Functional role of red (retro)-carotenoids as passive light filters in the leaves of Buxus sempervirens L.: increased protection of photosynthetic tissues?

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Abstract

Red (retro)-carotenoids accumulate in chloroplasts of Buxus sempervirens leaves during the process of winter leaf acclimation. As a result of their irregular presence, different leaf colour phenotypes can be found simultaneously in the same location. Five different colour phenotypes (green, brown, red, orange, and yellow), with a distinct pattern of pigment distribution and concentration, have been characterized. Leaf reddening due to the presence of anthocyanins or carotenoids, is a process frequently observed in plant species under photoinhibitory situations. Two main hypotheses have been proposed to explain the function of such colour change: antioxidative protection exerted by red-coloured molecules, and green light filtering. The potential photoprotective role of red (retro)-carotenoids as light filters was tested in Buxus sempervirens leaves. In shade leaves of this species the upper (adaxial) mesophyll of the lamina was replaced by the equivalent upper part of a different colour phenotype. These hybrid leaves were exposed to a photoinhibitory treatment in order to compare the photoprotective effect exerted by adaxial parts of phenotypes with a different proportion of red (retro)-carotenoids in the lower mesophyll of a shade leaf. The results indicated that the presence of red (retro)-carotenoids in the upper mesophyll did not increase photoprotection of the lower mesophyll when compared with chlorophyll, and the best protection was achieved by an upper green layer. This was due to the fact that the extent of photoinhibition was proportional to the amount of red light transmitted by the upper mesophyll and/or to the chlorophyll pool located above. These results do not exclude a protective function of carotenoids in the upper leaf layer, but imply that, at least under the conditions of this experiment, the accumulation of red pigments in the outer leaf layers does not increase photoprotection in the lower mesophyll.

Key words: Common box, leaf reddening, photoinhibition, photoprotection, retro-carotenoids, winter acclimation.

Introduction

There is an evolutionary convergence to accumulate red compounds (mainly anthocyanins, but also retro-carotenoids and betacyanins) in photosynthetic organs under unfavourable conditions such as suboptimal temperatures, pathogen attacks, nutrient deficiencies, or ultraviolet radiation (Steyn et al., 2002). Despite the wide distribution of this characteristic among higher plants, there is still great controversy regarding its photoprotective function. Most studies point to two main hypotheses: (i) a potential role as passive light filters that would reduce light intercepted by chlorophyll (Chalker-Scott, 1999; Feild et al., 2001; Close and Beadle, 2003; Manetas et al., 2003; Neil and Gould, 2003; Williams et al., 2003); and (ii) the protection from reactive oxygen species (Rice-Evans et al., 1997; Neil et al., 2002; Steyn et al., 2002).
The first is based on the fact that red anthocyanins are mostly located in the outer cell layers of the leaf (Steyn et al., 2002). This character could increase photoprotection of the lower mesophyll by increasing the reflectance of red light in the surface of leaves (Burger and Edwards, 1996; Woodall et al., 1998) by absorbing harmful blue wavelengths (Feild et al., 2001), or by attenuating green light that can penetrate more deeply into the leaf and excite shade-adapted chloroplasts in the lower mesophyll cells (Nishio, 2000).

Although anthocyanins are more frequently responsible for leaf reddening, some higher plants are also able to accumulate red (retro)-carotenoids under photoinhibitory conditions. This is the case of rhodoxanthin in Aloe vera (Diaz et al., 1990), Thuja plicata (Weger et al., 1993), and Cryptomeria japonica (Han et al., 2003, 2004), and eschscholtzixanthin, monoanhydroeschscholtzixanthin, and anhydroeschscholtzixanthin that accumulate in leaves of Buxus sempervirens during winter (Ida et al., 1995; Hormaetxe et al., 2004). The location of retro-carotenoids in plastoglobuli of the outer mesophyll cells (Koiva et al., 1986; Toyama and Funazaki, 1971) also suggests that light interception by these pigments is effectively shielding chloroplasts from excess light energy (Han et al., 2003). Contrasting with higher plants, the accumulation of large amounts (as much as 4% dry weight) of red carotenoids (astaxanthin, canthaxanthin, and myxoxanthophyll) under stress conditions is widespread among green algae (Masojídek et al., 2000) and in cyanobacteria (Miskiewicz et al., 2000).

The similar location of anthocyanins and red (retro)-carotenoids in the outer cells of the mesophyll, and their induction by photoinhibitory conditions, could easily lead to some of the functions of anthocyanins being extrapolated to carotenoids. However, the analogy between both kinds of compounds should be done with care, since there are some essential differences between these compounds: anthocyanins are hydrophilic compounds that accumulate in the vacuole, while carotenoids are lipophilic molecules present in chloroplasts. This chloroplastic location could imply a more dynamic physiological role (Han et al., 2003, 2004), especially as retro-carotenoids and xanthophyll cycle pigments are biosynthetically related.

The aim of the present study was to analyse to what extent light shielding by red (retro)-carotenoids, independently of other functions, protects chloroplasts from photoinhibition. Much of the research done with red pigments is based on the comparison of red and green phenotypes, but this design has an intrinsic limitation since red and green leaves are developmentally or phenologically different, and fluorescence techniques (the usual indicator of photoinhibition) only measure the upper cell layers. To overcome these difficulties, common box (Buxus sempervirens L.) was used as the model species. This is a submediterranean evergreen tree characterized by a wide ecological tolerance. That evergreen leaves are able to adapt to a wide range of environmental conditions must be due, in part, to high antioxidant and xanthophyll cycle pools (García-Plazaola et al., 2000, 2003). In parallel with the synthesis of photoprotective compounds, leaves of this species also accumulate red (retro)-carotenoids during stress conditions (Hormaetxe et al., 2004). Leaves of this species possess two unique traits that can help to clarify the role of red (retro)-carotenoids as light filters: (i) they can easily be split into two parts (adaxial and abaxial) because there is limited connection between the upper and lower mesophyll; and (ii) they show a large phenotypic variability, and plants with different colour patterns [due to different proportions of red (retro)-carotenoids] occur simultaneously in the same location. Based on both traits, it has been possible to compare the photoprotective effect exerted by each phenotype by replacing the adaxial mesophyll of shade leaves with the equivalent adaxial part of a coloured sun leaf. Shade leaves were used as the experimental model to increase the susceptibility to photoinhibition.

Materials and methods

Plant material and experimental design

Common box (Buxus sempervirens L.) is a sclerophyllous evergreen species that forms small trees or shrubs. It shows a remarkably wide ecological amplitude and stress tolerance, and so it is able to grow in highly contrasting habitats such as the deep shade of the beech forests and the sunny slopes of the Mediterranean mountains at high altitudes. In the present study, different colour phenotypes (green, brown, orange, red, and yellow) of B. sempervirens were obtained in winter from two sun-exposed localities: Buggedo (42° 36’ N; 13° 01’ W; 500 masl) and Criales (42° 53’ N; 3° 19’ W; 650 masl). A complete description of pigment composition of whole leaves of these phenotypes is presented in Hormaetxe et al. (2004). Shade leaves were obtained from B. sempervirens plants growing in the understory of a pine (Pinus sylvestris) forest in Criales. Shade leaves were collected and kept in the dark for 12 h at room temperature (20–22°C) to allow a complete recovery from reversible photoinhibition and to provide comparable conditions. In each shade leaf, a disc (diameter 6 mm) of the upper mesophyll was removed and replaced by the equivalent adaxial disc of a coloured sun leaf (Fig. 1). The effect of this procedure on optical properties was tested in shade leaves before and after grafting their own upper mesophyll; no significant differences were found at P < 0.001 for the wavelength range 400–750 nm. The pigment composition of the upper sun discs and lower mesophyll layer of shade leaves is shown in Table 1. Temporary foliar hybrids constructed in this way were called GH, BH, RH, OH, and YH when the shade abaxial part was covered by green, brown, red, orange, or yellow adaxial discs, respectively. These leaves were exposed to mild photoinhibitory light conditions (30 min at 25 °C and 1000 μmol photons m−2 s−1) as described in Dodd et al. (1998). Other photoinhibitory treatments were also conducted at suboptimal temperatures (15 °C) or supraoptimal temperatures (40 °C), as well as in the presence of the herbicide paraquat (1,1’-dimethyl-4, 4’-bipyridinium chloride) in order to exacerbate photoinhibitory effects and the photoprotective potential of each phenotype. When this herbicide was used, 50 μl of 10 μM paraquat was added directly to the lower mesophyll of shade leaves. After treatments, these adaxial discs were removed and immediately
Microscope (Wetzlar, Germany) fitted with a carotenoids was examined under bright field microscopy and the different sun phenotypes. The histological location of red (retro)-carotenoids was calculated as 1–reflectance–transmittance.

Analytical methods
Pigment composition was studied in adaxial and abaxial leaf discs after photoinhibitory treatment, as well as in five samples from whole leaves of each phenotype. Photosynthetic pigments were extracted and measured by reverse-phase HPLC following the method of García-Plazaola and Becerril (1999) with the modifications described in García-Plazaola and Becerril (2001). Red (retro)-carotenoids were identified by their respective spectra and retention time, and quantified by the extinction coefficients reported by Ida et al. (1995).

Fluorescence
Chlorophyll a fluorescence was measured as described in Hormaetxe et al. (2004). The maximal photochemical efficiency of photosystem II was estimated by the ratio $F_{v}/F_{m}=\left(F_{m}-F_{n}\right)/F_{m}$ and the degree of photoinhibition as the percentage of decrease of the initial $F_{v}/F_{m}$ values after the photoinhibitory treatment. Initial $F_{v}/F_{m}$ of the abaxial mesophyll of shade leaves before the treatments was on average 0.78 ($n=15$), indicating that shade plant material was not suffering winter chronic photoinhibition (Werner et al., 2002) when sampled in the field. As occurred with optical properties the effect of grafting on chlorophyll fluorescence was also negligible in shade leaves.

Leaf reflectance and transmittance
Leaf optical properties were measured in whole leaves and detached adaxial and abaxial surfaces on five plants per phenotype with a UNISPEC, spectroradiometer FieldSpec UV/NIR portable spectral analysis system (PPsystems, Amesbury, USA) with the optic fibre, leaf-clip holder, and reference provided by the manufacturer. Five replicates per phenotype were made at 3.3 nm intervals over a range of 400–750 nm. The standard error of these measurements represented, on average, 9.4% of the mean. Leaf reflectance and transmittance are defined, respectively, as the proportion of incident light that is reflected from, and transmitted through, the leaf. Absorptance is calculated as 1–reflectance–transmittance.

Microscopy
Transverse hand-cut sections of fresh leaves were taken from the different sun phenotypes. The histological location of red (retro)-carotenoids was examined under bright field microscopy and the presence of chlorophyll by epifluorescence microscopy. Light and epifluorescence microscopy were performed using a Leica DM2500 microscope fitted with a ×20 PI-Fluor objective and a Leica filter set for ultraviolet (UV) (set A with BP 340–380 excitation, RKP 400 chromatic beam splitter, and LP 430 barrier filter). Red fluorescence indicates the presence of chlorophyll.

Simultaneous bright field and epifluorescence pictures were taken of each leaf sample.

Statistics
Linear regression was used to analyse the relationships between light absorption by the lower mesophyll layer and the degree of photoinhibition for the whole wavelength range (400–750 nm) and at 50 nm intervals. The same approach, after logarithmic transformation of chlorophyll content values, was used to establish the relationship between the degree of photoinhibition and chlorophyll content. Calculated $P$ values, coefficients, and regression lines are indicated on the figures or tables whenever significant at $P<0.05$.

Results
Sun phenotypes used in this study showed marked differences in structure and composition (Table 1). Colour differences were mainly based on a different proportion of chlorophyll and yellow/red carotenoids, and this plant material was the basis for this comparative study. This pattern of pigment distribution allowed four main groups to be differentiated: green leaves without red (retro)-carotenoids and high chlorophyll content, yellow leaves with low chlorophyll and red (retro)-carotenoid contents, brown leaves with high chlorophyll and red (retro)-carotenoid contents, and orange and red leaves with a high pool of red (retro)-carotenoids and low chlorophyll. In green leaves, chlorophyll was distributed uniformly across a leaf section (Fig. 2) while, in the other phenotypes, it was located mainly in the lower mesophyll. In the case of yellow and orange phenotypes, epifluorescence was lower and chlorophyll was exclusively present in the deeper parts of the mesophyll. On the other hand, red pigments (when present) were mostly located in the upper palisade mesophyll.

Differential patterns of pigment distribution and composition among phenotypes resulted in important differences in leaf optical properties (Fig. 3). Thus, reflectance in the red region (630–690 nm) was higher in all phenotypes when compared with green leaves. However, transmittance in the same region was also 3.2-, 9.5-, 10.1-, and 14.8-fold higher in brown, red, orange, and yellow phenotypes, respectively, when compared with green leaves. In all phenotypes, transmittance at wavelengths lower than 500 nm was negligible, indicating that the yellow pigments and chlorophyll are sufficient to protect from harmful blue radiation.
Table 1. Pigment composition (chlorophyll, red carotenoids, neoxanthin, lutein, xanthophyll cycle, β-carotene and xanthophyll esters) of adaxial discs (upper mesophyll) from sun leaves of the five phenotypes studied and abaxial disc (lower mesophyll) from control green shade leaves.

Pigment concentration is shown in μmol pigment m⁻² leaf area. Each value is the mean of five replicates ± standard error.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Chl a+b (μmol/m²)</th>
<th>Red carot. (μmol/m²)</th>
<th>Neoxan. (μmol/m²)</th>
<th>Lutein (μmol/m²)</th>
<th>Xan. cycle (μmol/m²)</th>
<th>β-Carot. (μmol/m²)</th>
<th>Xan. esters (μmol/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red upper</td>
<td>45.8±6.5</td>
<td>64.8±6.6</td>
<td>2.2±0.3</td>
<td>30.6±1.6</td>
<td>17.0±3.8</td>
<td>5.9±1.6</td>
<td>14.1±14</td>
</tr>
<tr>
<td>Brown upper</td>
<td>93.1±14.2</td>
<td>24.6±5.2</td>
<td>3.1±0.5</td>
<td>32.3±4.0</td>
<td>32.4±6.1</td>
<td>10.3±2.0</td>
<td>5.8±1.9</td>
</tr>
<tr>
<td>Orange upper</td>
<td>57.4±9.4</td>
<td>29.9±4.4</td>
<td>1.9±0.4</td>
<td>24.4±2.2</td>
<td>26.2±4.2</td>
<td>8.1±1.0</td>
<td>21.0±2.1</td>
</tr>
<tr>
<td>Yellow upper</td>
<td>28.9±6.9</td>
<td>3.1±1.6</td>
<td>0.9±0.3</td>
<td>13.9±2.7</td>
<td>16.3±3.7</td>
<td>3.3±0.7</td>
<td>4.3±1.2</td>
</tr>
<tr>
<td>Green upper</td>
<td>188.1±11.9</td>
<td>0</td>
<td>7.3±0.4</td>
<td>45.5±3.2</td>
<td>37.6±3.3</td>
<td>20.1±0.7</td>
<td>0</td>
</tr>
<tr>
<td>Shade lower</td>
<td>86.5±4.5</td>
<td>0</td>
<td>3.5±0.2</td>
<td>10.7±0.9</td>
<td>3.5±0.1</td>
<td>6.4±0.6</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 2. Transverse sections of fresh B. sempervirens leaves of red, brown, orange, yellow, and green phenotypes under bright field (upper panels) or epifluorescence microscope (lower panels).
In the foliar hybrids experiment, transmittance of the upper mesophyll was proportionally higher than in entire leaves, but their spectral characteristics remained basically identical (Fig. 4A). Blue light transmittance was almost zero in all phenotypes. For all wavelengths the highest transmittance was observed in yellow adaxial parts. In the other phenotypes there was a shift of the maximum from the green to the red region that moved to higher wavelengths as the pool of red (retro)-carotenoids increased. The total amount of radiation that passed through green and brown leaves was quantitatively similar at wavelengths lower than 650 nm, but differed markedly in the red region, thereby allowing the comparative study of the protective effects of green light filtering. By multiplying transmittance of the adaxial part by the absorptance of the abaxial part of the shade leaves (Fig. 4B) for each wavelength, the proportion of photons that are effectively absorbed by the lower mesophyll (Fig. 4C) in the hybrid leaves experiment was obtained. On average, this proportion ranged between 1.5% in GH and 5.2% in YH. In all hybrid leaves, the highest wavelength absorption was between 580 and 700 nm (red light mainly). In the blue light range, light absorption by the lower mesophyll layer was negligible in GH, BH, and RH, but its use was much higher in the green region in leaves covered by YH or OH. Light absorption by the lower mesophyll layer by RH and OH was fairly similar at wavelengths higher than 550, but was 4-fold higher at lower wavelengths in OH. The comparison of RH and OH on the one hand, and GH and BH on the other, allows the photoprotective effect of filtering blue-green light to be studied.

When the total light absorption by the lower mesophyll layer and degree of photoinhibition induced by several photoinhibitory treatments were plotted separately for each experiment, the correlation was significant except at 40 °C (Fig. 5), indicating a direct relationship between photon absorption and photoinhibition. These correlations imply, for example, that, when PPFD is 1000 μmol photons m⁻² s⁻¹, illumination with only 50 μmol photons m⁻² s⁻¹ is able to generate a photoinhibition of 21% at 25 °C in YH. When the same analysis was performed but with light absorption by the lower mesophyll layer considered at 50 nm intervals (Table 2), there was no significant correlation at all at 40 °C, and significance was only obtained at wavelengths higher than 600 nm, except at 15 °C when the significance of the regression extended to the interval 550–700 nm.

Since leaves covered by green upper mesophylls showed the lowest energy use, and the best correlation between photoinhibition and light absorption by the lower mesophyll layer was in the red region, it was predicted that the chlorophyll content of the upper part would be the main component responsible for light absorption and photoprotection of the lower mesophyll. To confirm this hypothesis, the chlorophyll content of the upper disc was plotted against the degree of photoinhibition in the abaxial part for each individual sample (Fig. 6). Significant correlations were obtained in all experiments except in the 40 °C treatments. At high temperatures, heat-induced damage is probably higher than light-induced photoinhibition.

**Discussion**

Previous results (Hormaetxe et al., 2004) showed the accumulation of red (retro)-carotenoids in B. sempervirens leaves in parallel with the activation of photoprotection mechanisms. This finding, and the location of red (retro)-carotenoids in chromoplasts of the outer cell layers (Koiwa et al., 1986), led to the speculation that these pigments could play a protective role by decreasing light interception by chloroplasts which are situated below and chlorophyll overexcitation. Previous studies on the protective role of red pigments (Dodd et al., 1998; Woodall et al., 1998; Gould et al., 2000; Neill et al., 2002; Pietrini et al., 2002; Close and Beadle, 2003; Han et al., 2003, 2004; Manetas et al., 2003; Neill and Gould, 2003) compare green and red leaves that usually correspond to different phenotypes or phenological states, implying that the same plant material is not being compared. In the present experiments,
advantage was taken of the different leaf colour phenotypes (green, brown, red, orange, and yellow) of *B. sempervirens* that occur simultaneously in the same location under stress conditions as a result of the different proportions of chlorophyll and yellow/orange carotenoids. However, the main goal of the present experimental design was to assay photoinhibition in the mesophyll of the same type of shade leaves so that side-effects related to intrinsic phenotype or developmental differences could be avoided. This was due to the unique morphological traits of the common box leaves (described previously) that allow the construction of foliar hybrids (Fig. 1).

In all phenotypes, red (retro)-carotenoids (when present) accumulated in cell layers just below the epidermis, while chlorophyll was more concentrated in the lower mesophyll. This pattern has been typically described in other species possessing red leaves (Woodall *et al.*, 1998; Gould *et al.*, 2000; Pietrini *et al.*, 2002; Steyn *et al.*, 2002; Manetas *et al.*, 2003) and supports a role for red pigments attenuating visible light. This would generate shade conditions in the lower mesophyll. In fact, these characteristics of shade leaves have been described in red leaves of *Quercus coccifera* (Manetas *et al.*, 2003) and *Quintinia serrata* (Gould *et al.*, 2002). However, in yellow phenotypes [with a very low content of red (retro)-carotenoids] chlorophyll was also located in the lower mesophyll (Fig. 2), indicating that the distribution of red pigments in the adaxial surface and chlorophyll in the lower mesophyll is not a conclusive clue to the protective role of carotenoids but, furthermore, is...
a general feature of overexcited leaves. Moreover, as has been shown in other species (Burger and Edwards, 1996; Woodall et al., 1998), red leaves of *B. sempervirens* reflected more radiation at 630–690 nm (this range corresponds with wavelengths strongly absorbed by chlorophyll) than green leaves (1.8-fold), but transmittance in the same wavelength range for the upper mesophyll was also 5-fold higher in red leaves.

The results of the experiment with foliar hybrids did not fully support the initial hypothesis that red (retro)-carotenoids may serve an auxiliary photoprotective role by light shielding (Hormaeche et al., 2004). Thus the best protection of the photosynthetic machinery in the abaxial part was obtained, in all experiments, when a green layer was above, and the degree of photoinhibition after the experiments was proportional to the chlorophyll content of the upper disc (Fig. 6). In fact, when green and brown leaves are compared [the latter contain a large amount of chlorophyll and red (retro)-carotenoids and filter more effectively green light], no benefit was observed for the lower mesophyll covered by brown upper discs, and the chlorophyll content was again the main determinant of photoprotection. This is not the first study that is inconsistent with an auxiliary photoprotective role for red pigments. In other species, red leaves experience greater levels of chronic photoinhibition than green leaves (Dodd et al., 1998; Choinski et al., 2003; Williams et al., 2003) or an enhanced antioxidant content (Neil et al., 2002) indicative of higher photoprotective demand. The lack of association of anthocyanins with an auxiliary photoprotective role has also been suggested in the evergreen *Quintinia serrata* (Gould et al., 2000).

These results should be taken with care since blue light penetration was almost zero in this species, but this is not necessarily a general feature of leaves. In any case the experimental approach is limited to the abaxial mesophyll (not to the whole leaf) and this does not exclude a direct protective function of red carotenoids in the adaxial mesophyll that in fact absorbs most of the incident light. Moreover, when yellow and orange or red leaves are compared, a beneficial light-filtering effect by possessing red (retro)-carotenoids can be seen. Since all these phenotypes seem to survive winter stress, they probably represent different strategies to achieve photoprotection. Despite these limitations, and assuming the higher protection as a light filter exerted by chlorophyll in the upper mesophyll, the question should be why is there is no green pigment in the plant kingdom able to protect chlorophyll by effective absorption of blue and red light and why is the presence of red pigments associated with photoinhibitory situations.
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