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Seasonal changes in xanthophyll composition and photosynthesis of cork oak (*Quercus suber* L.) leaves under mediterranean climate

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Abstract

Seasonal changes in pigment composition of sun and shade leaves of cork oak (*Quercus suber*) were studied under field conditions in Portugal. Expanding leaves showed a high concentration of xanthophyll cycle components, violaxanthin, antheraxanthin and zeaxanthin. The pool of violaxanthin plus antheraxanthin plus zeaxanthin (V + A + Z) varied greatly between the seasons, being higher at the end of summer and in winter when photosynthesis was limited by water stress and cold, respectively. The size of V + A + Z pool was associated to synthesis of zeaxanthin in response to an excess of light. In sun leaves, midday A + Z relative content was positively correlated with the V + A + Z pool, whereas in shade leaves A + Z decreased with leaf ageing. In both leaf types A + Z was positively correlated with the non-photochemical quenching (NPQ) of chlorophyll *a* fluorescence. However, in winter NPQ did not change significantly throughout the day, whereas the (A + Z)/(V + A + Z) increased following the typical daily trend observed in other seasons.

Key words: Chlorophyll fluorescence, pigments, *Quercus suber*, thermal dissipation, xanthophylls.

Introduction

Cork oak (*Quercus suber* L.) is a sclerophyllous evergreen tree species native of the western mediterranean. The climate of this region is characterized by summer drought and mildly cold winters. Most of the woody plants in the region are evergreen. Therefore, their photosynthetic

apparatus must acclimate to a wide variety of environmental stress factors, including water stress and high or low temperatures. When carbon assimilation is limited by stress excess light could lead to photoinhibition whenever the plants are unable to avoid those conditions or to dissipate excess photon energy safely.

A correlation between the ability to dissipate such excess excitation energy and the concentration of zeaxanthin has been shown in several species (Demmig *et al.*, 1988). This relationship strongly suggests that this xanthophyll has a central role in the non-radiative energy dissipation process (Bilger and Björkman, 1990; Demmig *et al.*, 1987). Although at present the precise role of zeaxanthin in energy dissipation is not entirely understood, different mechanisms have been postulated: firstly, zeaxanthin can quench the chlorophyll triplet excited state, thereby preventing singlet oxygen production (Demmig-Adams, 1990) or can quench singlet oxygen directly (Frank *et al.*, 1994) and secondly, xanthophylls seem to control the structure of the light-harvesting complex, such as aggregation (Horton *et al.*, 1994).

Zeaxanthin is formed in response to an increase in the incident sunlight (Adams and Demmig-Adams, 1992) by de-epoxidation of violaxanthin via antheraxanthin. This reaction occurs in the lumen of thylakoids, and it is modulated by the availability of ascorbate and the acidification of the lumen (Neubauer and Yamamoto, 1994). Epoxidation back to violaxanthin occurs when light absorption is no longer in excess. Thus, during the afternoon and night, the back reaction takes place (Adams and Demmig-Adams, 1992), completing the so-called xanthophyll cycle.

The xanthophyll cycle carotenoids seem to be highly flexible, adjusting rapidly to light conditions. For example, under some circumstances, the $V+A+Z$ pool is apparently able to increase by midday at the expense of β-carotene (Demmig et al., 1988; Demmig-Adams et al., 1989). In addition to daily changes, a seasonal pattern has been described, with major changes in the $V+A+Z$ pool content and composition occurring between summer and winter in some evergreen coniferous trees (Adams and Demmig-Adams, 1994). Within the same species and environmental conditions sun leaves contain more $V+A+Z$ pigments (4–5-fold higher) than shade leaves (Demmig-Adams and Adams, 1993; Thayer and Björkman, 1990) allowing for a larger dissipation of excess light energy in sun leaves (Demmig-Adams and Adams, 1994). Demmig-Adams (1990) reported an increase in the concentration of $V+A+Z$ when shade-grown plants were transferred to high light. The leaf angle with the horizontal is also correlated with the $V+A+Z$ content, as observed in mangroves (Lovelock and Clough, 1992). The xanthophyll cycle also responds to other stress factors that increase the ratio of light energy/photosynthetic carbon assimilation, such as water stress (Demmig et al., 1988); salinity (Lovelock and Clough, 1992); cold (Adams and Demmig-Adams, 1994) and nutrient limitations (Morales et al., 1994). All these observations support the photoprotective role of xanthophylls under field conditions and a strong modulation of this process by the environment.

The aim of this study was to assess the seasonal and daily patterns in the concentration of the xanthophyll cycle components and other photosynthetic pigments and their relationship with the physiological state of sun and shade leaves of adult cork oak trees, growing under field conditions in Southern Portugal.

Materials and methods

Field site, plant material and experimental design

The study was carried out in a stand of 40-year-old *Quercus suber* trees at Azaruja (Herdade do Paço de Camões) located near Évora, in southern Portugal (lat. 38° 34′ N; long. 7° 54′ W; alt. 320 m). This area is characterized by a Mediterranean type climate, with dry and warm summers. Annual precipitation, 642.6 mm on average, occurs mainly in autumn and winter. The average precipitation in mid-summer is 3 mm per month. Temperatures are high during summer with an average daily maximum temperature of 29.9 °C, and moderate in winter. Frost is rare with an average occurrence of 7.2 d per year.

The experiments were conducted on clear days of 1994 and 1995, considering the seasonality of canopy development in *Quercus suber*. In *Q. suber* the shoot growth and the development of new foliage begins in April and is terminated by June. Most older leaves (1-year-old) fall during the early part of shoot growth and only a very small number of leaves last longer than one year (Pereira et al., 1987). Therefore, taking into account the seasonal pattern for changes in photosynthesis in *Quercus*

suber described by Tenhunen et al. (1987), diurnal cycles were studied in five different critical periods: (i) late June, when new leaves were completely developed, temperature and irradiance were high, trees were not yet severely stressed and had a high photosynthetic rate during the morning; (ii) September, when trees were most stressed after the summer drought and stomatal closure strongly restricted CO_2 assimilation; (iii) November, after the autumn rainfall when trees recovered from the summer stresses, and temperatures were warm enough to allow high rates of photosynthesis; (iv) January, when low temperatures limited photosynthesis and frost could occur; and (v) late April, immediately before the flushing and new leaf development, when temperatures were moderate and soil moisture plentiful. Data were obtained at four times of the day, defined as: (i) pre-dawn (immediately before sunrise; this can be considered as the control before any light or temperature stress takes place), (ii) morning (3 h after sunrise, when light intensity and temperatures were moderate or low), (iii) midday (12 h solar time) when light intensity and temperatures reach the highest values) and (iv) evening (3 h before sunset; when temperatures were still high but light intensity had decreased to a great extent).

All measurements were done in sun and shade leaves of the same branches of 3–6 trees. Sun leaves were considered those facing south, receiving direct solar light during most of the day; shade leaves were located in the inner part of the crown and did not receive direct solar radiation. The average maximal and minimal temperatures and the midday photosynthetic photon flux density ($PPFD$) during the experimental period are shown in Table 1 for sun and shade leaves. Leaf discs of individual samples were collected directly from their light environment, immediately frozen in liquid nitrogen and stored at $-80\,°C$ until biochemical analysis.

Methods

Leaf water potential (ψ) at pre-dawn and midday was determined using a pressure chamber (PMS Instrument Co., Oregon, USA) (Scholander et al., 1965).

Gas exchange measurements were made under natural light conditions using an open-system portable CO2/H2O analyser (CI-301, CID Inc., USA). Carbon assimilation rates (P_n) and leaf conductance (g_w) were calculated using the standard equations.

Chlorophyll *a* fluorescence was measured using a portable pulse amplitude modulation fluorometer (PAM 2000, Walz, Effeltrich, Germany). Initial (F_o) and maximal fluorescence (F_m) were measured in dark-adapted leaves (30 min) with a total saturation pulse time of 0.8 s. The maximal apparent efficiency of photosystem II (PSII) photochemistry was estimated by the fluorescence ratio ($F_v/F_m = (F_m - F_o)/F_m$). Non-photochemical fluorescence quenching (NPQ) was estimated from the Stern–Volmer parameter, that represents a relative measurement of thermal dissipation at the PSII level, calculated according to

Table 1. *Predawn and midday air temperature and midday* PPFD *in sun and shade conditions during the measurements*

Month	Air temperature (C)		PPFD ($\mu mol\ m^{-2}\ s^{-1}$)	
	Predawn	Midday	Sun	Shade
June	20	33	1800	52
September	13	34	1590	78
November	7	23	1310	113
January	5	14	1100	74
April	10	30	1720	77

the equation $NPQ = F_m/F'_m - 1$ (Cornic, 1993), that quantifies the lowering of fluorescence emission from the level of F_m (yield of maximal fluorescence after dark adaptation) to a new (quenched) level F'_m (yield of maximal fluorescence from a leaf that is absorbing light from an actinic light source).

Pigments were extracted from leaf discs frozen in liquid nitrogen immediately after detachment from the branch and ground in a mortar with acetone (≈ 0.2 cm^2 of leaf tissue per ml of solvent) in the presence of sodium ascorbate. The extract was kept in the darkness at $-80\,°C$ until analysis. Pigments were analysed by the HPLC method as described by Rivas *et al.* (1989). The amounts of violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z) present in leaves were also expressed as a percentage of A + Z and total V + A + Z pool.

Two-way analysis of variance (ANOVA) was used to test for the effects and interactions of time (month and time of the day) and leaf type (sun or shade) on pigment concentrations and chlorophyll fluorescence. Sun and shade leaves were also analysed separately for time (month or time of the day) effects by a one-way analysis of variance. All the significant differences discussed in this work were significant at $P < 0.05$ in Fisher's least significant difference (lsd) tests between means.

Results

Seasonal changes in photosynthesis

Leaf water potentials measured at predawn and midday after, during and before the period of summer drought (Table 2) indicate that plants were only severely water-stressed in September, at the end of the summer drought. Nevertheless, in June there was a substantial midday depression in gas exchange (Table 3). Photosynthesis decreased to 30% of the morning value by midday, in parallel with changes in stomatal conductance. High temperatures and high water vapour pressure deficit between leaves and air may explain these responses. Photosynthetic rates were also low in September when

stomatal conductance remained very low throughout the day. Gas exchange was depressed in January and April, presumably as a result of the low temperatures and leaf ageing (Table 3).

Seasonal changes in pigment composition

The seasonal changes in pigment concentration of sun and shade leaves were followed throughout the first year of measurements, beginning with the new expanding spring leaves, and ending with the 12 month-old leaves in the second spring (Fig. 1). In April, the new expanding leaves showed the lowest concentrations (in a leaf area basis) for all pigments (neoxanthin, lutein, β-carotene and chlorophyll) except those of the V + A + Z pool. However, when expressed on a chlorophyll basis, the values were as high as when they reached their maxima later in the year. All pigment concentrations had increased by June, except for the V + A + Z pigments, which decreased by 20%. At the end of the summer, a significant reduction in all the pigments (12–20%) was observed (except for the V + A + Z pool that doubles in sun leaves). From September to April, chlorophyll content remained constant in both leaf types, although the chlorophyll a/b ratio increased significantly in sun leaves. Similarly, levels of lutein and β-carotene increased significantly. During all seasons total chlorophyll and neoxanthin concentrations were similar in sun and shade leaves. However, from June to April sun leaves exhibited significantly higher concentrations of β-carotene, chlorophyll a and especially V + A + Z, when compared to shade leaves. In shade leaves, the total V + A + Z concentration followed a seasonal pattern of variation simillar to that found in sun leaves, even though the absolute amounts were much lower.

Daily changes in xanthophyll cycle composition

In all seasons the lower antheraxanthin plus zeaxanthin (A + Z) content was observed at predawn (Fig. 2). During the hours of light, the conversion of violaxanthin into A + Z in sun leaves proceeded between dawn and the morning, and in September, January and April also continued between the morning and midday. At midday
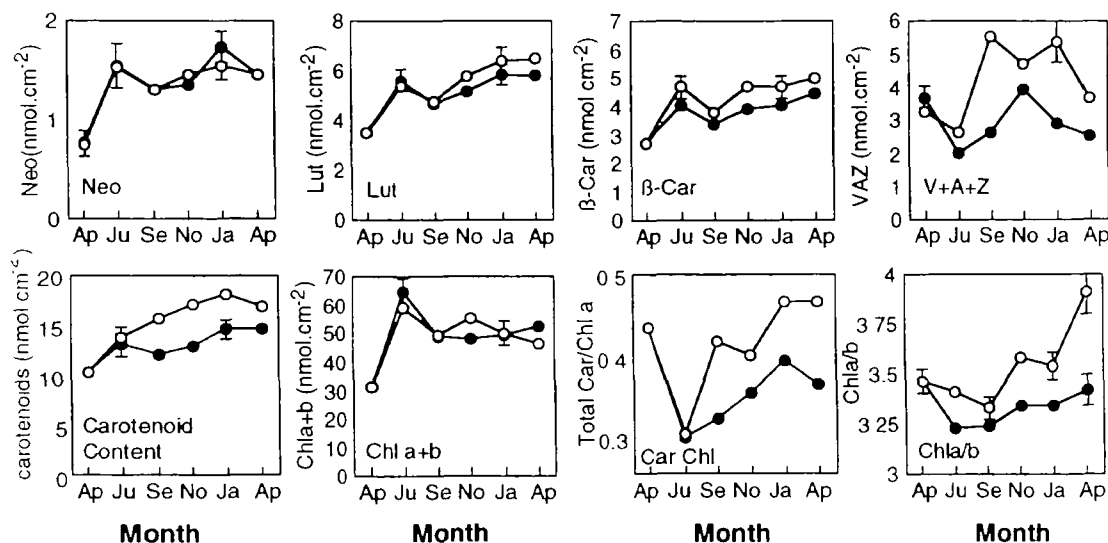
Table 2. *Predawn and midday leaf water potential (MPa) in June, September and November*

Each value is the average of six determinations ± standard error.

Month	Predawn	Midday
June	-0.31 ± 0.01	-2.26 ± 0.25
September	-1.72 ± 0.24	-2.85 ± 0.13
November	-0.27 ± 0.01	-1.96 ± 0.23

Table 3. *Morning and midday photosynthetic rate (*P_n*: μmol CO_2 m^{-2} s^{-1}), stomatal conductance (*g_w*: mmol H_2O m^{-1} s^{-1}), leaf temperature (°C) and PPFD (*μmol m^{-2} s^{-1}*) in sun leaves of* Q. suber

MO, morning and MD, midday. Each value is the average of 20–30 determinations ± standard error.

Month	P_n (MO)	g_w (MO)	Leaf temperature (MO)	PPFD (MO)	P_n (MD)	g_w (MD)	Leaf temperature (MD)	PPFD (MD)
June	12.78 ± 1.85	100.0 ± 20.1	30.2 ± 1.3	1796 ± 27	3.98 ± 0.55	25.5 ± 7.3	35.6 ± 0.24	1622 ± 47
September	4.12 ± 0.82	22.0 ± 2.8	35.0 ± 1.4	1577 ± 31	0.55 ± 0.04	15.8 ± 1.4	39.5 ± 0.38	1873 ± 8
November	13.58 ± 0.12	86.0 ± 2.4	26.5 ± 2.3	1364 ± 11	11.47 ± 0.11	104.7 ± 2.0	30.6 ± 0.95	1707 ± 4
January	5.80 ± 0.62	77.8 ± 5.8	15.3 ± 1.3	1127 ± 12	7.00 ± 0.50	47.3 ± 4.3	21.7 ± 1.40	1235 ± 99
April	6.78 ± 0.09	42.3 ± 4.3	23.8 ± 3.0	1302 ± 11	5.06 ± 0.34	51.8 ± 6.1	26.2 ± 1.75	1432 ± 65

Fig. 1. Annual changes in pigment composition in sun (white symbols) and shade (black symbols) leaves. First AP represents the new April leaves, while the second AP represents old April leaves, JU represents June, SE represents September, NO represents November, and JA represents January. Each value is the average of 12–20 determinations ± standard error

the amount of $A + Z$ relative to total $V + A + Z$ pool ranged between a minimum in June, without major environmental constrains and maxima in September and January, when either water or cold stress were present (Fig. 3). In these months, de-epoxidation to zeaxanthin continued at a high rate between midday and the evening. The midday $A + Z$ relative content and the $V + A + Z$ pool concentration were highly correlated in sun leaves ($r^2 = 0.924$) (Fig. 4).

The proportion of $A + Z$ relative to the $V + A + Z$ pool at midday between seasons was more pronounced in shade than in sun leaves. In September $A + Z$ represented as much as 67% of the $V + A + Z$ in shade leaves, while in April it was only 12% (Fig. 3). This variation was mostly related with leaf age ($r^2 = 0.868$) (Fig. 5) and was not related to maximal *PPFD*, maximal temperature and midday or predawn leaf water potential.

Relationship between xanthophylls cycle carotenoids and chlorophyll fluorescence

The daily patterns of variation of the chlorophyll fluorescence parameters F_v/F_m and *NPQ* were studied on each of the dates considered and related with the $(A + Z)/(V + A + Z)$ ratio (Fig. 6). The apparent photochemical efficiency of PSII, estimated by the ratio F_v/F_m, remained almost constant (0.80–0.83) during the daily cycle in shade leaves, but in sun leaves this ratio decreased during midday with an apparent recovery in the evening. However, in September, sun leaves did not recover in the evening and in January they were not able to recover even after 11–13 h of darkness (Fig. 6). The $(A + Z)/(V + A + Z)$ ratio followed the inverse pattern of F_v/F_m in sun leaves. However, in January the predawn

F_v/F_m in sun leaves decreased to 0.69, but the predawn $(A + Z)/(V + A + Z)$ was only 28%; the same as in other months. During the summer months, when photosynthesis had a midday depression, $(A + Z)/(V + A + Z)$ of shade leaves increased during midday, whereas F_v/F_m remained at 0.8–0.83. This occurred though the light conditions for those leaves were approximately constant (about 50 μmol m^{-2} s^{-1}).

Non-photochemical quenching (*NPQ*), was lower in the morning and increased by midday, except in January and September when *NPQ* values did not change significantly during the day (Fig. 6). *NPQ* was positively correlated with $A + Z$ ($r^2 = 0.796$) (Fig. 7), but the correlation between these parameters increased significantly if the January data were not considered ($r^2 = 0.886$) because $A + Z$ content was not correlated with *NPQ* in that month.

Discussion

Based on previous work (Tenhunen *et al.*, 1987), two critical periods for the photosynthetic activity in cork oak leaves could be expected under Mediterranean climate conditions (September and January). In the first one, stomatal closure due to summer drought would be the main limiting factor, whereas in the second, low temperatures would limit mesophyll photosynthetic activity (Table 3). A third period in April when water availability and temperatures were favourable for photosynthesis was also detected, but the 1-year-old leaves probably exhibited an important loss of photosynthetic capacity. Photosynthetic activity has been shown to decrease with leaf age in other sclerophylls (Field and Mooney, 1983).
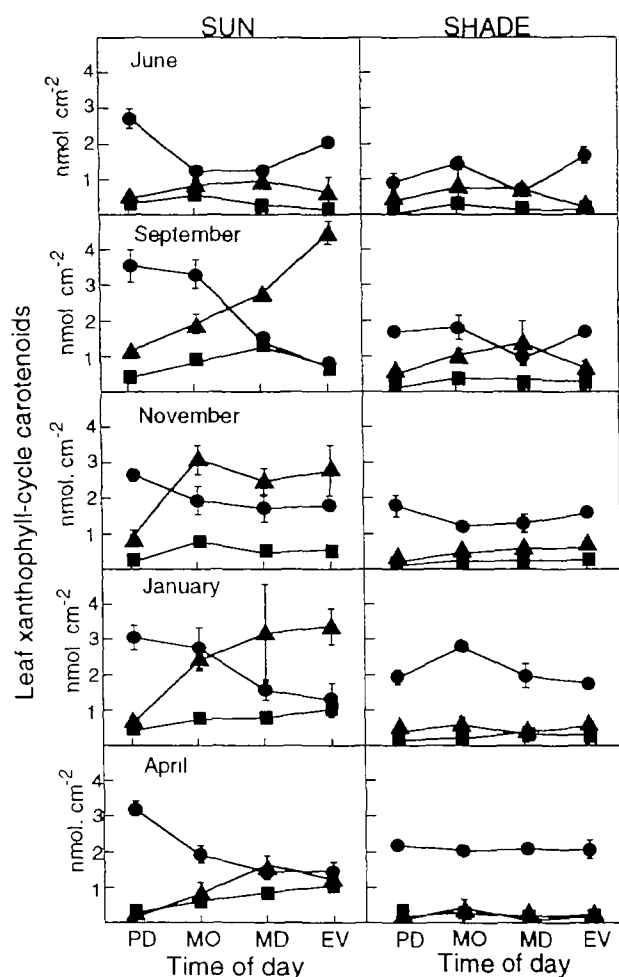
As the predawn measurements of maximal photochem-

Fig. 2. Daily changes of violaxanthin (circles), antheraxanthin (squares) and zeaxanthin (triangles) contents in sun (left column) and shade leaves (right column). PD, predawn; MO, morning; MD, mid-day, and EV, evening. Each value is the average of 3–6 determinations ± standard error.

ical efficiency remained high in the range of 0.79 to 0.83, except in January, it was concluded that the effects of excess light on photosynthesis during the day were not long-lasting. In January, the predawn F_v/F_m value in sun leaves was 0.69, indicating possible damage to the photo-synthetic apparatus or a process of down-regulation as observed in other tree species in the field (Adams and Demmig-Adams, 1994, 1995). As discussed below, the low concentrations of antheraxanthin and zeaxanthin measured at predawn would suggest that this low F_v/F_m was not due to a down-regulation process, but to photoin-hibition. This may imply that $Q.$ *suber* leaves are better adapted to cope with the excess of *PPFD* under high temperatures and water stress than with low temperatures. This seems logical since, in this region, winters are norm-ally not cold enough to cause severe damage to the photosynthetic apparatus. This strategy might also be useful to maximize photosynthesis in early summer, when

temperatures are high, but there is still some water available and the foliage consists of recently expanded leaves.

The carotenoid composition of cork oak sun and shade leaves changed during the season. In expanding leaves, carotenoid and chlorophyll concentrations were extremely low whereas the V + A + Z pool was similar to that of 1-year-old leaves. The V + A + Z/chlorophyll a ratio was therefore quite high in young leaves (0.137 mol/mol). The existence of large a pool of xanthophyll cycle caroten-oids (V + A + Z) per chlorophyll has been reported in leaves of chlorophyll-deficient mutants (Schindler *et al.*, 1994) and iron-deficient plants (Morales *et al.*, 1994). As these circumstances could lead to a high level of damage to the photosynthetic apparatus by excessive light, they suggest a role for zeaxanthin in the protection against excessive light intensities, which may be important during leaf development.

As leaves become older, lutein, β-carotene and chloro-phyll a increased progressively, except during the period of water stress. In fully grown leaves lutein, neoxanthin, β-carotene and V + A + Z concentration per unit leaf area or per unit of mass chlorophyll, were in the range described for other woody plants (Demmig *et al.*, 1988; Thayer and Björkman, 1990; Abadia *et al.*, 1996).

The concentration of V + A + Z pigments varied widely between seasons in sun leaves. Increases in the V + A + Z pool occurred simultaneously with periods when CO_2 assimilation was limited by environmental stress. On the other hand, there was a positive correlation between the total V + A + Z pool and the midday relative A + Z con-tent within the xanthophyll cycle pool of pigments (Fig. 4). These results could mean that the daily produc-tion of zeaxanthin may be related in the same way with the long-term accumulation of V + A + Z, as reported for mangroves (Lovelock and Clough, 1992). This may also be understood in the context of two different pools of VAZ cycle pigments, the increase in the total pool con-cerning mainly interconvertible pigments (Horton *et al.*, 1996).

The V + A + Z pool was always in a highly epoxidated state at predawn. This was different from the maintenance of high antheraxanthin and zeaxanthin concentrations observed under low temperatures (Adams and Demmig-Adams, 1994, 1995), water stress (Demmig-Adams *et al.*, 1989) or nutrient deficiencies (Morales *et al.*, 1990). The occurrence of low epoxidation index after cold nights was interpreted as a process of down-regulation (Adams and Demmig-Adams 1994, 1995). In the sun leaves of $Q.$ *suber*, the de-epoxidation to antheraxanthin and zeaxan-thin took place in parallel with daily changes in *PPFD*, but surprisingly some de-epoxidation was also detected in shade leaves in spite of low *PPFD*.

The maximal apparent photochemical efficiency (F_v/F_m) followed the inverse pattern as the ratio
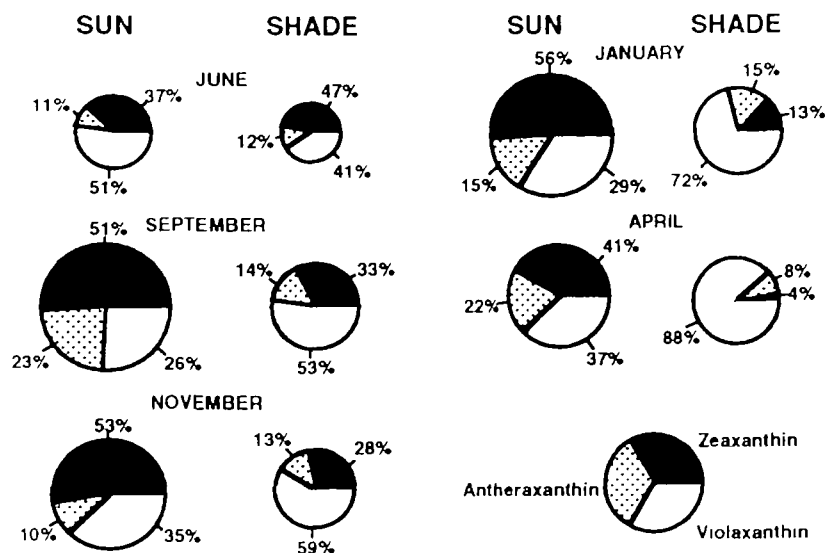
Fig. 3. Midday V+A+Z cycle composition (expressed as a percentage of total V+A+Z). Black frames represent zeaxanthin, white frames represent violaxanthin and dotted frames represent antheraxanthin The size of pie charts are proportional to the leaf xanthophyll cycle composition.
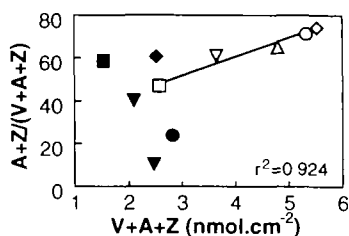

Fig. 4. Relationship between midday A+Z calculated as a percentage of (V+A+Z) and total V+A+Z pool in sun leaves. Measurements were done in June (squares), September (diamonds), November (triangles), January (circles) and April (inverted triangles).
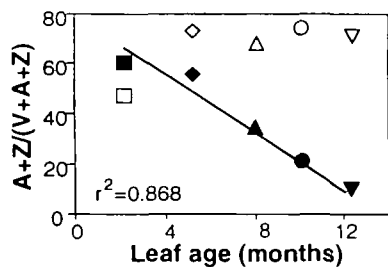
Fig. 5. Relationship between midday A+Z calculated as a percentage of (V+A+Z) and leaf age in shade leaves. Symbols are the same as used in Fig. 4.

(A+Z)/(V+A+Z) in sun leaves, except in January when a 30% decrease in predawn F_v/F_m was not accompanied by a night retention of antheraxanthin and zeaxanthin, as discussed above. These data could indicate that a process of photoinhibition to the photosynthetic apparatus took place. On the other hand, F_v/F_m and NPQ did not change significantly in shade leaves throughout the day, contrasting with the significant changes observed in (A+Z)/(V+A+Z) between June and November. This observation partlly agrees with the model of Bilger and

Björkman (1994) that suggests that thylakoid ΔpH is necessary for the development of non-photochemical quenching (NPQ), but is also in apparent contradiction with this model as the same ΔpH is necessary for the de-epoxidation of violaxanthin. Under some extreme circumstances low PPFD (50 μmol m^{-2} s^{-1}) may become excessive and lead to the synthesis of high amounts of A+Z, as in the case of dehydrating stems of *Selaginela lepidophylla* (Casper *et al.*, 1993). However, this is unlikely to have occurred in shade leaves of cork oak, considering that F_v/F_m was unchanged and that formation of A+Z in shade leaves also took place in periods when no stress was apparent.

NPQ followed approximately the same pattern as the A+Z concentration in sun leaves, with the exception of January. The general observation agrees with previous works in other species by Bilger and Björkman (1990, 1994) and Demmig-Adams (1990), who suggested a relationship between both parameters. In fact, it has been shown that zeaxanthin (plus antheraxanthin) could play a role in the de-excitation of singlet excited chlorophyll and the removal of excess light energy from the PSII antennae (Demmig-Adams, 1990; Frank *et al.*, 1994). In January, NPQ did not change throughout the day as temperature rose, whereas the (A+Z)/(V+A+Z) ratio was highest at midday, which resulted in a lack of correlation between these parameters during that period. On the other hand, F_v/F_m declines by midday as a result of a lower F_m. It could be argued that half an hour of dark adaptation by midday was not enough for the total relaxation of fluorescence quenching in highly stressed leaves; however, even after the night period F_v/F_m is still below the optimal values observed in the other months.

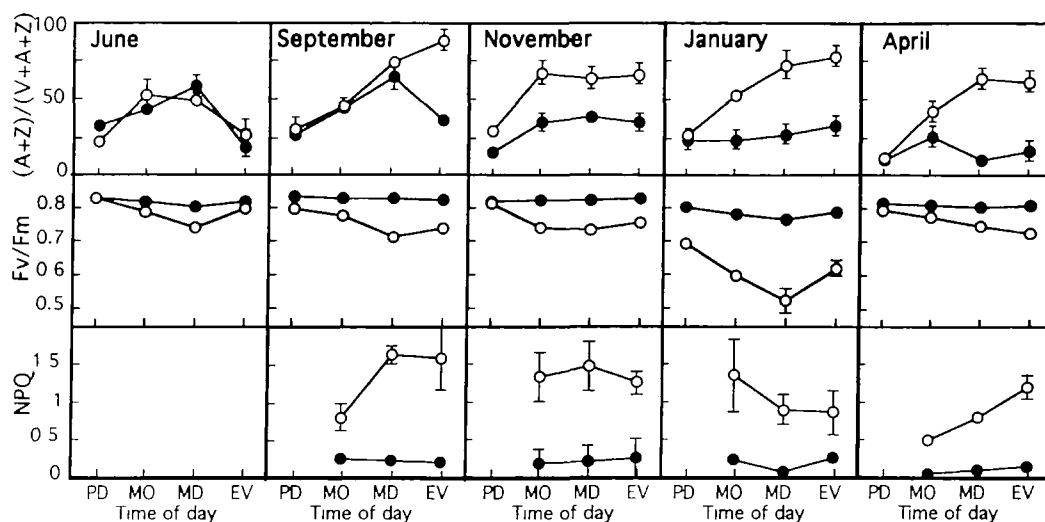In conclusion, these results showed that the V+A+Z

Fig. 6. Daily courses of $(A+Z)/(V+A+Z)$, F_v/F_m and NPQ in sun (white circles) and shade leaves (black circles) NPQ for June was not measured. Each value is the average of 3–6 determinations $\pm$ standard error.
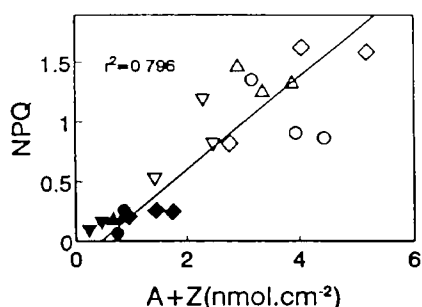

Fig. 7. Relationship between $A+Z$ content and NPQ in sun (white symbols) and shade (black symbols) leaves. Symbols are the same as used in Fig. 4.

pool varied between seasons, being higher during the periods of stress, and that the size of the $V+A+Z$ pool is associated to the synthesis of zeaxanthin in response to an excess of energy during the day. Nevertheless in winter, trees were obviously unable to fully dissipate energy which resulted in an apparent photoinhibition. The model proposed by Bilger and Björkman (1994) was also indirectly tested for non-radiative dissipation of the excess of light energy under a wide variety of ecological situations. Although these data basically agree with that model, an apparent exception is reported. In shade leaves, in the absence of excess *PPFD*, and consequently of a large thylakoidal ΔpH, de-epoxidation of violaxanthin took place during the day. In these leaves, the midday formation of $A+Z$ is apparently dependent on age, being zero in older leaves (January and April).

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