Strength of winter leaf redness as an indicator of stress vulnerable individuals in *Pistacia lentiscus*

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**Abstract**

Leaf anthocyanins are believed to afford protection against photoinhibition, yet the dependence of protection on actual anthocyanin concentrations has not been investigated. To that aim, non-invasive optical methods (spectral reflectance and chlorophyll fluorescence) were used to assess the levels of anthocyanins and chlorophylls as well as photosystem II photochemical efficiency in hundreds of leaves from the mastic tree (*Pistacia lentiscus*), which displays in the field a continuum of leaf tints during winter from fully green to fully red. Contrary to expectations based on the photoprotective hypothesis, the strength of leaf redness was positively correlated to the extent of mid-winter chronic photoinhibition and negatively correlated to leaf chlorophyll contents. Hence, a photoprotective role for anthocyanins is not substantiated. Instead, we argue that winter leaf redness may be used reliably, quickly and non-invasively to locate vulnerable individuals in the field.

**Introduction**

During some developmental stages or under environmental stress, the leaves of some plant species accumulate anthocyanins at levels enough to mask the green chlorophyll color. Since anthocyanins absorb visible light without being photosynthetic, their presence entails a photosynthetic cost due to photon loss. Nevertheless, the transient red leaf character has been preserved and several compensating functions have been proposed (see Archetti et al., 2009; Gould, 2004; Manetas, 2006; Steyn et al., 2002 for reviews). Among physiologists, the most popular hypothesis is that of photoprotection. It maintains that whenever the balance between light absorption and further processing of redox energy is perturbed and the usual means of overexcitation avoidance (or tolerance) are surpassed, the accumulation of anthocyanins may function as a passive light screen and/or detoxifying reactive oxygen species (see reviews above). However, empirical evidence is contradicting, with some reports supporting (Feild et al., 2001; Hughes et al., 2005; Hughes and Smith, 2007; Manetas et al., 2002), but others finding no evidence for a photoprotective role (Burger and Edwards, 1996; Esteban et al., 2008; Karageorgou and Manetas, 2006; Kyparissis et al., 2007). Moreover, in the case of winter leaf reddening in the Mediterranean shrub *Cistus creticus*, it was shown that the red-leaf phenotype was physiologically inferior and less tolerant than the green phenotype to the combination of low temperature and high light in the field (Kytridis et al., 2008; Zeliou et al., 2009).

In all the above studies, the behavior of fully red versus green leaves, species or phenotypes was compared. However, the effects of the concentration of accumulated anthocyanins have been ignored up to now. We argue that the extent of protection would depend on the effectiveness of the screen and/or its anti-oxidative potential, both apparently being concentration-dependent. Alternatively, if leaf redness characterizes individuals inherently vulnerable to photoinhibition, the extent of vulnerability may also depend on the amount of accumulated anthocyanins. During preliminary field observations for the location of appropriate plants, we found that *Pistacia lentiscus* (mastic tree) does not simply display a clear green/red phenotypic distinction during winter, but a continuous range of visual tints (i.e. anthocyanin levels) under apparently similar field conditions. We report herewith on correlations between anthocyanin concentrations, chlorophyll levels and extent of chronic photoinhibition of photosystem II and discuss the use of winter leaf redness as a possibly reliable indicator of photosynthetic vigor.

**Materials and methods**

**Plant material and sampling site**

*Pistacia lentiscus* L. (Anacardiaceae) is a Mediterranean evergreen shrub with sclerophyllous leaves. Leaf duration is 2–3 years with old leaf fall occurring at late spring/early summer (Orshan,
1989). In preliminary field observations we noticed that, during winter, leaves of some individuals may turn red while neighboring plants, under the same apparent environmental conditions (i.e. similar exposure and on the same soil) remain green. Considerable variation exists in the strength of redness, both on a population and individual plant basis. Thus, some plants display a visibly homogeneous leaf color ranging from fully green to fully red, while others may display variation in leaf color on the same individual. Leaf sampling from the latter plants was avoided. Leaf redness remains up to early spring and the leaf color during late spring, summer and autumn is invariably green in all plants. A well established sand dune population located in South-West Greece (38°09’ N, 21°22’ E, 3 m a.s.l.) was used. On 24th February 2007, forty three green and nineteen red plants of various tints were tagged and four equally exposed leaves per plant (i.e. a total of 248 leaves) were sampled. Leaves from each plant were separately put in air-tight small plastic envelopes lined internally with moist filter paper and transferred to the laboratory in a dark box. Chlorophyll fluorescence was measured from both the right and left adaxial side after dark adaptation for three hours. Then, spectral reflectance in the visible/near infra-red band was measured on the same spots and the means for each plant (n=2 × 4=8) were computed.

In late winter of the following year (2008), the study site was visited again and a sub-sample of 12 plants (i.e. 6 green and 6 red) were re-tagged and leaves sampled, to confirm the results obtained during the previous winter of 2007. A third sampling was performed from the same 12 plants in mid-autumn of 2008, i.e. during the “green” period of the year to see whether the patterns observed during the winter-stress period are maintained during the favorable period of the year.

Chlorophyll fluorescence in dark-adapted leaves was measured with a Mini-PAM (Walz, Effeltrich, Germany) fluorimeter. After measuring fluorescence with all PS II centers open by means of a weak (0.05 μmol m⁻² s⁻¹) modulated beam (Fₒ), a saturating pulse of 8,000 μmol m⁻² s⁻¹ was applied to close all PS II reaction centers and drive fluorescence yield at its maximum (Fm). The PS II photochemical efficiency was computed as Fm/Fm=(Fm-Fₒ)/Fm according to Maxwell and Johnson (2000). Leaf spectral reflectance in the range 400–1100 nm was measured with a spectroradiometer (UniSpec, PP Systems, Haverhill, MA, USA) equipped with an internal light source, a bifurcated optical fiber applied directly on the leaf surface and a diode-array detector. A Spectralon (reflectance > 97%) standard was used as a reference and the spectra were dark corrected for stray light with the internal source off. The following spectral indices were computed:

1. Anthocyanin reflectance index (ARI) as

\[ \text{ARI} = \frac{(R_{550})^{-1} - (R_{700})^{-1}}{2} \]

where \((R_{550})^{-1}\) and \((R_{700})^{-1}\) are inverse reflectances at 550 and 700 nm, respectively. ARI is linearly and positively correlated to leaf anthocyanins and is independent of chlorophyll levels (Gitelson et al., 2001).

2. Normalized difference index (NDI) as

\[ \text{NDI} = \frac{(R_{750} - R_{705})}{(R_{750} + R_{705})} \]

where \(R_{750}\) and \(R_{705}\) are reflectances at 750 and 705 nm, respectively. NDI is linearly and positively correlated to leaf chlorophyll and is independent of anthocyanin levels (Gitelson and Merzlyak, 1994).

Regression lines for the various measured parameters were drawn with the help of SPSS (v.15.0) statistical package. When needed (see Table 1), significance of differences in the measured parameters between phenotypes within a season or between seasons were assessed by one-way ANOVA and the same statistical package.

### Results

In both sampling years, naked eye evaluation of redness roughly coincided with mean tree ARI above 0.035 and up to 0.220. Corresponding ARI values of visibly green plants ranged between ca. 0.004–0.025 (Fig. 1). Hence, judging a posteriori, shrubs subjectively assigned to the red phenotype had, indeed, higher ARI values. Fig. 1 shows that green plants displayed considerable variation in maximum PS II photochemical efficiency (as \(Fm/Fm\)), ranging from roughly 0.70–0.83. Red plants displayed generally lower \(Fm/Fm\) and a significant negative correlation between leaf redness and PS II photochemical efficiency. The same was true for the anthocyanin versus chlorophyll correlation (Fig. 2). Accordingly, as the leaves become redder, the risk of photoinhibition increases and the levels of chlorophyll decrease. As a result, a strongly positive correlation was obtained in the \(Fm/Fm\) versus NDI curves (Fig. 3).

Results in Figs. 1–3 refer to the sampling of winter 2007 and were confirmed by a second sampling in winter of 2008 (Table 1). Hence, during two consecutive years, red plants displayed lower leaf chlorophyll levels and higher risk of chronic photoinhibition. The possibility that winter-red plants maintain lower chlorophyll levels and PS II photochemical efficiencies even during the favorable, “green” period of the year was rejected since sampling from the same plants in autumn of 2008 revealed similar values for all measured parameters in both phenotypes (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>February</th>
<th>October</th>
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<tr>
<td>Fm/Fm</td>
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<td>0.730 ± 0.019b</td>
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<tr>
<td>NDI</td>
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### Fig. 1

Maximum PS II photochemical efficiency (\(Fm/Fm\)) versus leaf anthocyanin content (ARI) in 43 visibly green (●) and 19 red (○) plants of various tints. Parameters measured during the “red” period of the year (i.e. late winter). Data points are means from 4 leaves (two measurements per leaf). \(p^2=2.98 \times 10^{-12}; r^2=0.55\).
anthocyanins. Yet, as shown in Fig. 1 for red plants (empty circle) since light attenuation apparently increases with the level of light. If vulnerability to stress is linked to a physiological risk induced by cold temperatures and high light. Accordingly, we assume that red phenotypes are more vulnerable to stress than green (full circle). ARI means from 4 leaves (two measurements per leaf). p=2.74 × 10^{-15}; r²=0.52.

![Fig. 2. Chlorophyll content (as NDI) versus anthocyanin content in 43 visibly green (●) and 19 red (○) plants of various tints. Parameters measured during the "red" period of the year (i.e. late winter). Data points are means from 4 leaves (two measurements per leaf).](image)

![Fig. 3. Maximum PS II photochemical efficiency (Fv/Fm) versus chlorophyll content (NDI) in 43 visibly green (●) and 19 red (○) plants of various tints. Parameters measured during the "red" period of the year (i.e. late winter). Data points are means from 4 leaves (two measurements per leaf). p=9.6 × 10^{-15}; r²=0.42.](image)

Discussion

In spite of predictions set by the photoprotective hypothesis, red leaves displayed lower dark-adapted PS II photochemical efficiencies during winter in both sampling years (Fig. 1). Accordingly, we assume that red phenotypes are more vulnerable to photoinductive risk induced by cold temperatures and high light. If vulnerability to stress is linked to a physiological inferiority of the red phenotype (as recently proposed for another, winter-red, Mediterranean shrub i.e. Cistus creticus, Kytidis et al., 2008), one could expect a positive correlation between leaf redness and dark-adapted PS II yield within the red phenotype, since light attenuation apparently increases with the level of anthocyanins. Yet, as shown in Fig. 1 for red plants (empty circles), a strong decreasing trend in Fv/Fm is evident as ARI increases, indicating that the more red the leaves are, the more vulnerable they are to stress. Accordingly, if anthocyanins protect against photoinduction, the protection is weak and cannot alleviate the inherent physiological inferiority of the red phenotype.

Many Mediterranean plants suffer from a winter drop in PS II photochemical efficiency (García-Plazaola et al., 2000; Karavatas and Manetas, 1999) and this is also evident in P. lentiscus, where a season effect was observed in both phenotypes (Table 1). Yet, our results indicated that this drop is a statistical attribute of the population, consisting of a mixture of relatively resistant and more vulnerable individuals. It happens in this plant that the more vulnerable individuals are those exhibiting also the winter-red leaf character.

Vulnerability to stress in the red phenotype is also evident by the decrease in chlorophyll levels with anthocyanin increase (Fig. 2). Transient reduction of chlorophylls has been reported in some evergreens both during winter and summer and the adaptive significance of lowering the excitation pressure on the photosynthetic machinery during the unfavorable periods by some chlorophyll loss has been discussed (Kyparissis et al., 1995; Varone and Gratani, 2007). Hence, a relief from chlorophyll overexcitation could be afforded by the combined decrease of target molecules and increase in the light attenuating anthocyanins. However, it seems that the result of the assumed double action is not enough to maintain the light capturing efficiency of PS II to optimal levels in the red phenotype.

Since our results do not support the alleged photoprotective function, we may turn to the alternative ecological hypotheses, which consider red leaf color as a warning signal against insect herbivory (for reviews see Archetti et al., 2009, and Manetas, 2006). Yet, this is also unlikely, since a signal, either honest or mimetic (Karageorgou et al., 2008), might be useless during the winter period, when herbivorous insect pressure is at minimum.

Regardless of anthocyanin function, our results suggest that leaf redness may be a sensitive indicator of high photoinductive risk and physiological weakness. Apparently, the idea applies only to the species tested and should be confirmed with more winter-red species in the field. Also, the idea may not apply to redness observed in young or senescing leaves, although Sinkkonen (2008) reported a higher probability of autumnal leaf redness in drying branches of Acer platanoides. In any case, spectral reflectance measurements are quickly reliable and non-invasive. They can be applied on a leaf or, remotely, on a population or ecosystem basis with ground or satellite-based spectrometers (Hatfield et al., 2008). Location of vulnerable individuals and monitoring of plant, population or ecosystem health and quality are possible applications.

References


Manetas, Y., 2006. Why some leaves are anthocyanic and why most anthocyanic leaves are red? Flora 201, 163–177.


