PHOTOSYNTHETICA 42 (4): 591-596, 2004

Carotenoid composition of peridermal twigs does not fully conform to a shade acclimation hypothesis

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Abstract

The photosynthetic pigments of twigs in five tree and shrub species possessing chlorenchyma under a well developed, stomata-less, and highly photon absorptive periderm were analysed and compared to those of the corresponding canopy leaves. We asked whether the unavoidable shade acclimation of corticular chlorenchyma results in photosynthetic pigment complements typically found in shade leaves. As expected, chlorophyll (Chl) *a/b* ratios in twigs were consistently low. However, carotenoid (Car) analysis did not confirm the initial hypothesis, since twigs generally contained increased Chl-based pool sizes of the xanthophyll cycle components. The contents of photo-selective neoxanthin and lutein were high as well. Yet, β-carotene content was extraordinarily low. In addition, twigs retained high pre-dawn ratios of the de-epoxidized antheraxanthin and zeaxanthin, although environmental conditions were not pre-disposing for such a state. The unexpected Car composition allows the conclusion that other micro-environmental conditions within twigs (hypoxia, increased red to blue photon ratios, and extremely high CO_2 concentrations) are more important than shade in shaping the Car profiles.

Additional key words: *Arbutus*; chlorophylls; leaves; *Pistacia*; *Populus*; *Prunus*; *Quercus*; sun/shade acclimation; twig internal micro-environment; xanthophyll cycle.

Introduction

Photosynthesis is not an exclusive leaf privilege, since petioles, twigs, stems, and even flowers of many plants may contain active chloroplasts. In the case of stems, we may distinguish between stem photosynthesis *per se* and corticular photosynthesis. Stem photosynthesis takes place in green stems bearing abundant and functional stomata and lacking a well-developed periderm (Nilsen 1995). In this case, stem CO_2 assimilation is considerable and is believed to assist in the whole plant carbon gain from the atmosphere, or being the only source of assimilated carbon during the leafless period of the year. Corticular photosynthesis (Pfanz *et al.* 2002) is performed by chlorenchymatous tissues located beneath a highly photon-absorptive periderm lacking stomata. Accordingly, diffusion of gases and transmittance of radiant energy are considerably limited. Diffusion limitations and the high ratio of respiring to CO_2 assimilating tissues result in extremely high internal CO_2 concentrations with corresponding reduction in O_2 concentrations (Pfanz and Aschan 2001, Pfanz *et al.* 2002). Published values range from 1 to 26 % CO_2 and 21 to 5 % O_2, depending on plant species and season (see Pfanz and Aschan 2001 and the literature there-in). In addition, spectral data consistently indicate a preferential attenuation of blue radiation while penetration of longer wavelengths is not appreciably impeded (Kauppi 1991, Kharouk *et al.* 1995, Solhaug *et al.* 1995).

Leaf photosynthetic pigment contents and especially their ratios are acclimated to the prevailing irradiation (Anderson 1986). As a rule, pigments or processes that tend to increase the efficiency of photon capture are favoured under shade, while pigments and processes that harmlessly handle the consequences of over-excitation are favoured under high irradiance, especially with co-occurring water shortage or temperature extremes. Thus, chlorophyll (Chl) *a/b* ratios are low in shade due to the high ratios of light-harvesting complexes per reaction centres (Anderson 1986). In addition, shade chloroplasts display lower Chl-based pool sizes of the potentially photo-protective β-carotene and the components of the xanthophyll cycle (Demmig-Adams *et al.* 1989, Thayer and Björkman 1990, Rosevear *et al.* 2001), while the supposedly photo-selective neoxanthin and lutein are not so responsive (Hansen *et al.* 2002).

Corticular Chl contents have been measured in many plants (see Pfanz *et al.* 2002 and the literature there-in)

Received 31 May 2004, *accepted* 23 August 2004.

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Abbreviations*:* A = antheraxanthin; Car = carotenoid; Chl = chlorophyll; PS = photosystem; V = violaxanthin; Z = zeaxanthin.

and the consistently low Chl *a/b* ratios have been considered indicative of the shade characters of corticular chloroplasts. In addition, the irradiance dependency of CO_2 fixation (Wittmann *et al.* 2001) and electron transport rates along photosystem 2 (PS2) (Manetas 2004) are compatible with a deep shade adaptation. Corresponding information on carotenoid (Car) pigments in twigs is scarce and concerns only total Car contents assessed from absorbance measurements in crude extracts (Pilarski 1999, Wittmann *et al.* 2001). Therefore, the aim of the present study was to analyse twig Cars and assess whether the shade acclimation syndrome can be confirmed with twig Car profiles as well. Given that this was not the case, we discuss possible reasons for this discrepancy.

Materials and methods

Plants: Preliminary surveys were conducted in the vicinity of the Patras University campus to locate plants whose twigs have well developed periderm, lacking stomata, and green tissues beneath the periderm. Both cultivated and wild trees and shrubs were examined. The presence of chlorenchyma was assessed visually by simply scratching off the periderm and the absence of stomata was verified under a scanning electron microscope (see below). Five species were selected on the basis of the above prerequisites. *Populus deltoides* Bartr. *ex* Marsh. (eastern cottonwood) and *Quercus coccifera* L. (kermes oak) with grey periderm, and *Pistacia lentiscus* L. (mastic tree), *Prunus cerasus* L. (sour cherry), and *Arbutus unedo* L. (strawberry tree) with brown-red periderm. Five individuals from each species were sampled. On each sampling date, an adequate number of leaves and twigs from each individual were harvested. Pigment extraction and analysis were run in duplicate (with at least 6 leaves or twigs extracted) and the mean values were considered characteristic for the individual. The sampling period lasted from early September to late October 2001, well before leaf senescence and shedding, which occurred gradually after mid-November in the two winter-deciduous trees (*P. deltoides* and *P. cerasus*). The rest of the plants are evergreens. Twigs and leaves initiated during the previous (spring) growth period (*i.e.* 7–8 months old) were used.

Pigment extraction and analysis: For pigment extraction, we adopted the hot DMSO (dimethylsulfoxide) method proposed by Wittmann *et al.* (2001) as modified by Levizou *et al.* (2004). After centrifugation of the crude extract, Chls *a* and *b* were determined in the supernatant spectrophotometrically using a *Shimadzu UV-160A* double beam spectrophotometer and the equations of Wellburn (1994). For further Car analysis, the supernatant was further cleared by passing through 0.45 µm filters and pigments were separated using a *Shimadzu LC-10 AD HPL* chromatograph equipped with a non-endcapped *Zorbax ODS* (4.6×25 mm) column (*Rockland Technologies*, Chadds Ford, PA, USA) calibrated against purified β-carotene (*Sigma Chemical*, St Louis, MO, USA) and freshly prepared xanthophylls by thin layer chromatography as described by Kyparissis *et al.* (1995). Development was performed isocratically at 16.7 mm^3 s^{-1} (20 min with acetonitrile : methanol, 85 : 15 v/v and 20 min with methanol : ethyl acetate, 68 : 32 v/v), according to Thayer and Björkman (1990). Pigments were detected by a *Shimadzu SPD-M10A*$_{VP}$ UV-VIS photodiode array detector and further analysed by a *Shimadzu Class-VP* version *6.1* software package.

Our initial intention was to make both pre-dawn and mid-day samplings in order to observe possible diurnal xanthophyll cycle inter-conversions. Yet, accurate measurements of xanthophyll cycle components in the light-adapted state require instant deep-freezing and subsequent quick extraction of the samples. The unavoidable and time consuming need for stripping off the periderms before extraction (Levizou *et al.* 2004) made such handlings impractical, especially in the field. Therefore, the plant material was sampled at pre-dawn, immediately transferred in the laboratory in dark containers, and subsequent handling and extraction were performed under dim light (<0.1 µmol m^{-2} s^{-1}).

Microscopy: After sputter coating with gold, twig segments were observed under a *Jeol 6300* scanning electron microscope (SEM).

Statistics: When needed, significance of differences between twigs and corresponding leaves for each species were assessed by Student *t*-tests (*SPSS 9.0* statistical package).

Results

Observations with the SEM confirmed the absence of stomata in all peridermal twigs (not shown). Lichens, which could interfere with pigment analysis, were also absent.

Chl contents in the twigs were low. Expressed on an area basis, they ranged from 10 % (*A. unedo*) to 34 % (*Q. coccifera*) of the corresponding leaf values (Table 1). In addition, the Chl *a/b* ratio was characteristically low in twigs, ranging from 1.42 in *A. unedo* to 2.07 in *Q. coccifera*, while corresponding leaf values were always higher than 2.7 (Table 1).

Chromatographic analysis revealed that twigs contained the usual chloroplast Cars. Yet, considerable quantitative differences were found between twigs and leaves. Expressed on a Chl basis, neoxanthin content was higher in twigs of all five species (Fig. 1*C*), lutein in four (Fig. 1*D*), and the xanthophyll cycle components (V+A+Z, violaxanthin+antheraxanthin+zeaxanthin) in three of them (Fig. 1*B*). Yet, β-carotene was absent or very low in the twigs of four species, while in *Q. coccifera* its content, although appreciable, was lower than in leaves (Fig. 1*E*). As a result, total Cars were higher in the twigs of four species (Fig. 1*A*). Even in cases where the differences were not statistically significant, a trend for higher Car/Chl ratios in twigs was always evident (with the exception of β-carotene).

As expected for fully dark-adapted leaves, the major component of the xanthophyll cycle pool was V, while A and Z were either absent or found in traces. Yet, A and, to a lesser extent, Z contents in twigs were considerable. As a result, the ratio (A+Z)/(V+A+Z), which indicates the conversion state of the system, was higher in the twigs of three species (*P. deltoides*, *P. cerasus*, and *A. unedo*) while similar trends in *P. lentiscus* and *Q. coccifera* were not statistically significant (Fig. 1*F*).

Table 1. Total chlorophyll (Chl) contents and Chl *a/b* ratios in leaves and twigs of the indicated species. Means ± SE from five individual plants. All differences between twigs and the corresponding leaves are statistically significant at $p<0.001$.

	Chl (*a*+*b*) [mg m^{-2}]		Chl *a/b*	
Species	Twigs	Leaves	Twigs	Leaves
Arbutus unedo	52.0 ± 6.7	502.2 ± 78.9	1.42 ± 0.15	2.76 ± 0.05
Pistacia lentiscus	78.7 ± 9.1	505.0 ± 10.5	1.78 ± 0.07	2.94 ± 0.08
Populus deltoides	55.1 ± 12.4	340.7 ± 8.4	1.70 ± 0.20	3.47 ± 0.03
Prunus cerasus	58.6 ± 8.2	418.3 ± 33.2	1.94 ± 0.09	3.36 ± 0.12
Quercus coccifera	188.7 ± 12.9	554.0 ± 22.4	2.07 ± 0.05	3.01 ± 0.13

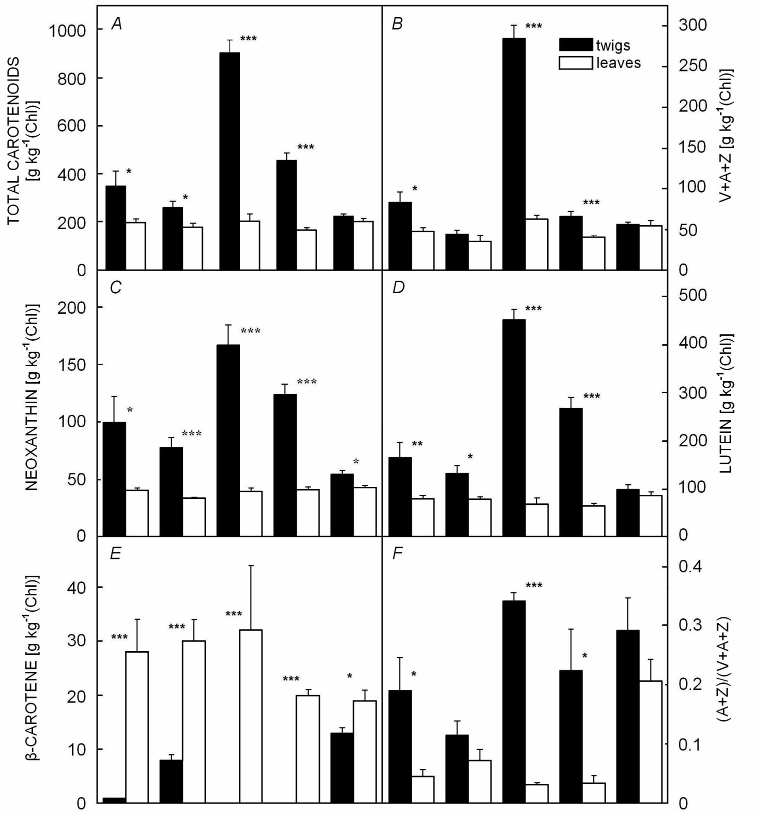

Fig. 1. Chlorophyll-based contents of total and individual carotenoids in twigs and leaves. (*A*) Total carotenoids, (*B*) V+A+Z, (*C*) neoxanthin, (*D*) lutein, (*E*) β-carotene, (*F*) the ratio of de-epoxidized (antheraxanthin+zeaxanthin) to total xanthophyll cycle components, (A+Z)/(V+A+Z). Statistically significant differences between twigs and leaves: at $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$, $n = 4$.

Discussion

Results from many investigations (Pearson and Lawrence 1958, Pilarski 1984, 1999, Kharouk *et al.* 1995, Solhaug *et al.* 1995, Schmidt *et al.* 2000, Aschan *et al.* 2001, Wittmann *et al.* 2001) indicated that twigs may contain *ca*. 30–100 % of the leaf Chl on an area basis. In our study, only the results of *Q. coccifera* fell within this range while the rest of our test species had lower Chl contents. Twig Chl content may vary with season (Kauppi 1991), exposure (Glase and Granet 1978, Wittmann *et al.* 2001), and age (Pearson and Lawrence 1958, Aschan *et al.* 2001). However, the variations found by these authors are not enough to account for the differences observed in our study. Therefore, we have to assume a high interspecies variation in twig Chl contents. Yet, in agreement with all mentioned previous studies, a low Chl *a/b* ratio was found, indicating shade characteristics of twig chloroplasts. Indeed, the development of photosynthetic tissues under low irradiance leads to higher ratios of light-harvesting complexes per reaction centres and, accordingly, to low Chl *a/b* ratios (Anderson 1986). In twigs, this is compatible with the low transmittance of periderms (see Pfanz and Aschan 2001 and the literature there-in). In addition to the low Chl *a/b* ratios, shade chloroplasts usually display lower Car/Chl ratios (Demmig-Adams *et al.* 1989, Thayer and Björkman 1990, Rosevear *et al.* 2001). The above ratio has been calculated in the twigs of three species based on absorbance spectra of crude extracts without further chromatographic analysis (Pilarski 1999, Wittman *et al.* 2001). In one case (Pilarski 1999) the ratio was higher in twigs while in the two other cases no differences were found (Wittman *et al.* 2001).

Concerning chromatographically analysed Cars in our test plants, not only their total Chl-based contents were higher in twigs (Fig. 1*A*), but also the contents of individual compounds were not compatible to that expected from the shade acclimation syndrome. The content of β-carotene, an effective scavenger of oxy-radicals (Durrant *et al.* 1990, Choudhury and Behera 2001), is indeed lower in shade leaves, yet the magnitude of the observed reductions in many plants is only a few percent compared to corresponding fully exposed leaves (Johnson *et al.* 1993, Rosevear *et al.* 2001). In twigs, however, only traces of β-carotene were found (Fig. 1*E*). Unexpectedly, the pool sizes of the xanthophyll cycle components were considerably higher in twigs (Fig. 1*B*), indicating the need (or the potential) of a highly effective system for harmless dissipation of excess excitation energy (Demmig-Adams and Adams 1996, Choudhury and Behera 2001), in spite of the strong and permanent shading of twig chlorenchyma by the periderm (Pfanz *et al.* 2002) and the apparently shaded position of twigs under the leaf canopy. Moreover, the contents of photo-selective neoxanthin and lutein, which may be slightly lower (Demmig-Adams *et al.* 1989, Thayer and Björkman 1990, Garcia-Plazaola *et al.* 1999), slightly higher (Rosevear *et al.* 2001), or not responsive (Hansen *et al.* 2002) under shade, in the twigs were considerably higher (Fig. 1*C,D*). Therefore, with the exception of the low Chl *a/b* ratio, the Car composition of twig chlorenchyma is more reminiscent of a "sun" than a "shade" type of photosynthetic tissue. The reasons for this discrepancy are not apparent from the results of this study. One plausible explanation could be that part of the extracted Cars may not be engaged in either photo-selective or photo-protective functions, if they are stored in plastoglobuli which are extracted in the organic solvent and which may be abundant in corticular chloroplasts. Plastoglobuli have been detected in such chloroplasts, yet it is not certain whether they are more or less abundant compared to corresponding plastoglobuli in leaf chloroplasts (Buns *et al.* 1993, Pfanz *et al.* 2002). In addition, earlier reports on the occurrence of Cars in plastoglobuli (Bailey and Whyborn 1963) have been questioned in more recent studies (Steinmüller and Tevini 1985). On the basis of the above, we may reject the explanation of an artifactual high Car/Chl ratio in twigs due to the presence of plastoglobuli.

In seeking for an alternative explanation, we may mention that low irradiance is not the only parameter characterizing the microenvironment within a peridermal stem. Due to the high diffusive resistance of the periderm, respired CO_2 is trapped in stems and published data indicate extremely high concentrations, ranging *ca*. 1–25 % (see Schaedle 1975, Pfanz and Aschan 2001, and the literature there-in). At the upper part of this range, CO_2 may acidify chloroplast stroma (Yin *et al.* 1993, Bligny *et al.* 1997) inhibiting the pH sensitive reactions of the reductive pentose phosphate cycle (Pfanz and Heber 1986). Accordingly, photosynthetic CO_2 assimilation may not be an effective electron sink under these conditions, raising the need for a strong, xanthophyll cycle-dependent, photo-dissipative capacity even at low irradiance. Indeed, the pool size of the xanthophyll cycle components in the leaves of field grown plants are elevated when the Calvin cycle activity is reduced by drought (Munné-Bosch and Alegre 2000) or cold (Kyparissis *et al.* 2000) stress. A high CO_2 stress is impossible in leaves under ambient conditions, but may well occur in peridermal twigs. Indeed, evidence for such a stress was recently obtained in twigs with removed periderms. Thus, high CO_2 suppressed considerably the linear electron transport rate along PS2, apparently increasing the proportion of excess excitation energy in the pigment bed and the need for enhanced photo-protective non-photochemical quenching even at low irradiance (Manetas 2004).

Although the above may explain the high pool sizes of the xanthophyll cycle components, they are apparently contradictory when the extremely low contents of β-carotene in twigs are taken into account. Yet, the mechanisms of photo-protective functions of xanthophyll cycle components and β-carotene are considered different. Thus, A and Z are thought to mediate the direct quenching of Chl over-excitation (Young *et al.* 1997) whereas β-carotene rather scavenges oxygen radicals produced by triplet-state Chl (Choudhury and Behera 2001). Oxygen concentrations in stems can be as low as 5 % (Ziegler 1957, Levy *et al.* 1999) and the apparently hypoxic conditions may lessen the risk of oxy-radical formation and, accordingly, allow lower β-carotene levels.

Finally, whereas the shade condition is usually applied on a leaf by a reduction on all visible wavelengths, a red-enriched shade is encountered within stems. Indeed, all published spectral transmittance data of periderms indicate a preferential absorbance of blue-violet radiation but high penetration of red-yellow wavelengths (see Introduction). In our case, transmittance of periderm in *P. cerasus* was more than 80 % in the 600–700 nm region, but less than 3 % in the 400–500 nm region (Manetas 2004). Continuous irradiation of leaves with red compared to blue radiation results in considerably higher contents of Cars, especially xanthophylls (Lichtenthaler *et al.* 1980). This effect, which is possibly photomorphogenetic, may have the adaptive significance of increasing the efficiency of energy capture at the violet/blue region of the spectrum which is less lavishly provided.

Our results also showed high contents of both A and Z in twigs, in spite of the fully dark-adapted state of our samples. Such over-night sustained high ratios of (A+Z)/(V+A+Z) have been observed under cool nights in the leaves of some plants (Adams and Demmig-Adams 1995), including species examined in the present study (Kyparissis *et al.* 2000). The phenomenon was ascribed to suppression under low night temperatures of the enzymic system responsible for the conversion of A and Z to V, allowing a high photo-dissipative potential (*i.e.* photo-protection) during early morning. It was observed during the coldest winter days in the leaves of *A. unedo* and *P. lentiscus* but not during the autumn (Kyparissis *et al.* 2000, see also Fig. 1*F*), which is considered as a favourable period for Mediterranean evergreen sclerophylls. Since the experiments of the present study were performed during autumn, we cannot interpret our findings in an analogous way. Yet, xanthophyll cycle interconversions are pH sensitive as well and possible perturbations of the chloroplast pH buffering capacity due to high CO_2 may affect the function of this cycle (Demmig-Adams and Adams 1996). Whatever the reason(s) for the abnormal contents of A and Z in twigs at pre-dawn, a sustained high photo-dissipative potential is usually correlated with low maximum PS2 photochemical efficiencies. Indeed, F_v/F_m of twigs in our test plants at pre-dawn was low (0.546–0.709, depending on plant species) during autumn, compared to normal (*ca.* 0.820) values obtained from the corresponding leaves (Manetas 2004).

We conclude that although the Chl composition of twigs is compatible with the unavoidable shade acclimation of cortex chlorenchyma, the Car composition, at least in the species tested, may reflect additional needs raised by the occurrence of photosynthesis under extremely high CO_2 concentrations, hypoxia, and photosynthetically active radiation preferentially depleted in blue photons.

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