variation in greenness and redness can be exploited in conjunction with the ongoing debate concerning the photoprotective function of leaf anthocyanins.

We may discuss the results for each test plant separately. In *C. sanguinea* at early October, the photoinhibitory risk under field conditions seems to increase with leaf redness (Fig. 2*B*), a finding which is in apparent contrast to the photoprotective hypothesis. However, the photoinhibitory risk increases with decreasing Chl (Fig. 3), hence the results neither reject nor confirm

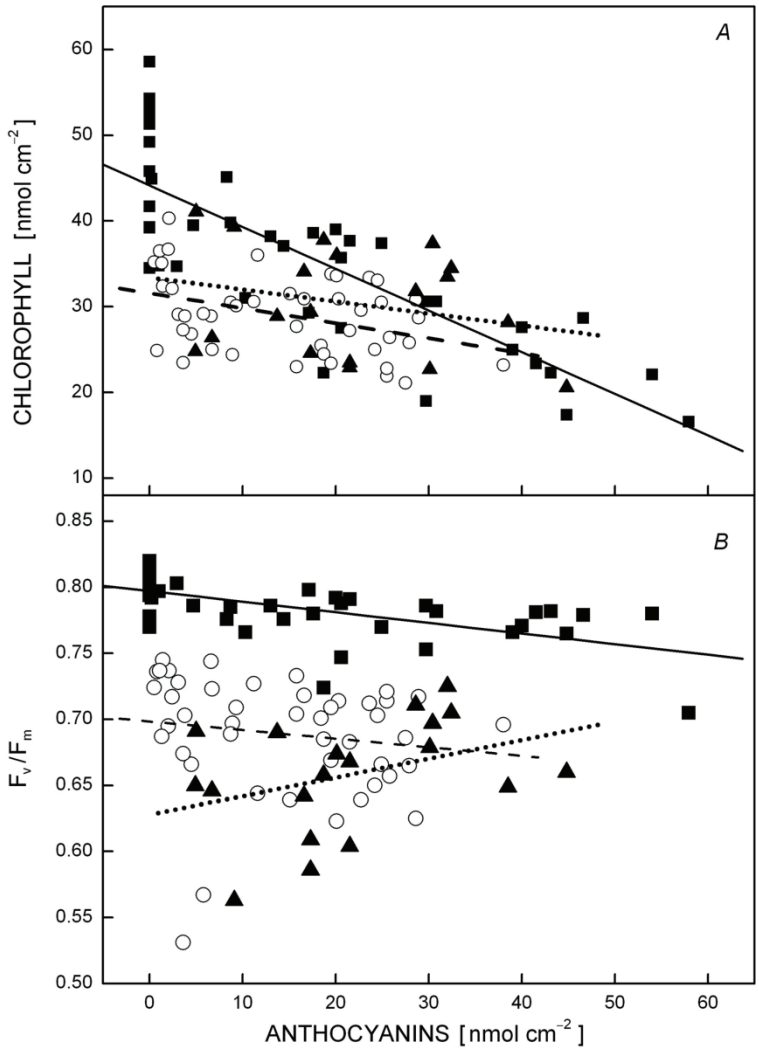

Fig. 2. Anthocyanin *vs*. chlorophyll concentrations (*A*) and maximum PSII photochemical efficiency (F_v/F_m) (*B*) in senescing leaves of *C. sanguinea* sampled at early (■, *solid line*), mid (○, *dashed line*) and late October (▲, *dotted line*). Each point represents a separate leaf. Corresponding levels of significance for (*A*) are $p<0.001$, $p=0.008$ and $p=0.300$, respectively. Corresponding levels of significance for (*B*) are $p<0.001$, $p=0.328$ and $p=0.120$, respectively.

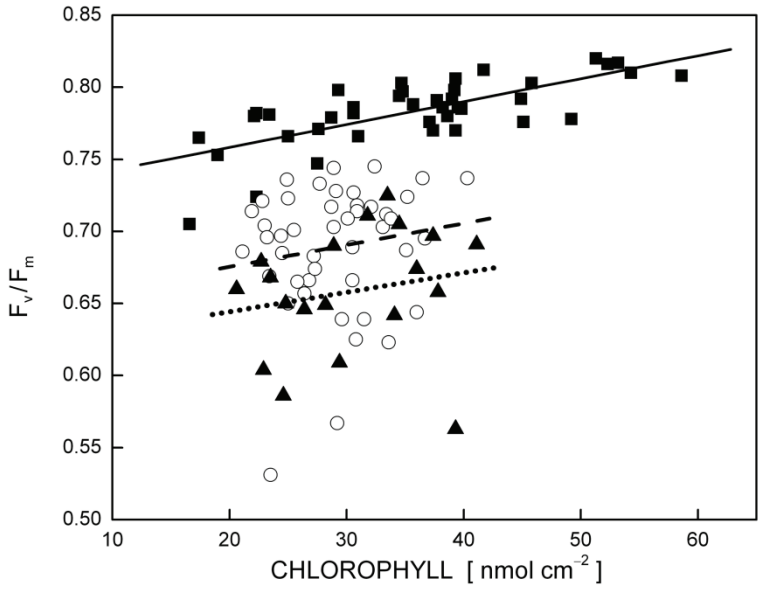

Fig. 3. Maximum PSII photochemical efficiency (F_v/F_m) *vs*. chlorophyll concentration in senescing leaves of *C. sanguinea*. Sampling and symbols as in Fig 2. Corresponding levels of significance are $p<0.001$, $p=0.315$ and $p=0.420$, respectively.

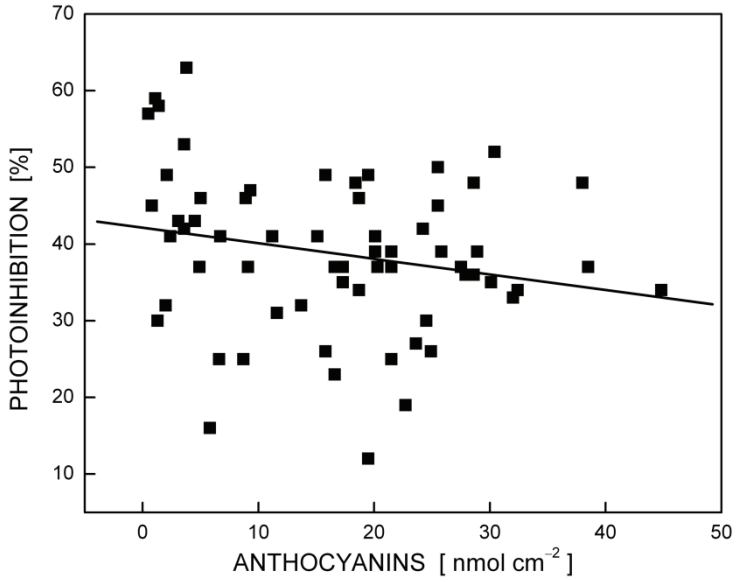

Fig. 4. Percent reduction of F_v/F_m *vs*. anthocyanin concentrations in senescing leaves of *C. sanguinea* at mid-October. Each point represents a separate leaf. Photoinhibitory treatment at 1,700 μmol m^{-2} s^{-1} for 1 h at room temperature. $p=0.083$.

the photoprotective hypothesis. After the cold event of mid-October, however, the negative correlation between anthocyanin levels and F_v/F_m changes to neutral and further on to slightly positive (Fig. 3). We may interpret this result as indicating a slight photoprotective function of anthocyanins, which becomes evident only during the cold period. This conclusion is partially supported by the results of the laboratory photoinhibitory treatment. Here, the percent loss of PSII trapping efficiency appears to be independent of the extent of Chl degradation. However, although the confounding effects of Chl degradation on PSII trapping efficiency are removed and the photoinhibitory treatment is rather strong (*i.e.* 50% higher light level compared to that naturally enjoyed by the leaves), only a slight, nonsignificant trend for alleviation of PSII damage in red leaves is observed (Fig. 4). Accordingly, a protective function of anthocyanins against PSII photo damage is questionable for *C. sanguinea*.

As far as field measurements are concerned, the situation with *P. quinquefolia* is even less substantiating for the photoprotection hypothesis, since the F_v/F_m *vs.* anthocyanin line became steeper after the mid-October cold event, indicating higher sensitivity of the red leaves. This could be due to an inherently higher stress vulnerability of the red leaves, as recently proposed for Mediterranean winter-red shrubs (Zeliou *et al.* 2009, Nikiforou and Manetas 2010). Alternatively, it could be due to the confounding effect of higher sensitivity of PSII in leaves with advanced senescence (and, accordingly, more red), to cold weather (Fig. 1). Whatever the case, short-term photoinhibitory conditions applied in the laboratory revealed that in both plants and within certain limits of Chl content, the extent of PSII damage was independent of Chl content (Fig. 6*B*) but negatively

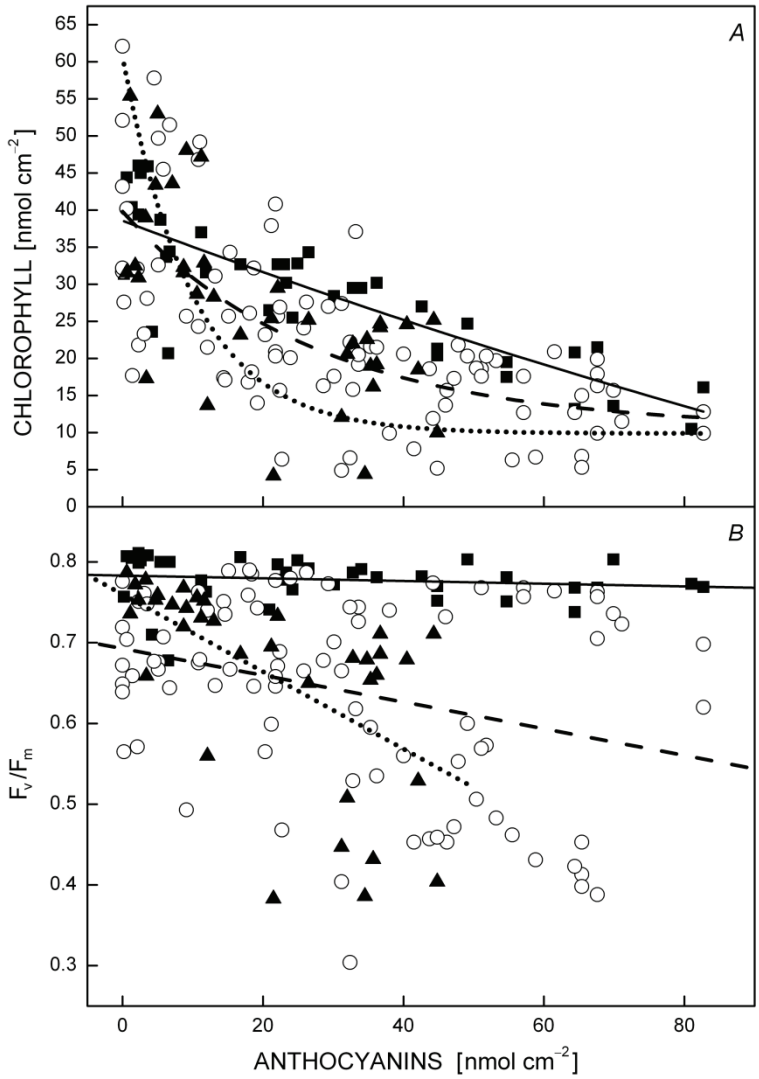

Fig. 5. Anthocyanin *vs*. chlorophyll concentrations (*A*) and maximum PSII photochemical efficiency (F_v/F_m) (*B*) in senescing leaves of *P. quinquefolia*. Sampling and symbols as in Fig 2. Each point represents a separate leaf. For all cases, in (*A*), $p<0.001$ and for mid- and late October sampling, R^2 = 0.483and R^2 = 0.700, respectively. Corresponding levels of significance for (*B*) are p=0.002, p=0.003 and p=0.001, respectively.

linked to leaf redness (Fig. 7). The degree of protection, however, was slight and a comparatively high anthocyanin concentration was needed for a few percent less PSII damage.

In view of the above results, we conclude that, for the species investigated in the present study, the protection afforded by anthocyanins against photoinhibition of PSII is marginal, if any, especially under field conditions. One cannot exclude, however, that during particularly cold autumns, protective effects may be more significant. Hence, if protective, leaf redness constitutes a measure taken in advance of a possible stress and remains after the stress has passed away. This lack of flexibility has been emphasized in the past (Manetas 2006) and contrasted to other means against photoinhibition, both behavioral (leaf and chloroplast movements) and physiological (xanthophyll cycle), which are engaged as long as needed. Anthocyanins, on the other hand, not only appear well in advance, but degrade very slowly after the removal of stress. In the mean time, they passively absorb visible light which is lost for photosynthesis. Apparently, the cost of lost photons is higher when natural light is not saturating for photosynthesis, *i.e.* during autumn with increasing solar zenith angle. It may also be noted that leaf senescence is an energetic developmental stage and, although the majority of chloroplasts loose gradually their photosynthetic function, some remain functional for a longer time (Keskitalo *et al.* 2005).

Another argument against the photoprotective hypothesis is that the absorption spectrum of anthocyanins does not match the action spectrum of photoinhibition, as one would expect for an ideal sunscreen device (Manetas 2006). We may therefore postulate that photosynthetic pigments may not be the target of photoprotection by anthocyanins and research may probably turn to other candidate molecules satisfying two requirements. First, needing continuous protection and, second, displaying an absorption spectrum more or less similar to that of anthocyanins. One such case has already been described and concerns the defensive, photodynamic molecule

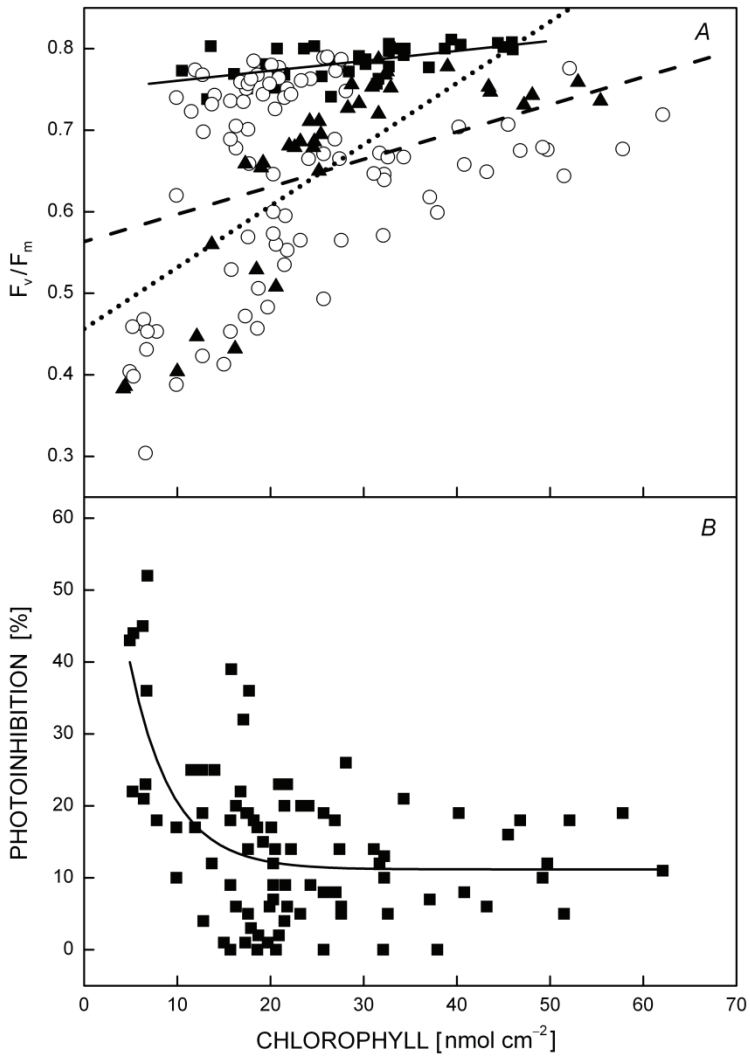

Fig. 6. Chlorophyll concentration *vs*. maximum PSII photochemical efficiency (F_v/F_m) (*A*) and percent reduction of F_v/F_m (*B*) in senescing leaves of *P. quinquefolia*. Each point represents a separate leaf. In (*A*), sampling and symbols as in Fig 2. In (*B*), the photoinhibitory treatment applied as in Fig 4. In both (*A*) and (*B*), levels of significance are $p<0.001$ in all cases. In (*B*), $R^2 = 0.359$.

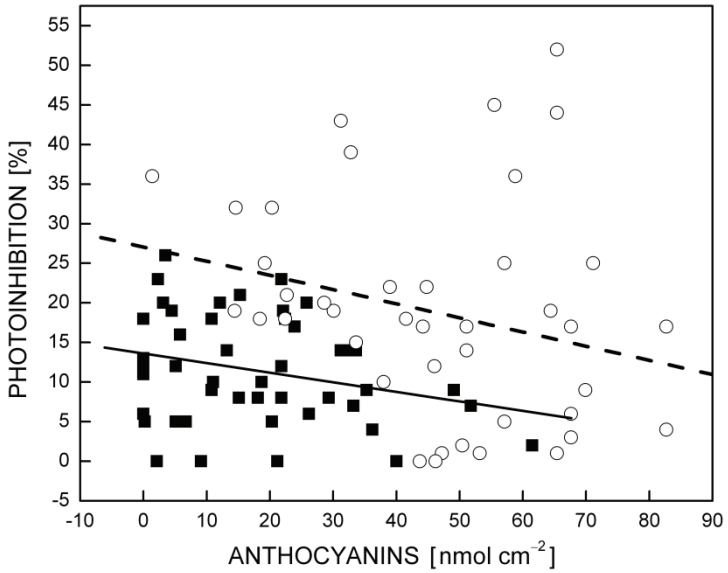

Fig 7. Percent reduction of F_v/F_m *vs*. anthocyanin concentrations in senescing leaves of *P. quinquefolia* with high- (>20 nmol cm^{-2}, ■, *solid line*) or low (<20 nmol cm^{-2}, ○, *dashed line*) chlorophyll levels. Photoinhibitory treatment as in Fig. 5. Corresponding levels of significance are $p=0.073$ and $p=0.096$, respectively.

thiarubrin in the stems and petioles of *Ambrosia chamissonis* (Page and Towers 2002). Another candidate could be the highly toxic, green absorbing, red Chl catabolite (RCC) continuously produced during Chl catabolism in senescing leaves (Hortensteiner 2006).

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