RESEARCH PAPER

Three pools of zeaxanthin in *Quercus coccifera* leaves during light transitions with different roles in rapidly reversible photoprotective energy dissipation and photoprotection

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Abstract

Under excess light, the efficient PSII light-harvesting antenna is switched into a photoprotected state in which potentially harmful absorbed energy is thermally dissipated. Changes occur rapidly and reversibly, enhanced by de-epoxidation of violaxanthin (V) to zeaxanthin (Z). This process is usually measured as non-photochemical quenching (NPQ) of chlorophyll (Chl) fluorescence. Using instrumentation for instantaneous leaf freezing, NPQ, spectral reflectance, and interconversions within the xanthophyll cycle with time resolution of seconds were recorded from *Quercus coccifera* leaves during low light (LL) to high light (HL) transitions, followed by relaxation at LL. During the first 30 s of both the LL to HL and HL to LL transitions, no activity of the xanthophyll cycle was detected, whereas 70–75% of the NPQ was formed and relaxed, respectively, by that time, the latter being traits of a rapidly reversible photoprotective energy dissipation. Three different Z pools were identified, which play different roles in energy dissipation and photoprotection. In conclusion, ΔpH was crucial to NPQ formation and relaxation in *Q. coccifera* during light transitions. Only a minor fraction of Z was associated to quenching, whereas the largest Z pool was not related to thermal dissipation. The latter is proposed to participate in photoprotection acting as antioxidant.

Key words: Antheraxanthin, light transitions kinetics with seconds time resolution, non-photochemical quenching, photoprotection, *Quercus coccifera*, rapidly reversible photoprotective energy dissipation, zeaxanthin.

Introduction

Under high light (HL), the photosynthetic antenna switchs into a thermal dissipation state instead of efficient sunlight utilization (Horton *et al.*, 1996). This phenomenon, triggered by ΔpH, is measurable as non-photochemical quenching (NPQ) of chlorophyll (Chl) fluorescence (Briantais *et al.*, 1979). Rapidly reversible photoprotective energy dissipation (or ΔpH-related quenching, qE) is assigned to thermal deactivation of singlet-excited Chl (^{1}Chl*) in the PSII antenna (Horton *et al.*, 1996; Niyogi, 1999). qE accounts for *c.*80% of NPQ (Li *et al.*, 2000; Nilkens *et al.*, 2010). In addition to

ΔpH, qE is governed by zeaxanthin (Z) (Demmig-Adams and Adams, 1992) and photosystem II subunit S protein (PsbS) (Funk *et al.*, 1995; Li *et al.*, 2000, 2004; Niyogi *et al.*, 2005).

Upon illumination, leaves show absorption changes centred at 530–535 nm (A_{535}) (Heber, 1969). A_{535} shows fast followed by slow increases that reverse within 2–3 min of darkness or LL (Krause, 1973). Upon darkening, ΔA_{535} is partially irreversible, indicating that 505 nm absorbance changes (A_{505}) are observable at 535 nm (Bilger *et al.*, 1989; Morales *et al.*, 1990). ΔA_{535} correlates to qE rather than ΔpH formation *per*

se (Horton *et al.*, 1991; Ruban *et al.*, 1993) and therefore can be used to monitor qE.

Within the xanthophyll cycle, Z is formed by de-epoxidation of violaxanthin (V) via antheraxanthin (A). Demmig-Adams (1990) and Demmig-Adams and Adams (1992) provided the first evidences of a connection between Z and NPQ, suggesting Z as pigment responsible for quenching. In limiting light, the lumen pH is >6 (Kramer *et al.*, 1999), $c. 7.5 \pm 0.5$ (Takizawa *et al.*, 2007), decreasing to pH 5–5.8 in saturating light (Witt, 1979; Kramer *et al.*, 1999). When pH-activated (optimum pH 5.2) by protonation of carboxylate side chains, an ascorbate-dependent V de-epoxidase associates with the thylakoid lumenal surface (Gilmore, 1997; Morosinotto *et al.*, 2003), and A and Z are formed at expense of V in a stoichiometric relationship (Yamamoto, 1979; Bilger *et al.*, 1989). The reaction-limiting step is the liberation of V from light-harvesting complexes (LHCs) rather than enzyme activation (Morosinotto *et al.*, 2003). V de-epoxidase is released at pH 6.6 (Gilmore, 1997; Morosinotto *et al.*, 2003). Z formation half-time ranges from 1.5 to 10 min (Bilger and Björkman, 1990; Demmig-Adams, 1990). The Z to V back reaction occurs after returning to LL or darkness, catalyzed by a NAD(P)H- and O_2-dependent epoxidase located in the stromal side, thought to be constitutively active, with maximum activity at pH 7.5–8 (Yamamoto, 1979; Morales *et al.*, 1990; Müller *et al.*, 2001). Yamamoto *et al.* (1972) described an A_{505} that resembled the spectrum of Z minus V in organic solvents. Since then, A_{505} is extensively used as a non-destructive measure of xanthophyll cycle activity (Bilger *et al.*, 1989; Bilger and Björkman, 1990; Morales *et al.*, 1990; Ruban *et al.*, 1993).

NPQ requires PsbS, without which the NPQ rapidly reversible behaviour is lost (Li *et al.*, 2000) or largely slowed down (Johnson and Ruban, 2010; Nilkens *et al.*, 2010). PsbS was suggested as acting as qE- and putative Z-quencher-binding sites (Li *et al.*, 2000, 2004) and a ΔpH sensor (Dominici *et al.*, 2002; Li *et al.*, 2004). An alternative view is that PsbS acts indirectly as accelerator of conformational changes that bring about quenching within LHCII (Horton *et al.*, 2000), triggering NPQ (Johnson and Ruban, 2010). PsbS accelerates NPQ formation and relaxation in PsbS overexpressing lines (Crouchman *et al.*, 2006; Nilkens *et al.*, 2010; Zia *et al.*, 2011). Müller *et al.* (2001) proposed that PsbS activity is mediated by protonation of lumenal-exposed acidic residues, possibly two (Li *et al.*, 2002b, 2004), later identified as glutamate residues (Niyogi *et al.*, 2005). A-five subunit complex, composed of two monomeric Lhcb proteins (Lhcb4, CP29; Lhcb6, CP24) and the trimeric LHCII that connect the inner and outer moieties of the light-harvesting system, forms a stable supercomplex, whose dissociation upon HL is necessary for triggering NPQ (Holzwarth *et al.*, 2009; Betterle *et al.*, 2010). PsbS (Betterle *et al.*, 2009) and/or CP24 (Johnson *et al.*, 2011) was proposed to destabilize the supercomplex (PsbS increasing grana fluidity; Goral *et al.*, 2012), triggering a rapid and reversible change in the organization of grana membranes with disconnection of such supercomplex from the PSII reaction centre (Betterle *et al.*, 2009; Kereïche *et al.*, 2010) that suppress the efficient light-harvesting PSII array formation

and mediates the transition between the unquenched and quenched state.

Different models have been proposed for the qE site and mechanism. Aspects under debate are whether: (i) the quenching site is located in the major LHCII or minor LHC; (ii) quenching is caused by electron transfer from Z to Chl, energy transfer from Chl to lutein, or Chl–Chl charge transfer state formation; and (iii) ΔpH, PsbS, and Z act synergistically or independently (Horton *et al.*, 1991, 2000; Ruban *et al.*, 1993, 2012; Holzwarth *et al.*, 2009; Johnson *et al.*, 2009; Lambrev *et al.*, 2010; Nilkens *et al.*, 2010).

In addition to study plant NPQ capacity, it is insightful to characterize the transition to (formation) and from (relaxation) the NPQ state (Ruban and Johnson, 2010). Towards this aim, the pioneering works of Ruban *et al.* (1993) and Nilkens *et al.* (2010) should be highlighted. The former detected heterogeneous kinetics of Z and NPQ formation upon illumination, whereas the latter was the first one to analyse the exact time course of Z formation and link to different NPQ components. A key characteristic of qE is that it is induced in seconds after exposing plants to HL. For instance, ΔA_{535} reaches saturation within $c. 1$ min or less (Morales *et al.*, 1990; Johnson *et al.*, 2012). Kinetics investigations provide the chance to resolve whether single or different mechanisms can account for qE and to give clues as to which type of mechanisms are involved.

Detailed reflectance and fluorescence spectroscopy *in vivo* requires careful choice of plant material. Research of the qE mechanistic bases has been focused on *Arabidopsis*, whereas data from other species (such as long-lived slow-growing species, perennial evergreens, or sun and shade plants) are scarce (García-Plazaola *et al.*, 2012; Jahns and Holzwarth, 2012). *Quercus coccifera* has the advantage that responds to changes in light intensity with 95–100% displacement of the xanthophyll cycle towards Z (Morales *et al.*, 2002; Peguero-Pina *et al.*, 2008, 2009), higher than 20–46% in cotton and *Arabidopsis* (Demmig-Adams, 1990; Johnson *et al.*, 2009; Johnson and Ruban, 2010) and 49% in *Guzmania monostachia* (Ruban *et al.*, 1993), but close to 80–86% reported for *Arbutus unedo* or in other *Arabidopsis* works (Demmig-Adams, 1990; Li *et al.*, 2002a). The Z concentrations in the present experiments are below 50 mmol (mol Chl)$^{-1}$, are likely associated with pigment–protein complexes, and are putatively involved in NPQ. By contrast, at higher V+A+Z concentrations, Z should be in the 'free' form and should behave mostly as a chloroplast antioxidant (Havaux and Niyogi, 1999; Niinemets *et al.*, 2003). Therefore, it cannot be excluded that different pools were de-epoxidized in response to HL with different kinetics. Also, the amount of PsbS determines qE capacity (Li *et al.*, 2002a; Niyogi *et al.*, 2005). Long-lived, slow-growing evergreen species (Demmig-Adams and Adams, 2006), such as *Q. coccifera*, have a high capacity for NPQ (Peguero-Pina *et al.*, 2008; Vilagrosa *et al.*, 2010), associated to large PsbS/PSII ratios. Demmig-Adams *et al.* (2012) suggested evergreens as model species for photoprotection studies beyond the *Arabidopsis* model (García-Plazaola *et al.*, 2012).

This study undertook a detailed investigation of the induction and relaxation phases of NPQ in a time window of

seconds or minutes that enabled a comparison of the relative importance of A and Z in a LL–HL–LL transition. For this purpose, this study developed instrumentation for instantaneous freezing of leaf material which allowed NPQ, spectral reflectance changes, and A and Z concentrations to be followed kinetically.

Materials and methods

Plant material and experimental conditions

Q. coccifera L. plants were 5 years old with a Zaragoza (Spain) provenance. Measurements were made on fully mature current-year leaves at predawn. Leaves were exposed to LL (65 µmol m^{-2} s^{-1}) for 240 s and, subsequently, to HL (1850 µmol m^{-2} s^{-1}) for 180 s (induction). Finally, leaves were exposed to LL for 180 s (relaxation). The use of LL led to leaves with stomatal conductances of 160–220 mmol H$_2$O m^{-2} s^{-1} (data not shown) depleted of Z (see Results). The LL source was a halogen spotlight located 0.9 m above the experimental device. A heat trap was placed in front of the light to avoid air overheating. HL conditions were obtained by using a KL2500 LCD white light source (Schott, Mainz, Germany).

Sampling for determination of *in situ* dynamic activity of the xanthophyll cycle was made with a locally manufactured device which cuts a leaf disc from an attached leaf for freezing in liquid N$_2$. The device used an electromagnet to control a steel punch, which cored the leaf disc directly into a liquid N$_2$ container. Leaf discs were stored at –20ºC until HPLC determination. After 240 s of LL, simultaneous spectral reflectance measurements and sampling for pigments were started at 6-s (first min) and 12-s (second and third min) intervals, both during induction and relaxation kinetics. Leaves were therefore not repeatedly measured throughout the experiment because of the destructive sampling at each measuring time. NPQ was measured in separate experiments using the same illumination protocol and sampling frequency.

Spectral reflectance

Leaf reflectance was detected with a visible/near-infrared spectroradiometer USB-2000 (Ocean Optics, Dunedin, USA), expressed as spectral reflectance after standardization with white standard (Spectralon, Labsphere, North Sutton, NH, USA). Integration time was 100 ms. The physiological reflectance index (PRI$_{531}$) was calculated as PRI = (R$_{531}$ − R$_{570}$) / (R$_{531}$ + R$_{570}$), where R$_{531}$ and R$_{570}$ represent, respectively, the reflectance at 531 and 570 nm, the latter used as reference (Gamon *et al.*, 1990). Reflectance changes at 505 nm were followed using an alternative index (defined as PRI$_{505}$) replacing R$_{531}$ by R$_{505}$. PRI$_{531}$ and PRI$_{505}$ can be obtained non-destructively from attached leaves and were used as proxies for changes in A$_{535}$ and A$_{505}$, respectively.

Other reflectance indices are proposed to estimate photosynthetic activity. Dobrowski *et al.* (2005) reported that R$_{690}$/R$_{600}$ and R$_{740}$/R$_{800}$ correlated well with Chl fluorescence. Obviously, R$_{690}$ and R$_{740}$ contain Chl fluorescence. These ratios were used to monitor quenching at 690 and 740 nm during the LL–HL–LL transitions.

Blue light induces chloroplast rearrangements. The halogen white light used contained 18% blue light (337 µmol photons m^{-2} s^{-1} at HL, measured with the USB-2000), despite the dominant red colour of the halogen sources. Upon HL, chloroplasts arrange on the side cell walls, maximizing mutual shading (Inoue and Shibata, 1973) and decreasing the amount of light absorbed by chloroplasts. Brugnoli and Björkman (1992) quantified the effects of chloroplast movements on spectral changes and fluorescence. In the present work, chloroplast movements affected both NPQ measurements and PRI$_{531}$ and PRI$_{505}$ changes and they were corrected whenever possible (see Results). When compared to other techniques, such as fluorescence emission spectra often used to follow spectral changes associated to NPQ, reflectance changes are somewhat limited.

Chlorophyll fluorescence

Chl fluorescence was measured with a FMS II modulated fluorometer (Hansatech Instruments, Norfolk, UK). Initial Chl fluorescence in darkness (F$_O$) was measured at predawn by switching on the modulated light (0.6 kHz); leaf surface photosynthetic photon flux density was below 0.4 µmol m^{-2} s^{-1}. Maximal Chl fluorescence in darkness (F$_M$) was measured at predawn (20 kHz) with a 0.8-s pulse of 6000 µmol m^{-2} s^{-1} of white light. F$_O$ was measured in presence of far-red light that fully oxidizes the PSII acceptor side (Belkhodja *et al.*, 1998; Morales *et al.*, 1998). After that, a second pulse of high-intensity white light was used to determine the maximal Chl fluorescence in the light-adapted state (F'$_M$). In separate experiments (data not shown), no effects of the pulses frequency on NPQ values were observed. NPQ was calculated as (F$_M$/F'$_M$) – 1 (Bilger and Björkman, 1990).

Photosynthetic pigments

Leaf discs frozen in liquid N$_2$ were wrapped in aluminium foil and stored at –20 ºC. Pigments were extracted with acetone in presence of Na-ascorbate. Extracts were thawed on ice, filtered through a 0.45-µm filter and analysed by isocratic HPLC (Larbi *et al.*, 2004). Chemicals were HPLC quality. In some cases, Chl was estimated non-destructively using the TCARI/OSAVI reflectance index (Haboudane *et al.*, 2002).

Results

Photosynthetic pigment composition of
Q. coccifera leaves

Z changes diurnally with typically little Z in the morning (LL) and maximum Z levels at peak irradiance (HL) (Demmig-Adams, 1990). In this work, the (A+Z)/(V+A+Z) ratio changed from 0.13 (LL) to 0.97 (HL) (Table 1). Predawn A+Z/(V+A+Z) values in this species are 0.1–0.2 (Peguero-Pina *et al.*, 2009; Vilagrosa *et al.*, 2010). The V+A+Z pool was 30–34 mmol (mol Chl)$^{-1}$, with no detectable changes between LL and HL (Table 1). Lutein epoxide, described in *Quercus* species (García-Plazaola *et al.*, 2003), was detected at concentrations below 6.5 mmol (mol Chl)$^{-1}$ and did not change in response to HL (Table 1). Concentrations of other photosynthetic pigments are given in Table 1.

Induction of NPQ and associated reflectance,
antheraxanthin, and zeaxanthin changes

NPQ induction and relaxation kinetics are shown in Fig. 1A. Upon HL illumination, NPQ rose within the first seconds toward maxima values and declined back after few seconds of LL. This is energy-dependent quenching (qE) typical behaviour. When darkness was used instead of LL, ΔpH collapsed faster and consequently qE fully relaxed (data not shown), returning leaves to a non-dissipating condition.

Leaf reflectance contains Chl fluorescence (Buschmann and Lichtenthaler, 1988). Therefore, methods were developed to quantify fluorescence from reflectance, by measuring at wavelengths affected normalized by those not affected by

Table 1. Photosynthetic pigment composition and de-epoxidation state of the xanthophyll cycle in *Quercus coccifera* leaves in the low light-adapted and high light-adapted states. Samples were taken from 0 and 180 s after changing from LL to HL. Values are mean ± 1 SE of four or five replicates. Different letters indicate statistical difference between treatments (Student's t test $P < 0.001$). Chlorophyll compositions are given in µmol m^{-2}; carotenoid compositions are given in mmol pigment (mol Chl)$^{-1}$. A = antheraxanthin; V = violaxanthin; Z = zeaxanthin.

Light level	Chl *a*	Chl *b*	Neoxanthin	Lutein	Lutein epoxide	β-Carotene	V+A+Z	Chl *a/b*	(A+Z)/ (V+A+Z)
Low	361 ± 20	132 ± 7	29 ± 3	123 ± 2	4.8 ± 0.8	93 ± 3	30 ± 2	2.7 ± 0.1	0.13 ± 0.01[a]
High	293 ± 19	104 ± 3	33 ± 3	121 ± 1	6.5 ± 1.2	95 ± 4	34 ± 3	2.8 ± 0.1	0.97 ± 0.01[b]

fluorescence (e.g. R_{690}/R_{600} and R_{740}/R_{800}; Dobrowski *et al.*, 2005). Upon HL illumination, both R_{690}/R_{600} (open circles, Fig. 1B) and R_{740}/R_{800} (solid circles, Fig. 1B) ratios decreased with time, reflecting NPQ development. Quenching was larger at 690 than at 740 nm, due to PSI contribution to 740 nm fluorescence (Lambrev *et al.*, 2010). NPQ relaxation kinetics was not clearly seen when monitoring reflectance ratios (Fig. 1B).

In *Q. coccifera*, HL decreased PRI_{531} (solid circles, Fig. 1C) and PRI_{505} (open circles, Fig. 1C). PRI_{531} and PRI_{505} changes were partially reversible in the short term in LL (Fig. 1C). PRI_{531} and PRI_{505} half-times could not be calculated because of interferences due to chloroplast movements, as will be shown.

Fig. 2A shows reflectance spectra at times 0, 96, 108, and 168 s after HL. Up to 96 s, the HL-minus-LL difference spectra had negative peaks at 532 and 680 nm (Fig. 2B), matching the main peak of the NPQ state in the green (Johnson *et al.*, 2009, 2010) and fluorescence quenching in the Chl emission spectrum (Lambrev *et al.*, 2010). Differences in quenching between bands contributing more to PSII or PSI (Lambrev *et al.*, 2010) could not be distinguished from these spectra. Beyond 108 s, the HL-minus-LL difference spectra had a large positive band broad in the blue-green and red parts of the spectrum, and a blue-shifted red edge (difference spectra are not shown but see traces in Fig. 2A). These spectral characteristics suggest chloroplast movements. These reflectance changes are in opposite direction compared to those resulting from ΔpH and Z formation, and overlapped them from 108 to 180 s upon HL with two characteristic steps occurring at *c*.108 and 168 s (Fig. 2A). Chloroplast movements and changes in leaf absorbance during kinetics influence fluorescence and reflectance. Therefore, this study quantified the reduction in Chl seen by the fluorimeter and spectroradiometer by calculating the TCARI/OSAVI ratios as a measure of the *in vivo* Chl concentration (Haboudane *et al.*, 2002) in order to be as close as possible to actual NPQ and PRI values.

NPQ time-dependent changes after corrections for chloroplast movements were similar to those described by Morales *et al.* (1999). Thus, as shown in Fig. 3A, NPQ did lie somewhere (shaded area) between NPQ measurements before (uppermost limit of shaded area) and after (open circles) corrections for chloroplast movements. Half-times for NPQ formation and relaxation were 15 s and 45–20 s (before and after corrections), respectively. Nilkens *et al.* (2010) reported in *Arabidopsis* a fast phase of relaxation of 35–50 s.

Effects of chloroplast movements were also observable in the PRI_{531} and PRI_{505} kinetics. Leaf reflectance, including that at 505, 531, and 570 nm, increases with decreasing Chl (Richardson *et al.*, 2002). Chloroplast movements have an optical effect as loss of leaf Chl (i.e. the spectroradiometer sees less Chl), decreasing PRI_{531} and PRI_{505} as a result of increasing $R_{531}+R_{570}$ and $R_{505}+R_{570}$, respectively. This is what was observed in the kinetics of both PRI_{531} and PRI_{505} (Fig. 1C) after 108 s of HL (Fig. 2A), superimposed on the effects of ΔpH development and A+Z formation.

Chloroplast movement effect on leaf transmittance at 650 nm is approximately equal to that at 508 nm (Brugnoli and Björkman, 1992). Therefore, this study used 650 nm as reference instead of 570 nm for calculating PRI_{505}. $PRI_{505(ref 650)}$ changes were smaller in amplitude, more smooth but similar kinetically (solid circles, Fig. 3A). The same approach could not be used for PRI_{531} because it is more difficult to find an appropriate reference (Brugnoli and Björkman, 1992).

In order to check whether the NPQ induction kinetics was related to xanthophyll cycle activity, leaf discs were rapidly frozen in the light at different times of HL immediately after the reflectance spectrum was recorded. Furthermore, reflectance spectra and sampling for pigments were made after keeping HL pre-illuminated leaves for different times in LL. The resulting time-dependent changes in V, A, and Z are shown in Fig. 4. In response to HL, de-epoxidated forms A and Z were formed at expense of V, but with kinetics very different to that of NPQ, which indicates that the induction of NPQ and that of the activity of the xanthophyll cycle have substantially different time courses. Unlike NPQ, the time course of the V de-epoxidation was very slow and partial (10%, not statistically different at $P < 0.05$ using Student's t test) during the first 30 s in HL. A reached maximum level after 90 s in HL, time in which Z/(V+A+Z) ratios were relatively low (*c*.0.2–0.3). Beyond that time, Z increased linearly with time reaching Z/(V+A+Z) ratios of 0.9. After returning to LL, during 30 s, the V+A+Z pool remained as A+Z (0.95–0.97 A+Z/(V+A+Z)) and a relatively rapid but partial re-epoxidation of the V+A+Z pool occurred afterwards (*c*.40% of the pool remained as A+Z). De-epoxidation and epoxidation half-times were 150 and 35 s, respectively. The former was one order of magnitude slower than that of NPQ formation, as already shown.
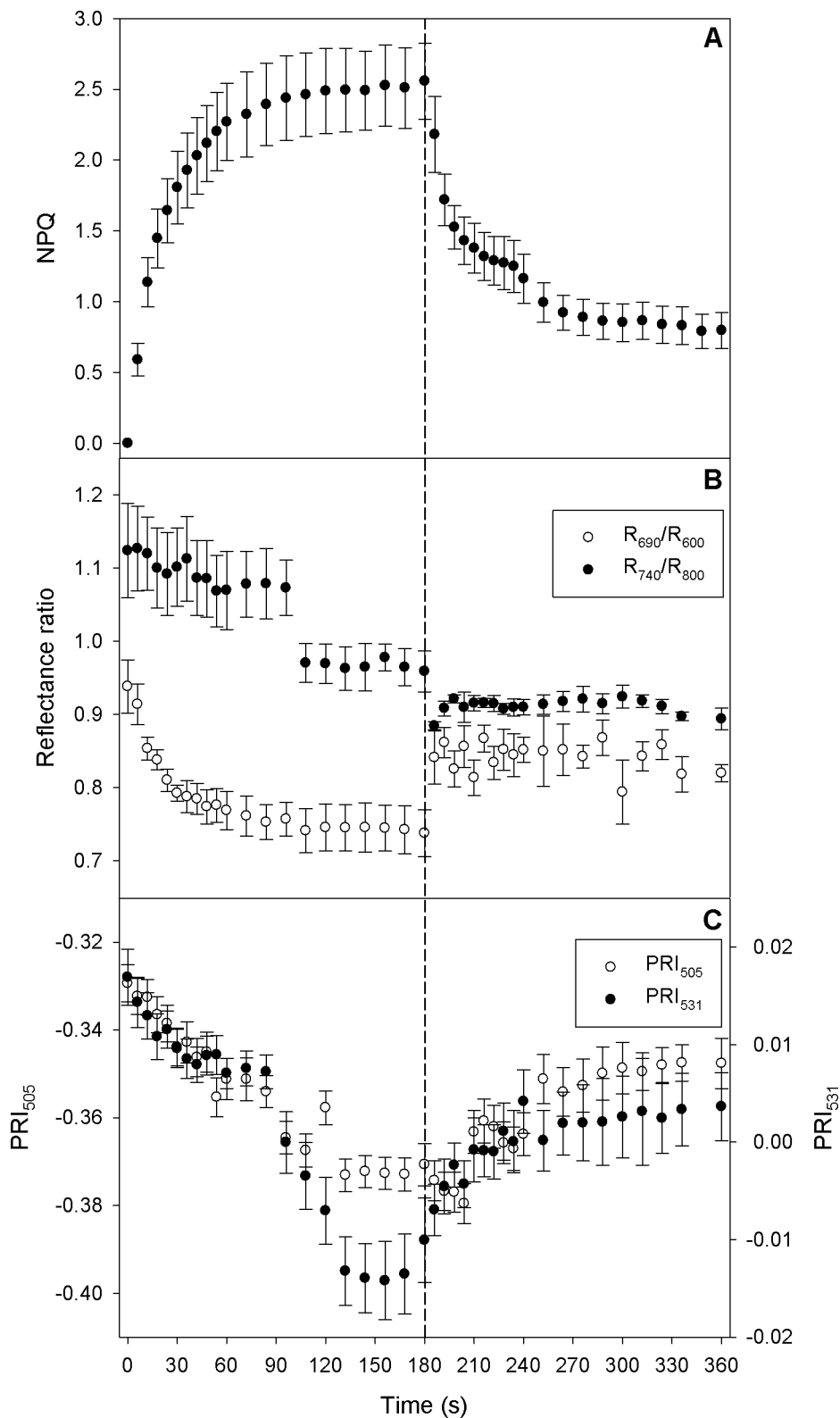
Fig. 1. (A) Development of non-photochemical quenching (NPQ) upon a transfer from low light (LL) to high light (HL) and relaxation at LL in *Quercus coccifera* leaves. (B) Reflectance ratios at wavelengths affected normalized by wavelengths not affected by fluorescence emission R_{690}/R_{600} and R_{740}/R_{800}. (C) Physiological reflectance indexes PRI_{531} and PRI_{505}. Plants were previously illuminated for 4 min at LL (65 µmol m^{-2} s^{-1}), illuminated with HL (1850 µmol m^{-2} s^{-1}) for 3 min, and then allowed to recover for 3 min at the referred LL. Data are mean ± 1 SE of five replicates. Vertical broken lines represent the transition from HL to LL.
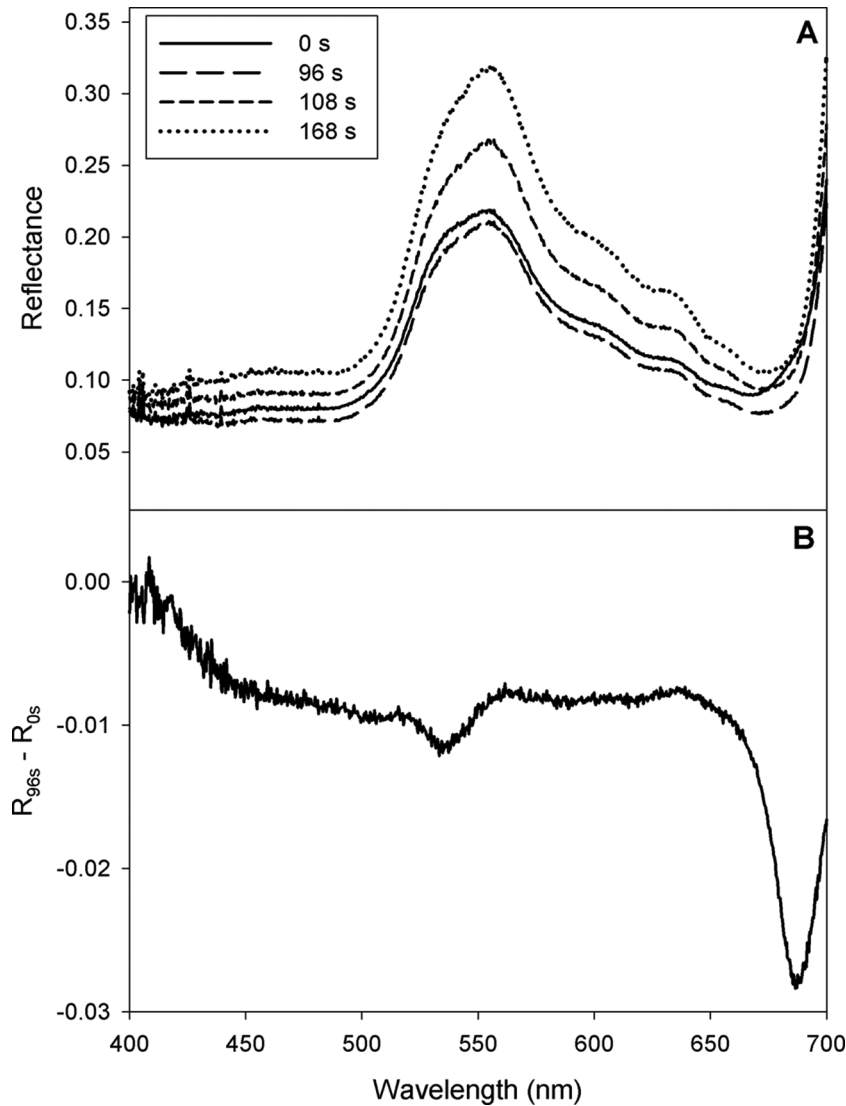
Fig. 2. Spectral changes upon a transfer from low light (LL) to high light (HL) in *Quercus coccifera* leaves. Illumination conditions were as in Fig. 1. (A) Representative reflectance spectra from 400 to 700 nm at several times after HL illumination. (B) The difference spectrum during 96–0 s of HL.

Relationship between NPQ and the antheraxanthin and zeaxanthin pool during the LL–HL transition and recovery at LL

This study used PRI_{505} as an *in vivo* estimation of de-epoxidation within the xanthophyll cycle. When the kinetics of NPQ formation and relaxation was plotted against the PRI_{505} changes, non-linear relationships were found both during the induction and the recovery (Fig. 3B). The slope of the NPQ versus PRI_{505} plot was remarkably low at the beginning of both the formation and relaxation of NPQ, indicating an apparently reduced sensitivity of NPQ to the de-epoxidized molecules within the xanthophyll cycle. However, slopes increased afterwards drastically, suggesting that NPQ sensitivity to such de-epoxidized forms has increased markedly.

When PRI_{505} changes were substituted by V, A, and Z changes in the relationship with NPQ, the data revealed valuable information (Fig. 5). During NPQ formation (Fig. 5A), three different phases were identified (separated by vertical dotted lines). A first one was characterized by large NPQ increases (from 0 to 1.7; 70% of the NPQ maximum capacity) with very small (no significant) increases of A+Z at expense of V. During the second phase, NPQ increased up to 2.5, reaching saturation, which was related to already significant increases of A+Z (up to 30–40% of A+Z/(V+A+Z)). Later on, the xanthophyll cycle was fully de-epoxidized (0.95–0.97 A+Z/(V+A+Z)) but those changes had no effects on quenching.

During NPQ relaxation (Fig. 5B), three kinetic behaviours were also recorded (separated by vertical dotted lines). During the first phase, NPQ relaxed from >2.5 to 1.40, with no detectable activity of the xanthophyll cycle, remaining as high as 0.95–0.97 A+Z/(V+A+Z). A second phase was characterized by large decreases in the A+Z/(V+A+Z) ratios, down to 0.50, with small NPQ effects (decreasing to 1.2–1.1).
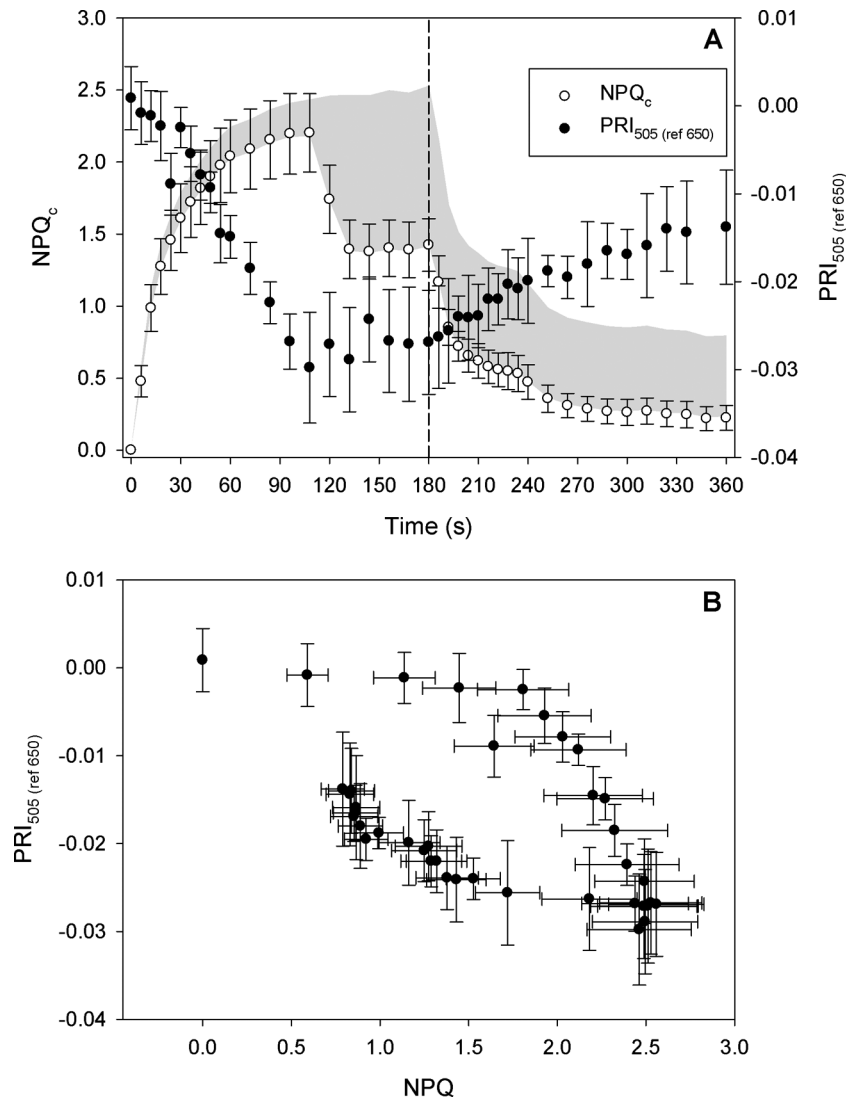
Fig. 3. (A) Time-course of changes in non-photochemical quenching (NPQ) and physiological reflectance index PRI_{505} upon a transfer from low light (LL) to high light (HL) and relaxation at LL in *Quercus coccifera* leaves after corrections for chloroplast movements. Illumination conditions were as in Fig. 1. Open circles represent limit NPQ values after corrections (NPQ_c) for chloroplast movements (left axis); actual NPQ values lie somewhere between the measured values shown in Fig. 1A and open circles (shaded area). Solid circles represent modified PRI_{505} index using 650 nm as reference wavelength instead of 570 nm (right axis). (B) Relationship between PRI_{505} (reference at 650 nm) and NPQ. Data are mean ± 1 SE of five replicates. Vertical broken line in Fig. 3A represents the transition from HL to LL.

During the last phase, no further changes could be observed within the xanthophyll cycle, only NPQ decreased slightly.

Discussion

The present manuscript characterizes the exact time course of xanthophylls conversion in both NPQ induction and relaxation using biochemical as well as *in vivo* spectroscopy methods in leaves of the evergreen plant *Q. coccifera* with time resolution of seconds. This paper provides insights into the role of xanthophylls in the NPQ process and enables the clear confirmation of the vague relationship between NPQ and Z, highlighting the indirect role of the latter in the establishment of the protective energy dissipation state. It is shown that

70–75% of the HL-induced qE develops and relaxes within the first 30 s of HL and LL, respectively. Ruban *et al.* (1993) and Nilkens *et al.* (2010) reported similar results. The present data indicate that ΔpH plays a determinant role during qE formation and relaxation. As early as 1979, Briantais *et al.* reported that NPQ is a ΔpH-dependent process. In addition, it is nowadays known that an enhanced ΔpH may restore qE in the absence of PsbS (Johnson and Ruban, 2011) and of lutein and xanthophyll cycle activity (Johnson *et al.*, 2012). It can be concluded, therefore, that under some conditions low lumen pH (<4.5–5.0) alone can cause maximum quenching (Ruban *et al.*, 1993, 2012). Z was shown to shift quenching activation at lumen pH of *c.*5.7–6.2, allowing simultaneously high photosynthetic electron transport rates (Ruban *et al.*,
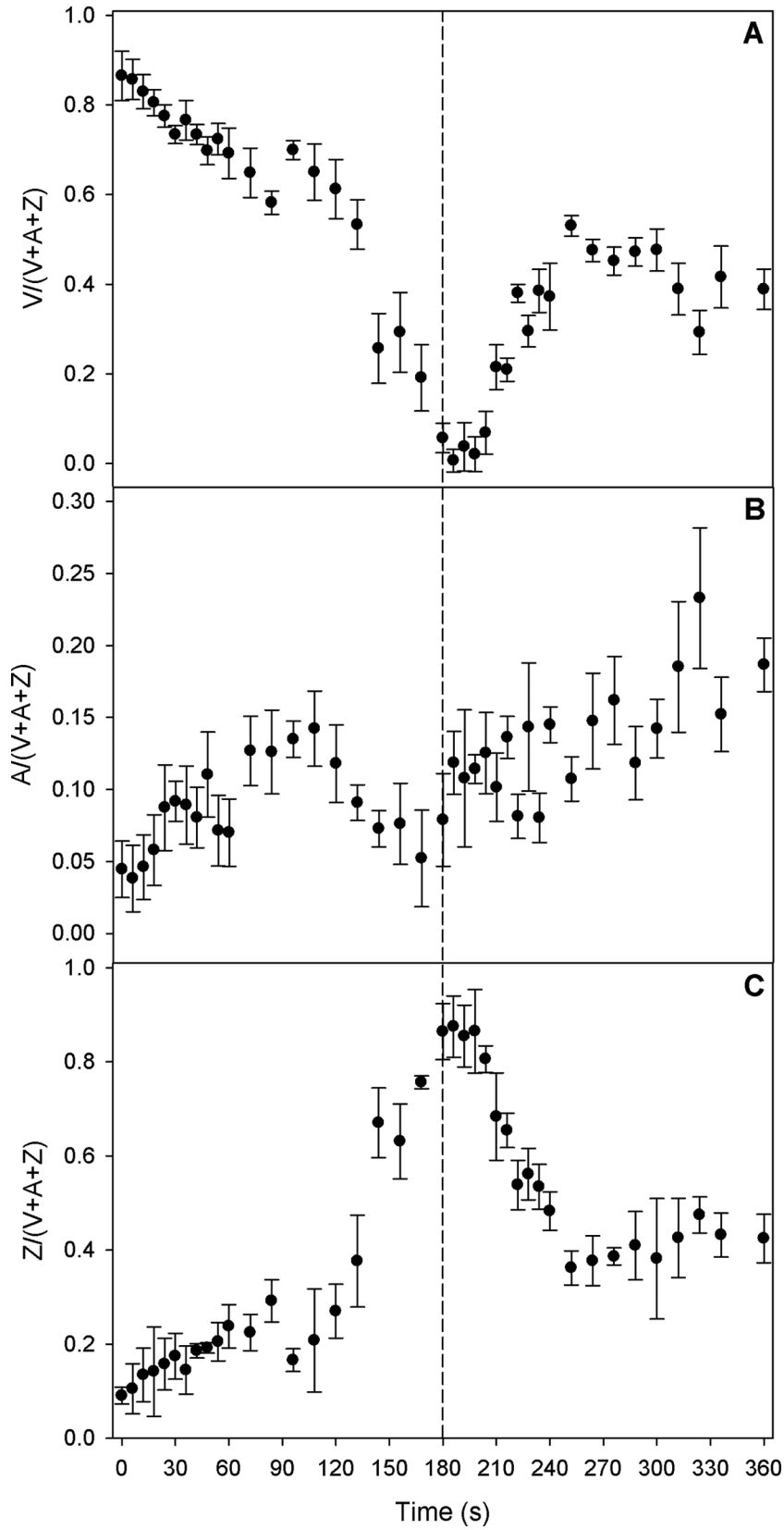
Fig. 4. Kinetic changes of the de-epoxidation state of the xanthophyll cycle upon a transfer from low light (LL) to high light (HL) and relaxation at LL in *Quercus coccifera* leaves. Illumination conditions were as in Fig. 1. (A) V/(V+A+Z). (B) A/(V+A+Z). (C) Z/(V+A+Z). Data are mean ± 1 SE of four or five replicates. Vertical broken lines represent the transition from HL to LL. A = antheraxanthin; V = violaxanthin; Z = zeaxanthin.
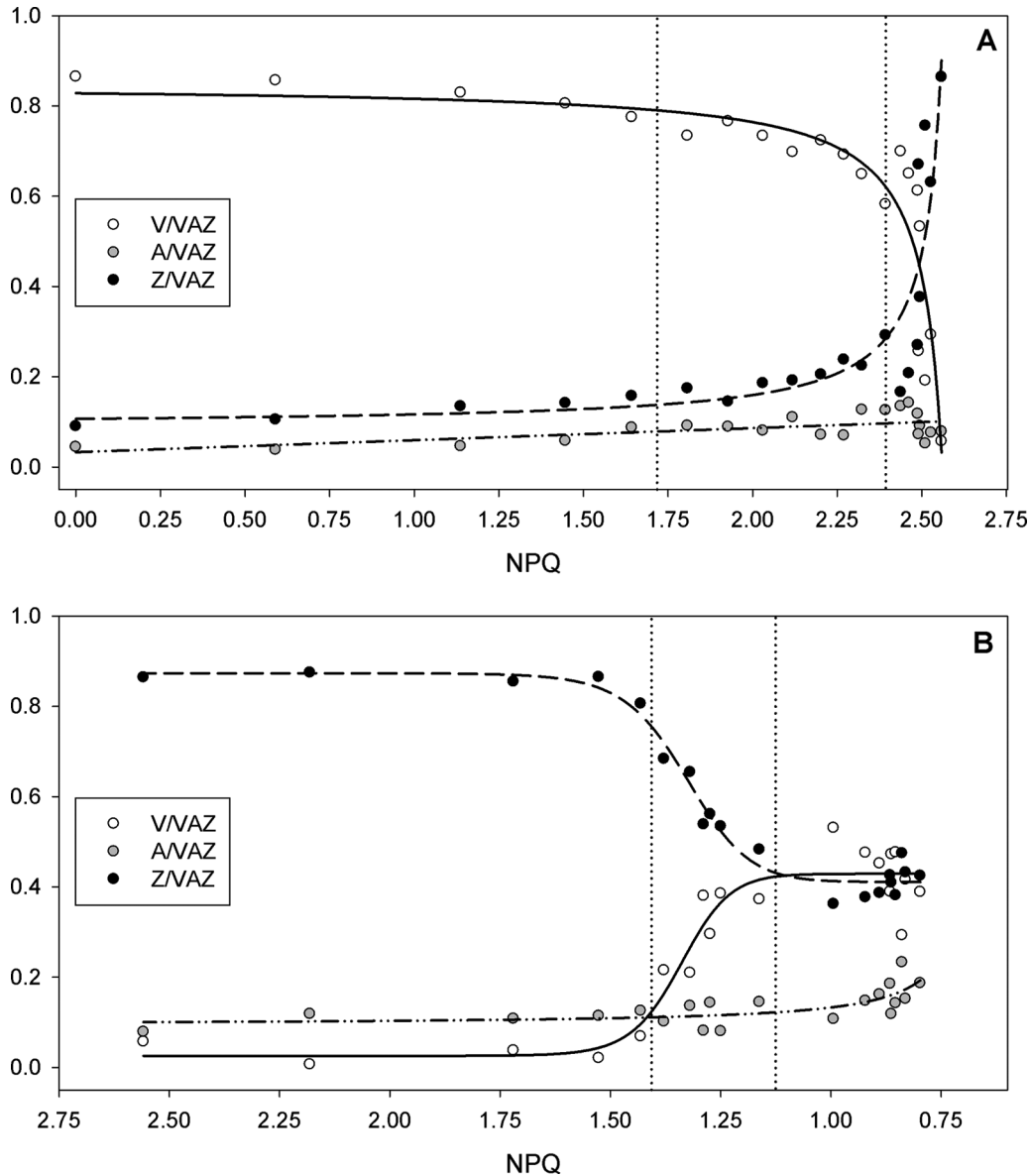
Fig. 5. Relationships between molecules of the xanthophyll cycle [V/(V+A+Z), A/(V+A+Z), and Z/(V+A+Z) ratios] and non-photochemical quenching (NPQ) upon transfer from low light (LL) to high light (HL) (A) and relaxation at LL (B) in *Quercus coccifera* leaves. For clarity purposes, only means are shown. The standard errors of these data are shown in Figs. 1 and 4. Vertical dotted lines separate distinct kinetic responses of the xanthophyll cycle. A = antheraxanthin; V = violaxanthin; Z = zeaxanthin.

2012). The rapid onset of qE causes a large fraction of all PSII to re-open (Morales *et al.*, 1999) as a result of excess energy dissipation and the consequential relief of the excitation pressure (Ruban *et al.*, 2012).

This study's approach has actually identified the existence of three functional pools of Z with very different roles in photoprotection in *Q. coccifera*: (i) a background pool that is essentially present already under predawn conditions in the unquenched state; (ii) a pool that grows after *c.* 30–90 s HL and contributes strongly in this time frame to NPQ; and (iii) a third pool that forms on a longer time scale but leads to little or no further increase in NPQ. Similar results (i.e. initial presence of low level of Z and negligible Z formation during the first 30 s of illumination) were reported when dark-adapted

G. monostachia leaves were illuminated (Ruban *et al.*, 1993). As a consequence, in *Q. coccifera* and under the present experimental conditions, maximum number of de-epoxidized xanthophylls related to quenching would be 11.5 mmol A+Z (mol Chl)$^{-1}$ (0.38 A+Z/(V+A+Z)); the rest [up to 33–34 mmol A+Z (mol Chl)$^{-1}$ (0.95–0.97 A+Z/(V+A+Z))] would have an antioxidant role, as will be discussed.

qE is kinetically heterogeneous. ΔpH causes protonation of LHCs (Ruban *et al.*, 1993; Walters *et al.*, 1994) and PsbS (Li *et al.*, 2004) and triggers the enzymatic de-epoxidation of V to A and Z (Yamamoto, 1979; Demmig-Adams and Adams, 1992). The exact interplay of these three factors remains the subject of great debate. A key step in understanding the mechanism is to decipher whether ΔpH, PsbS, and Z cooperate to

promote NPQ or act entirely independently. In the cooperative model, it is assumed that lumen pH, PsbS, and Z act synergistically to form qE (Horton *et al.*, 1991, 2000; Johnson *et al.*, 2009; Ruban *et al.*, 2012). In a recent review, Demmig-Adams *et al.* (2012) have concluded that rapid modulations of PSII efficiency and NPQ changes under natural conditions always occur in the presence of Z. However, the data presented here could also support the alternative, contrasting model (Holzwarth *et al.*, 2009; Nilkens *et al.*, 2010) of independent quenching action of PsbS and Z and contradict the cooperative model. This is particularly suggested by both fast induction (in the absence of Z formation) and relaxation (in presence of a large Z pool). Interestingly, during the first 30 s of qE formation and relaxation no changes were observed in the A+Z/(V+A+Z) ratios, which remained fairly constant at values of 0.13–0.26 (4–8 mmol A+Z (mol Chl)$^{-1}$) and 0.95–0.97 (33–34 mmol A+Z (mol Chl)$^{-1}$), respectively. During these periods, however, ΔpH and NPQ were largely formed and relaxed. Upon ΔpH formation in HL and collapse in darkness, PsbS appears to promote the fast establishment of the quenched (Holzwarth *et al.*, 2009) and restoration of the fluorescent (Zia *et al.*, 2011) states.

Another key question of debate is the relationship between NPQ and PSII photoprotection, in terms of avoiding PSII damage through reduction of Chl triplet (^{3}Chl) generation or quenching once generated, as recently modelled by Lambrev *et al.* (2012). Following their considerations, *Q. coccifera* leaves during the LL to HL transition would have three levels of photoprotection that correspond to the three mechanisms observed upon HL illumination from LL. The first one, up to 30 s of HL, is a ΔpH-regulated and PsbS-dependent qE type of quenching that operates by functional detachment of parts of the major LHCII from the PSII supercomplex (Betterle *et al.*, 2009; Holzwarth *et al.*, 2009; Nilkens *et al.*, 2010; Lambrev *et al.*, 2010, 2012). Horton *et al.* (1991) reported that qE can be observed without Z formation in isolated chloroplasts. This mechanism is important when the photosynthetic apparatus has to respond rapidly to changing light conditions (Lambrev *et al.*, 2012). The second one occurs kinetically from 30 to 90 s of HL and is a PsbS-independent and Z-dependent quenching, which photoprotects PSII by enhanced de-excitation in the minor PSII antenna (Holzwarth *et al.*, 2009; Lambrev *et al.*, 2010), possibly CP24 and/or CP29 because their absence decreases quenching by 30–60% (Andersson *et al.*, 2001; Kovacs *et al.*, 2006). Therefore, the present data do not support the suggestion that Z-dependent and Z-independent quenching may originate from the same PsbS-dependent mechanism (Johnson *et al.*, 2009) modulated by Z (Crouchman *et al.*, 2006). The first and the second mechanisms agree with the existence of two different quenching sites (Holzwarth *et al.*, 2009; Nilkens *et al.*, 2010) and could explain the proposed Z-independent and Z-dependent components of NPQ. The model shows that PSII photoprotection of antenna detachment and antenna quenching are additive – both have significant benefits for photoprotection – but Z-dependent antenna quenching is more effective than antenna detachment in providing photoprotection by reduction ^{3}Chl generation during prolonged periods of HL (Lambrev *et al.*, 2012). The third level of photoprotection,

beyond 90 s of HL, is a massive Z accumulation that would quench the generated ^{3}Chl acting as antioxidant (Havaux and Niyogi, 1999; Niinemets *et al.*, 2003).

Concerning this third pool of Z, the following may be considered. NPQ is a non-linear parameter that can theoretically go to infinity, whereas actual quenching (as measured by the fluorescence decrease) can at best be 100%. Thus, after a substantial initial fast qE phase, the sensitivity of NPQ to follow quenching is strongly reduced to further quenching effects due to Z increase. Therefore, this third Z pool might alternatively contribute to quenching or photoprotection but in a way that is not indicated by a change in the NPQ parameter (for further discussion see Lambrev *et al.*, 2012).

The exact pigments involved in quenching remain under debate, with both Chl–xanthophyll (Johnson *et al.*, 2011) and Chl–Chl (Holzwarth *et al.*, 2009) interactions suggested to be involved. Holzwarth and co-workers challenged the consensus about the involvement of carotenoids in qE. They found red-emitting states formed during NPQ *in vivo* and in LHCII aggregates suggested to be related to the formation of coherent Chl–Chl interactions with charge transfer character, with no evidence of carotenoid involvement in quenching (Muller *et al.*, 2010). These ideas are in line with earlier ones linking quenching to the appearance of red-shifted Chl absorption and 77K fluorescence bands, typical of excitonically coupled Chls (reviewed and discussed by Ruban *et al.*, 2012). Irrespective of the pigments involved, both hypotheses of work assume that a ΔpH-induced change activates the quenching pigment(s).

In summary, the kinetic data reported in this work may help to understand how plants are able to cope with excess light. Light is a very dynamic resource. Plants experience excess light during most of their life cycle and under a variety of stress conditions, which ranges in time span from seasons to seconds (Demmig-Adams *et al.*, 2012). ΔpH is determinant for maximal rates of qE formation and relaxation in the first 30 s of both switching to a dissipative state and returning to efficient light-harvesting antenna. Such a dynamic response is what one should expect for mechanisms acting in a highly variable light environment where cloud movements may expose plants from limiting to excess light, or the contrary, in only a few seconds.

Acknowledgements

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