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Seasonal changes in photosynthesis and photoprotection in a *Quercus ilex* subsp. *ballota* woodland located in its upper altitudinal extreme in the Iberian Peninsula

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Summary

Quercus ilex L. subsp. *ballota* (Desf.) Samp., a Mediterranean evergreen species growing in a continental Mediterranean climate, did not experience water stress and showed greater sensitivity to winter stress than to summer stress over a 12-month period. Net CO_2 assimilation rates and photosystem II (PSII) efficiency decreased markedly during the cold months and recovered completely in spring. Lutein, neoxanthin and β-carotene to chlorophyll (Chl) molar ratios all showed the same trend throughout the year, increasing from September to March. This increase was a result of increases in carotenoid concentrations, because Chl concentration per unit leaf area remained stable, and was higher at the end than at the beginning of the first growing season. Lutein-epoxide was a minor component of the total lutein pool. Thermal energy dissipation and non-photochemical quenching (NPQ) were associated with the de-epoxidated forms of the xanthophyll cycle pigments in the warm months. Photosynthetic rates decreased slightly at midday in summer. These changes were accompanied by decreases in maximum potential PSII efficiency (which recovered during the night), actual and intrinsic PSII efficiencies, photochemical quenching and increases in NPQ. Overall, our data indicate down-regulation of photosynthesis during the summer. The diurnal de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin occurred throughout the year, except in January. Antioxidant enzymatic activity increased in the winter months, especially during the coldest months, highlighting its key role in photoprotection against photo-oxidation. Structural and functional modifications protected PSII from permanent damage and allowed 1-year-old leaves to photosynthesize at high rates when temperatures increased in spring.

Keywords: antioxidant enzymatic activity, chlorophyll fluorescence, photosynthetic pigments, summer stress, winter stress.

Introduction

The carbon balance of evergreen species is often negative in extreme winter climates, and a combination of high temperatures and water stress in summer can also limit photosynthesis (Larcher 1969). In general, species are adapted to the temperature regime of their native environment. Adaptations to improve photosynthetic rates at low temperatures, however, generally worsen the response to high temperatures, and vice versa. Evergreen species from habitats characterized by large temperature variations during the growing season do not necessarily have a broad temperature optimum for photosynthesis, although they may have a high, genetically determined potential for photosynthetic acclimation to temperature (Berry and Björkman 1980). This characteristic, coupled with a longer leaf life span, offsets their generally lower photosynthetic rates compared with winter-deciduous species. Stressful environments may limit the evolution of species with high photosynthetic capacities that cannot be efficiently used and are therefore a wasted investment (Chabot and Hicks 1982).

Some evergreen oaks are exposed to low temperatures in winter and high temperatures in summer. In winter, low temperatures reduce enzyme activity, including the activities of photosynthetic enzymes, and thus the amount of light required to saturate photosynthesis in winter is reduced (Berry and Björkman 1980). At midday in summer, stomatal closure reduces stomatal conductance and CO_2 fixation, and more light is absorbed than is necessary for the photochemical reactions of photosynthesis (Tenhunen et al. 1981). Both high and low temperatures impose stress on the photosynthetic apparatus and may lead to photoinhibition (Demmig-Adams and Adams 1992, 1996*b*, Adams and Demmig-Adams 1994). When the photosynthetic rate decreases, the absorbed light energy not used in photosynthesis must be dissipated to avoid chloroplast damage caused by reactive oxygen species (Huner et al. 1993). There are several processes that protect the photosynthetic apparatus. A decrease in PSII efficiency during the light energy conversion process reduces the production of reactive oxygen

species (Demmig-Adams and Adams 1992, 1996*b*, Adams et al. 2004). The decrease in PSII efficiency and an increase in NPQ have been related to the accumulation of de-epoxidated forms of the xanthophyll cycle pigments, i.e., antheraxanthin and zeaxanthin, which participate in thermal dissipation of energy not used in photosynthesis (Adams and Demmig-Adams 1994). Furthermore, there is direct detoxification of reactive oxygen species by antioxidants and antioxidant enzymes, such as superoxide dismutase, ascorbate peroxidase, glutathione reductase and guaiacol peroxidase, which together decompose O_2^- and H_2O_2 (Foyer et al. 1994).

Quercus ilex L. is an Arcto-Tertiary species that survived the glaciations (Pignatti 1978). The species has a large bioclimatic range and a high plasticity in response to climate, with significant adaptability to thermal stress and to the high variability in precipitation, which is characteristic of the Mediterranean climate (Gratani 1996). Thus, *Q. ilex* is widely distributed in the semiarid, subhumid, humid and perhumid areas of the Iberian Peninsula (*sensu* Thornthwaite 1948), including the cold mountains of the Mediterranean and sub-Mediterranean regions. In the semiarid bioclimate, *Q. ilex* grows under continental conditions (cold to very cold winters) on infertile and poorly developed soils. However, *Q. ilex* grows best in a subhumid bioclimate, spreading from cold to mild regions. In the cold regions of the humid bioclimate, *Q. ilex* shares habitats with the deciduous oaks, *Quercus faginea* Lam. and *Quercus pyrenaica* Willd. In the perhumid bioclimate, although the deciduous oaks predominate, *Q. ilex* grows near the peaks of the mountains where the geopedological conditions are unsuitable for the growth of deciduous oaks.

In the Iberian Peninsula, two subspecies of *Q. ilex* have been described (Franco 1990). *Quercus ilex* subsp. *ilex* is distributed in littoral areas where the winter is relatively warm, whereas *Quercus ilex* subsp. *ballota* (Desf.) Samp. occupies continental areas with colder winters than those prevailing in littoral areas (Franco 1990). *Quercus ilex* subsp. *ilex* and *Q. ilex* subsp. *ballota* differ in their physiological characteristics, including vulnerability to water-stress-induced embolism (Corcuera et al. 2004).

The climate of the forest under study is transitional between that of the Mediterranean forest (evergreen oaks) and the nemoro-Mediterranean forest (deciduous oaks), a transitional forest between nemoral (temperate) and Mediterranean forest (Allué-Andrade 1990). In theory, the cold winters should favor deciduous oaks. However, the extremely thin soil over limestone outcrops, with a limited capacity to retain water, favors the predominance of species more tolerant to drought, such as the evergreen *Quercus ilex* L. subsp. *ballota*, which retains its leaves for 3 years (Corcuera et al. 2002). Despite the wide distribution of *Q. ilex*, Mitrakos (1980) proposed that summer water stress and the low temperatures of the coldest month of the year limit expansion of this species. However, it is not known to what extent photosynthetic activity is maintained during periods of summer drought and winter cold, and whether leaves that have been subjected to both summer stress and winter stress are able to photosynthesize when temperatures increase in spring. Previous studies have examined the impact of winter and summer stress on the photosynthetic apparatus of *Q. ilex* subsp. *ilex* under warm Mediterranean conditions (Gratani 1996, Faria et al. 1998, Gratani et al. 1998, García-Plazaola et al. 1999, Ogaya and Peñuelas 2003). The impact of summer stress on the photosynthetic apparatus of *Quercus suber* L. has also been reported (Faria et al. 1996).

Our objective was to characterize the seasonal variation in photosynthesis- and photoprotection-related parameters in leaves of *Q. ilex* subsp. *ballota* trees growing under continental conditions in a woodland habitat located in the upper altitudinal extreme for this species. For this purpose, we measured PSII efficiency, composition of photosynthetic pigments and enzymatic antioxidant activity during the first year of life (2000–2001) of the leaves.

Materials and methods

Study site and plant material

The work was carried out in a coppice dominated by *Q. ilex* subsp. *ballota* in the Sierra de Santa Cruz-Cubel, Zaragoza, NE Spain (1°39′ W, 41°07′ N, 1177 m a.s.l.). The phytoclimate of the study area corresponds to a transition from Mediterranean to nemoro-Mediterranean forest (deciduous oaks) with a tendency to sclerophylly (evergreen oaks) and a clear continental influence (Allué-Andrade 1990). The study site is located on poor soils developed over Tertiary limestone outcrops and is severly degraded by human activities.

The study site supports a coppice dominated by *Q. ilex* subsp. *ballota*, located at the upper altitudinal extreme (about 1200 m a.s.l.) for this species in the Iberian Peninsula. Temperature data were available from a meteorological station at Cubel-Casas Altas about 2 km from the coppice (1°38′ W, 41°06′ N, 1108 m a.s.l.). Mean annual temperature is 11.3 °C. Mean temperature in winter, spring, summer and autumn is 4.5, 11.6, 20.2 and 9.0 °C, respectively. Mean annual precipitation is 474 mm, with peaks in spring and fall. In the study area, the drought period in summer lasts from the end of June to early September. The study year (2000–2001) was drier and had minimal and maximal mean temperatures higher than the average ($P < 0.001$, Student's *t*-test, $n = 20$ years, data not shown). Extreme months in terms of maximal and minimal temperatures were August 2000 and February 2001, respectively (Figure 1). During the studied period, minimum temperature ranged from –5.6 °C in February 2000 to 18 °C in August 2000 (Figure 1A), whereas maximum temperature ranged from 4.5 °C in February 2000 to 36.5 °C in August 2000 (Figure 1B). February was the coldest month, with 13 days of frost.

New, fully developed leaves were sampled in July 2000 (leaves emerged in April 2000), followed by several samplings during fall, winter and spring (to verify the impacts of temperature on the photosynthetic apparatus and its potential recovery), and finally 12-month-old leaves were sampled in May 2001.

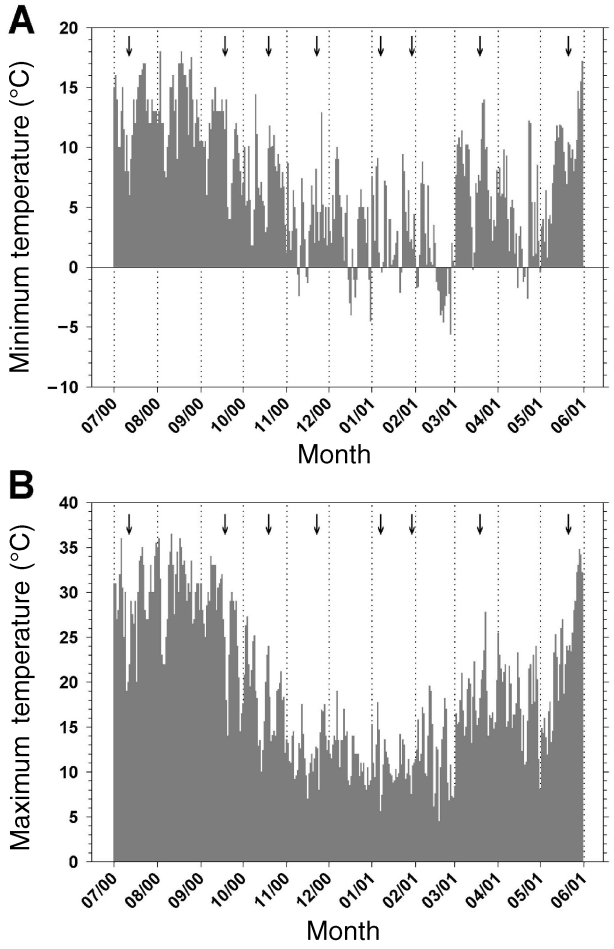

Figure 1. (A) Minimum and (B) maximum temperature from July 2000 to June 2001 near the study site in the Sierra de Santa Cruz-Cubel. Arrows represent sampling dates (July 13, September 20, October 19, November 24, January 9, February 1, March 20 and May 24).

Chlorophyll fluorescence, gas exchange and water potential measurements

Seven sampling dates were chosen throughout the year 2000 (July 13, October 19 and November 24) and 2001 (January 9, February 1, March 20 and May 24).

Chlorophyll (Chl) fluorescence was measured in situ before sunrise and at 0800 and 1200 h (solar time) with a PAM 2000 modulated portable fluorometer (Heinz Walz, Effeltrich, Germany). Measurements were taken on attached, sun-exposed leaves of the current year's growth. We used the experimental protocol for analysis of Chl fluorescence quenching described by Morales et al. (2000). Before sunrise, five leaves were chosen and covered with aluminum foil. Leaves were subjected to darkness by covering them with a black cloth for a few seconds during measurements of F_o (minimal Chl fluorescence yield in the dark), F_m (maximal Chl fluorescence yield in the dark) and F_o' (minimal Chl fluorescence yield during energization) (the period under the black cloth was too short to increase leaf temperature measurably). The same leaves were subsequently measured for Chl fluorescence. We measured F_o by switching on the modulated light at 0.6 kHz. We measured F_m and F_m' (maximal Chl fluorescence yield during energization) at 20 kHz with a 1-s pulse of 6000 μmol m^{-2} s^{-1} of white light. We measured F_o and F_o' during irradiation with far red light, to completely oxidize the PSII acceptor side. The actual (Φ_{PSII}) and intrinsic (Φ_{exc}) PSII efficiencies were estimated as $(F_m' - F_s)/F_m'$ and (F_v'/F_m'), respectively, where F_s is Chl fluorescence yield at steady-state photosynthesis and $F_v' = F_m' - F_o'$. Photochemical quenching (qP) was estimated as $(F_m' - F_s)/F_v'$. Non-photochemical quenching was estimated as $(F_m - F_m') - 1$.

Net photosynthetic rate (A; μmol CO_2 m^{-2} s^{-1}), stomatal conductance to water vapor (g_s; mmol m^{-2} s^{-1}), photosynthetically active radiation (PAR; μmol m^{-2} s^{-1}) and leaf temperature (T; °C) were measured with a gas exchange analyzer (CIRAS-1, PP-Systems, Herts, U.K.). Measurements were taken in situ, under natural conditions, at 0800, 1000 and 1200 h (solar time). Five south-facing sun-exposed leaves from the outer part of the crown were chosen from each of the three trees sampled (three replicates and five subsamples per replicate).

Five shoots from the outer part of the crown of five trees were cut with pruning shears and wrapped in plastic film; predawn water potential was measured immediately with a Scholander pressure chamber.

Analyses of photosynthetic pigments and antioxidant enzymes

Samples for analyses of chlorophylls and carotenoids and for assays of antioxidant enzyme activities were taken from sun-exposed, fully developed leaves of the current year's growth throughout the year 2000 (July 13, September 20, October 19 and November 24) and 2001 (January 9, February 1, March 20 and May 24).

Discs (0.5 cm^2 each) were cut with a cork borer at predawn and at 1000 and 1200 h (solar time), wrapped in aluminum foil, frozen in liquid nitrogen and stored (still wrapped in foil) at −80 °C. Nine different leaves were sampled on each date (three replications per time of the day). At midday, samples were taken from the same leaves in which modulated Chl fluorescence was measured. Pigments (one disc per sample) were extracted in a mortar with 100% acetone and a pinch of sodium ascorbate. Extracts were filtered through a 5-μm filter to remove insoluble residues and excess ascorbate and made up to the desired volume by the addition of acetone. There was about 1–2 cm^2 of tissue for every 5 ml of acetone (Abadía and Abadía 1993). Extracts were analyzed either immediately or after storage at –80 °C for no more than 24 h. Pigment extracts were thawed on ice, filtered through a 0.45-μm filter and analyzed by high performance liquid chromatography (HPLC) (Larbi et al. 2004). No pigment degradation was observed.

Sampling for antioxidant enzyme analysis was made at midday (1200 h, solar time). Whole leaves (three samples) were taken from three trees, wrapped in aluminum foil, frozen in

liquid nitrogen and (still wrapped in foil) stored at –80 °C. Antioxidant enzymes were extracted by homogenizing 500 mg of leaf fresh mass (without the central vein) at 5 °C in 10 ml of 100 mM phosphate buffer (pH 7.0) containing 0.1 mM ethylenediaminetetraacetic acid and 100 mg of polyvinylpyrrolidone in foliar fractions of 500 mg. The homogenized mixture was filtered through four gauze layers and centrifuged at 38,000 *g* for 10 min, after which the supernatant was removed and assayed for antioxidant enzyme activities. Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured as described in Becana et al. (1986), where a unit of SOD activity is defined as the amount of enzyme that produces a 50% inhibition in the Nitro Blue Tetrazolium (NBT) reduction. Total SOD activity was determined; measurements were performed in the absence of KCN and H_2O_2 because MnSOD is resistant to KCN and H_2O_2, FeSOD is resistant to KCN, but sensitive to H_2O_2 and CuZnSOD is sensitive to both compounds (Asada et al. 1975). Ascorbate peroxidase (APX; EC 1.11.1.11) activity was quantified according to Nakano and Asada (1981) with some modifications. The reaction medium (2 ml) was 80 mM phosphate buffer (pH 7.0) containing 1.0 mM ascorbate and 0.5 mM H_2O_2. Ascorbate oxidation was measured as the decrease in absorbance at 290 nm for 3 min and 30 s after adding 200 µl of extract. Glutathione reductase (GR; EC 1.6.4.2) activity was measured as in Schaedle and Bassham (1977) with some modifications. The reaction medium (2 ml) was 50 mM Tris-HCl buffer (pH 7.5) containing 0.15 mM NADPH, 0.5 mM oxidized glutathione (GSSG) and 3 mM $MgCl_2$. The NADPH oxidation was measured at 340 nm for 4 min after adding 400 µl of extract. Guaiacol peroxidase (GPX; EC 1.11.1.7) activity was measured according to Pütter (1974) with some modifications. The reaction medium (2 ml) was 100 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA, 20 mM guaiacol and 10 mM H_2O_2. Guaiacol oxidation was measured at 436 nm for 3 min and 15 s after adding 400 µl of extract.

Results

Seasonal changes in gas exchange, temperature, light and water potential

Seasonal changes in net CO_2 assimilation rates (*A*) (Figure 2A) and stomatal conductance to water vapor (g_s) (Figure 2B) showed similar patterns. Both *A* and g_s reached maximal values in spring (up to 12 µmol CO_2 m^{-2} s^{-1} and about 220 mmol m^{-2} s^{-1}, respectively), secondary maxima in fall (up to 9 µmol CO_2 m^{-2} s^{-1} and about 125 mmol m^{-2} s^{-1}, respectively) and minimal values in winter (0 or < 0 µmol CO_2 m^{-2} s^{-1} and 0–30 mmol m^{-2} s^{-1}, respectively). Leaf temperatures (*T*) (Figure 2C) were well correlated with *A* and g_s values, except in November 2000 and May 2001. November leaf temperatures were as low as in winter, whereas the *A* and g_s values were significantly higher than in winter. In May 2001, g_s was lower than in March 2001, showing a marked diurnal decrease. The *A* values were higher in May 2001 than in March 2001 in the early morning but decreased at midmorning and midday, probably because of the high temperature and the high leaf-to-air vapor pressure deficit. Photosynthetic photon flux (PPF) (Figure 2D) at the leaf level ranged from 1400 to 2000 µmol m^{-2} s^{-1} in winter and summer, respectively. Predawn water potential (Ψ_{pd}) (Figure 2E) was greater than –1.0 MPa during the entire study period, and it was therefore concluded that the sampled trees experienced no water stress although the study year (2000–2001) was drier than average.

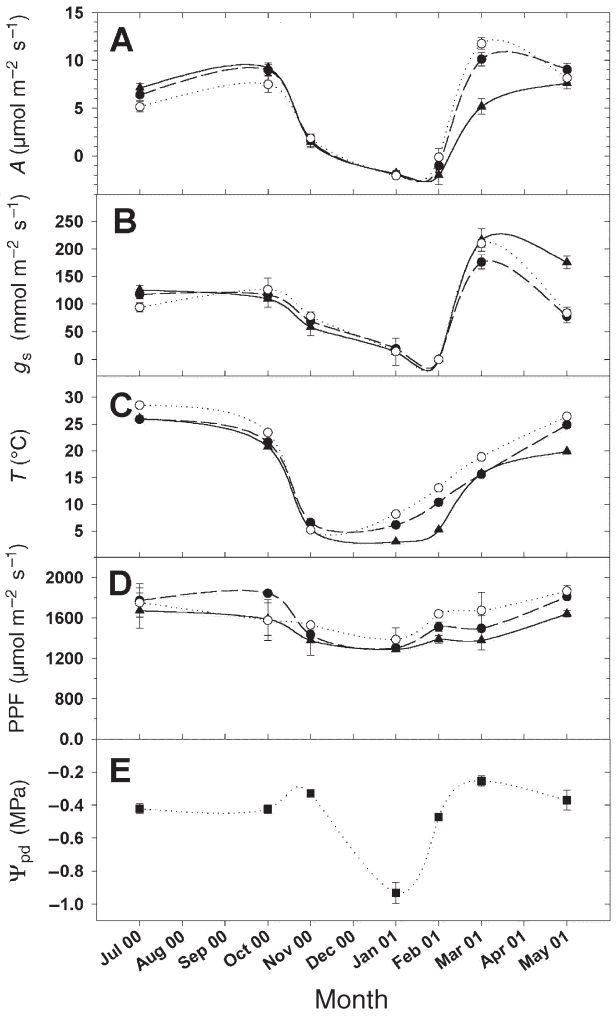

Figure 2. Seasonal changes in (A) net photosynthesis (*A*), (B) stomatal conductance to water vapor (g_s), (C) leaf temperature (*T*), (D) photosynthetic photon flux (PPF) and (E) predawn water potential (Ψ_{pd}) in *Quercus ilex* subsp. *ballota*. Sampling dates are those indicated by the arrows in Figure 1, except on September 20. Measurements were taken at 0800 (▲), 1000 (●) and 1200 h (○) solar time. Data are means ± SE.

Seasonal changes in chlorophyll fluorescence

Maximum potential PSII efficiency (Figure 3A), estimated as F_v/F_m, Φ_{PSII} (Figure 3B) and Φ_{exc} (Figure 3C) PSII efficiency, and the degree of closure of PSII reaction centers, estimated by qP (Figure 3D) followed similar trends. In most cases, all of these parameters exhibited minimal values in the winter (i.e., from November 2000 to February 2001) and maximal values

in the warm months in fall (October) and spring. Healthy plants adapted to darkness have F_v/F_m ratios ranging from 0.75 to 0.85 (Björkman and Demmig 1987); we used the lowest value of this range as a recovery threshold from temperature stress (straight line, Figure 3A). In July 2000, F_v/F_m values of about 0.8 were reached in the early morning, whereas at mid-morning and midday a marked decrease was observed (0.65). The F_v/F_m ratios decreased to about 0.55 in the coldest months of the year; however, these values recovered to above 0.75 in spring, the highest values being observed at midday (Figure 3A). The Φ_{PSII} values were lowest (values of about 0.1) in the cold months and at midday in summer and recovered in spring and October with values ranging from 0.2 to 0.35 (Figure 3B). The Φ_{exc} values were highest in spring and October (0.4–0.5) and reached their lowest values in summer and winter (0.24–0.29), with the most marked differences occurring at midday (Figure 3C). The qP values were highest in October and spring (0.6–0.7) and lowest in the coldest months (0.2–0.3) (Figure 3D). Non-photochemical quenching at midday was fairly stable (values of about 2.0), except in July 2000 and May 2001 when values increased to about 3 (Figure 3E); values tended to be lower in the early morning during the winter–spring period.

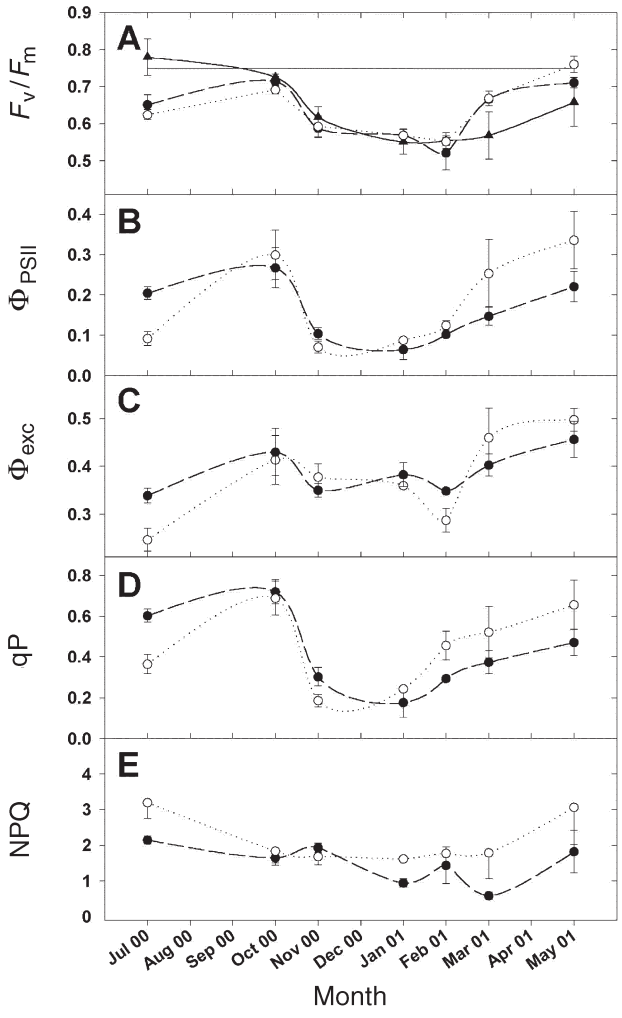

Figure 3. Seasonal changes in (A) maximum potential (F_v/F_m), (B) actual (Φ_{PSII}) and (C) intrinsic (Φ_{exc}) PSII efficiency, (D) photochemical quenching (qP) and (E) non-photochemical quenching (NPQ) in *Quercus ilex* subsp. *ballota*. Sampling dates are those indicated by the arrows in Figure 1, except on September 20. Measurements were taken at predawn (▲), 1000 (●) and 1200 h (○) solar time. Data are means ± SE. The horizontal straight line in Panel A indicates the lowest value of the F_v/F_m ratios found in healthy plants adapted to darkness (values ranging from 0.75 to 0.85; Björkman and Demmig 1987), which we used as the recovery threshold from temperature stress.

Photosynthetic pigment composition and antioxidant enzyme activity

The time course of the seasonal changes in leaf photosynthetic pigment composition was investigated throughout a year. Pigment concentrations on a leaf area basis followed a similar pattern to those on a Chl basis (data not shown). Lutein (Figure 4A), neoxanthin (Figure 4B) and β-carotene (Figure 4C) concentrations had similar time courses, with gradual increases from September 2000 to March 2001 and a decrease in May 2001; in most cases, concentrations increased from predawn to midday (Figures 4A–C) especially from November

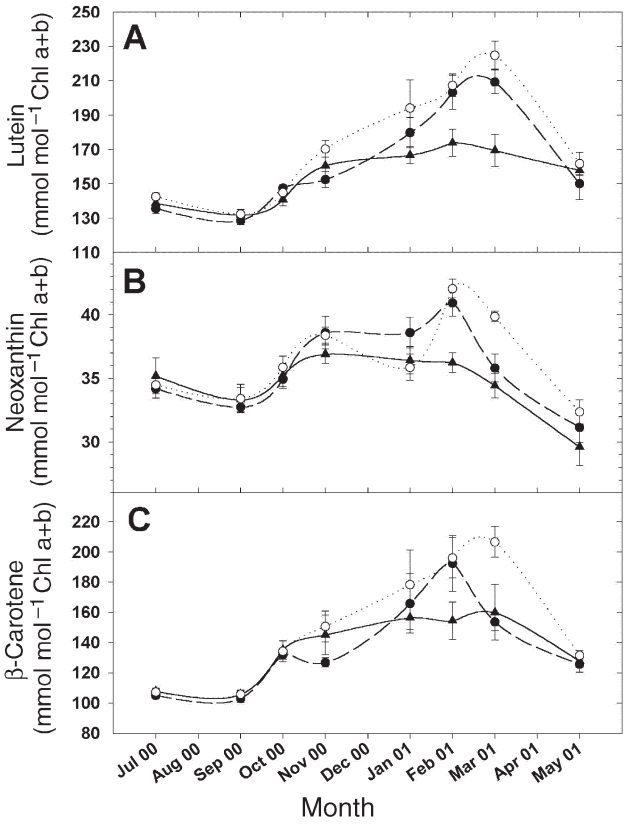

Figure 4. Seasonal changes in (A) lutein, (B) neoxanthin and (C) β-carotene to chlorophyll (Chl) molar ratios (mmol pigment mol^{-1} Chl) in *Quercus ilex* subsp. *ballota*. Sampling dates are those indicated by the arrows in Figure 1. Pigments were extracted from leaves collected at predawn (▲), 1000 (●) and 1200 h (○) solar time. Data are means ± SE.

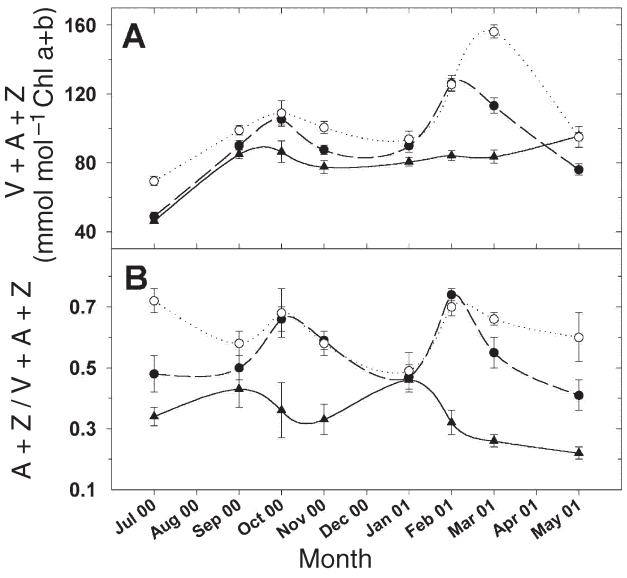

Figure 5. Seasonal changes in (A) violaxanthin (V) + antheraxanthin (A) + zeaxanthin (Z) to Chl molar ratios (mmol pigment mol^{-1} Chl), and (B) (A + Z)/(V + A + Z) ratios in the leaves of *Quercus ilex* subsp. *ballota*. Sampling dates are those indicated by the arrows in Figure 1. Pigments were extracted from leaves collected at predawn (▲), 1000 (●) and 1200 h (○) solar time. Data are means ± SE.

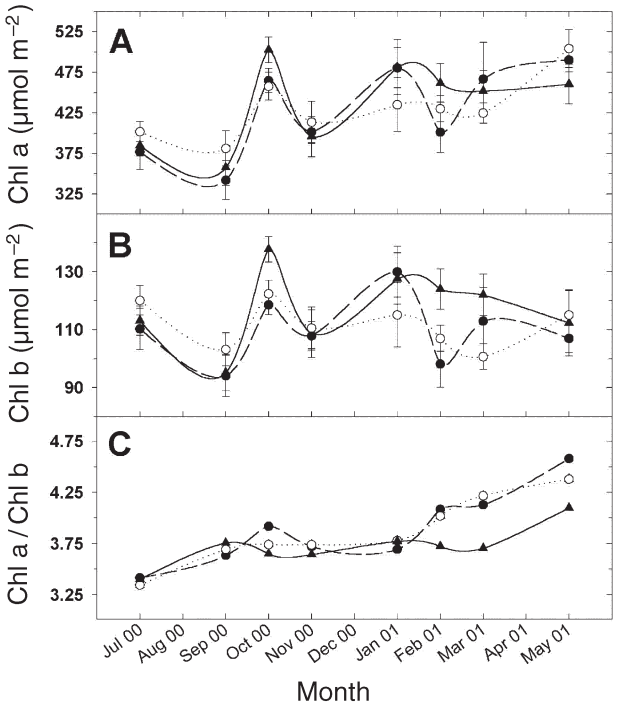

Figure 6. Seasonal changes in (A) Chl a and (B) Chl b concentrations and (C) Chl a/Chl b ratios in *Quercus ilex* subsp. *ballota*. Sampling dates are those indicated by the arrows in Figure 1. Pigments were taken at predawn (▲), 1000 (●) and 1200 h (○) solar time. Data are means ± SE.

2000 to March 2001. The violaxanthin (V) + antheraxanthin (A) + zeaxanthin (Z) to Chl ratios showed a similar time course; there was a tendency to increase throughout the year, with a decrease in May (Figure 5A). Throughout the year, the proportion of de-epoxidated xanthophylls within the VAZ cycle ((A + Z)/(V + A + Z)) was lower before dawn than in the early morning and at midday, except in January when it remained fairly constant throughout the day (Figure 5B). Lutein-epoxide accounted for a small proportion of the total lutein pool (data not shown). Chlorophyll a concentrations increased and showed maximal values in May 2001 (Figure 6A), whereas Chl b concentrations were much more stable throughout the year (Figure 6B). As a consequence, the Chl a/Chl b ratio increased and reached maximum values in May 2001 (Figure 6C).

The activities of SOD (Figure 7A) and guaiacol peroxidase (GPX) (Figure 7D) were positively correlated ($r^2 = 0.74$, $P < 0.05$). Their activities seemed to increase throughout the year, with the highest activities observed during the winter and in May 2001. Glutathione reductase (GR; Figure 7B) activity increased in summer and winter and at the end of May 2001; GR showed positive correlations with SOD and GPX only from November 2000 to May 2001. Ascorbate peroxidase (APX) (Figure 7C) activity did not respond to winter stress. The activities of SOD and APX showed an inverse correlation, although this was not significant ($r^2 = 0.64$, $P = 0.08$).

Discussion

Net CO_2 assimilation rate, g_s and PSII efficiency decreased markedly during the cold months in *Q. ilex* subsp. *ballota*, but recovered completely in spring. After cold episodes, CO_2 assimilation recovery has been reported in *Quercus ilex* subsp. *ilex* (Gratani 1996, Gratani et al. 1998), conifers (Westin et al. 1995, Lamontagne et al. 2000) and herbaceous species (Huner et al. 1993). We found no marked differences in A, g_s and PSII efficiency between the new, fully developed leaves (July 2000) and the 1-year-old leaves (May 2001). Fleck et al. (1998) reported similar stomatal conductance and photosynthetic rates in new and old *Q. ilex* subsp. *ilex* leaves in summer. We obtained evidence that *Q. ilex* subsp. *ballota*, a Mediterranean evergreen species, avoids damage to the photosynthetic apparatus during periods of low temperature (below and close to 0 °C) and relatively high irradiance (about 1400 µmol m^{-2} s^{-1}) that prevail in winter at the study site.

Values of Φ_{PSII} in *Q. ilex* subsp. *ballota* leaves never reached zero in winter (they were about 0.10), indicating that there was always some electron transport through PSII, even at the lowest temperatures and in bright sunlight. Electron transport through PSII under such conditions may reach 52–64 µmol electrons m^{-2} s^{-1}, taking into account an incident light availability of 1300–1600 µmol m^{-2} s^{-1}, a leaf absorptance of about 80% (Morales et al. 2002), about 50% of the light diverted to PSI, and a Φ_{PSII} of 0.1. However, under these conditions, A fell to zero (or below), indicating that dark respiration exceeded CO_2 fixation in these leaves. There are several possible pathways, in addition to CO_2 fixation, that can maintain some PSII electron transport including photorespiration, the

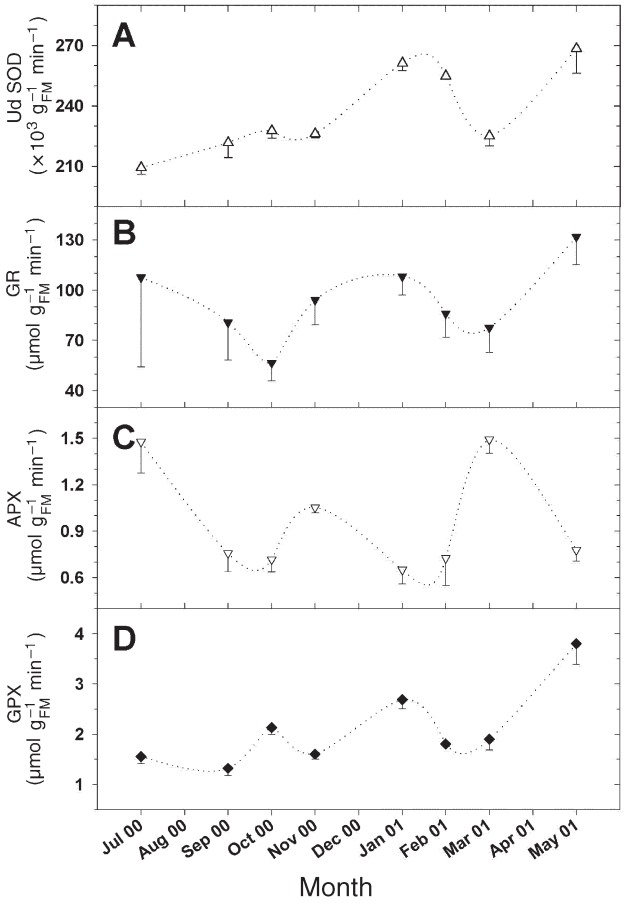

Figure 7. Seasonal changes in activities of the antioxidant enzymes (on a fresh mass basis) (A) superoxide dismutase (SOD), (B) glutathione reductase (GR), (C) ascorbate peroxidase (APX) and (D) guaiacol peroxidase (GPX) in *Quercus ilex* subsp. *ballota*. Sampling dates are those indicated by the arrows in Figure 1. Data are means ± SE.

water–water cycle and PSII charge recombination.

When PSII electron transport rate remains high relative to A, a great proportion of light energy is in excess and hence electrons may generate reactive oxygen species, in particular, superoxide radicals (O_2^-). Superoxide dismutase catalyzes the decomposition of O_2^-. We found that SOD activity was high in January and February 2001. Increases in SOD activity at low temperatures have been observed in other species (Schöner and Krause 1990, Polle and Rennenberg 1991). The GR activity also increased in the cold months, as reported by Schöner and Krause (1990). Plants with high SOD activity, and with simultaneous increases in GR and APX activities, have an increased ability to endure oxidative stress at low temperature (Kingston-Smith and Foyer 2000). However, we observed no increase in APX activity in winter, which agrees with data reported by Schöner and Krause (1990). The APX enzyme catalyzed the reduction of H_2O_2 formed by SOD to H_2O, and GR catalyzed the reduction of glutathione used in ascorbate regeneration. Nevertheless, because there was a high and positive correlation between the activities of SOD and GPX, we hypothesize that any H_2O_2 that was not reduced by APX could be reduced by GPX. Although the activities of all these enzymes would decrease in vivo in response to low temperatures, leaves with higher amounts of antioxidant enzyme activities would be more tolerant of stress on days with low positive temperatures (10–15 °C many days; see Figure 1A). In addition, no photo-oxidative stress was observed in the leaves, in accordance with the maintenance of stable Chl concentrations.

In winter, predawn maximum potential PSII efficiency, as estimated by F_v/F_m, reached values lower than those previously reported for *Q. ilex* subsp. *ilex* (Ogaya and Peñuelas 2003) and other evergreen species (Groom et al. 1991, Huner et al. 1993). Traditionally, low F_v/F_m values after cold nights have been ascribed to sustained NPQ, associated with the presence of antheraxanthin and zeaxanthin at predawn (Adams et al. 1995*a*). Also, low winter F_v/F_m values have recently been ascribed to changes in the chlorophyll pigment bed associated with the presence of antheraxanthin and zeaxanthin in the PSII antenna, which cause long-term PSII down-regulation (Gilmore and Ball 2000). On the one hand, low F_v/F_m values such as we observed could be ascribed in part to these findings, because predawn (A + Z)/(V + A + Z) ranged from 0.3 to 0.5. On the other hand, low F_v/F_m values could be related to a partially reduced PSII acceptor side at predawn, possibly because of the low mobility of plastoquinone in the thylakoid membrane at low temperature during illumination (authors' unpublished data). Thus, these minimal F_v/F_m values did not recover during the day in winter, but they recovered when the temperature increased in spring. Similar results have been reported in conifers (Westin et al. 1995). The predawn maximum potential PSII efficiency in winter was even lower than that observed at midday in summer in full sunlight, suggesting greater sensitivity to winter stress than to summer stress (a combination of high irradiances and temperatures, and low air relative humidities) in this Mediterranean species. This is in agreement with a previous study by Ogaya and Peñuelas (2003).

In the warmest month (July 2000), predawn F_v/F_m values were typical of an unstressed healthy plant. Photosynthetic rates and g_s decreased slightly at midday in summer in *Q. ilex* subsp. *ballota*. These changes were accompanied by decreases in F_v/F_m (which recovered during the night), Φ_{PSII} and Φ_{exc}, qP, and increases in NPQ. Photosynthesis also decreased (Fleck et al. 1998, Ogaya and Peñuelas 2003) and NPQ increased (Fleck et al. 1998) in summer in *Q. ilex* subsp. *ilex*. These data suggest the down-regulation of photosynthesis in *Q. ilex* subsp. *ilex* during the summer, as observed in several studies (Faria et al. 1998, Fleck et al. 1998, Ogaya and Peñuelas 2003). The limited impact of the summer on *Q. ilex* subsp. *ballota* photosynthetic apparatus might be associated with a lack of water stress.

Photosynthetic pigment composition of *Q. ilex* subsp. *ballota* leaves changed with leaf age. Lutein, neoxanthin and β-carotene to Chl molar ratios showed the same trend throughout the year, increasing from September 2000 to February–March 2001. This increase was caused by increased carotenoid concentrations, because Chl concentration per area re-

mained fairly stable, and was even higher at the end than at the beginning of the first growing season. Also, there were changes from predawn to midday. For instance, at predawn and midday in February–March (where differences between predawn and midday were high), Chl concentration was about 580 and 530 μmol m^{-2}, respectively, whereas V + A + Z carotenoid concentrations were 80 and 140 mmol mol^{-1} Chl (i.e., at midday and sunrise, respectively). These data indicate that most of the change was due to a change in carotenoid concentration and that the decrease in Chl concentration from predawn to midday was minor. Lutein was the main leaf carotene, accounting for 35–44% of the total carotenoids, followed by β-carotene (31–34%) and neoxanthine (6–11%). Similar carotenoid concentrations, on a leaf area basis, have been reported in *Q. ilex* subsp. *ilex* (Llorens et al. 2002) and *Quercus subpyrenaica* E. H. del Villar (Abadía et al. 1996). The xanthophyll cycle (V + A + Z) accounted for 10–27% of the total carotenoids; its concentration was similar to that observed in *Q. ilex* subsp. *ilex* (Faria et al. 1998). Carotenoids, apart from their role as secondary light-absorbing pigments (Malking and Niyogy 2000), prevent photo-oxidation of the photosynthetic apparatus by reducing the Chl triplet state, preventing the formation of singlet oxygen ($^1O_2$), or acting directly on its removal (Young et al. 1997). The high carotenoid/Chl ratios in winter may help leaves avoid photo-oxidation processes. The Chl a/Chl b ratio at the end of the first growing season was higher than at the beginning, perhaps the result of a reduction in the size of the PSII antenna, which could be considered an adaptation that reduces light absorption (especially in the cold months). In summer, similar ratios have been reported in both subspecies (Faria et al. 1998, Morales et al. 2002). *Quercus ilex* subsp. *ilex* maintains high Chl concentrations throughout most of the year (Gratani et al. 1998), with a decrease in Chl concentration from July to September (Faria et al. 1998).

Diurnal de-epoxidation of V to A + Z occurred throughout the year, except in January. We found no correlation between NPQ and (A + Z)/(V + A + Z) ratios during the year ($r^2 = 0.08$, $P = 0.33$). However, the relationship between NPQ and (A + Z)/(V + A + Z) seemed to differ between the warmest and coldest months. For instance in July 2000, thermal energy dissipation (up to about 75% of the incident ligh, estimated as $1 - \Phi_{exc}$; Demmig-Adams et al. 1996) and NPQ (up to about 3.2) were associated with the de-epoxidated forms (A + Z) of the V + A + Z cycle (up to 70%). Although many authors have reported close relationships between NPQ and A + Z (Adams et al. 1994, Demmig-Adams and Adams 1996*a*, Verhoeven et al. 1997, Fleck et al. 1998), others have not (e.g., *Q. suber* in January; García-Plazaola et al. 1997). It is possible that NPQ in winter is higher than the values reported. The absence of a correlation between NPQ and (A + Z)/(V + A + Z) ratios in winter could be associated with F_m at low temperatures being partially quenched as a result of the long-term PSII down-regulation proposed by Gilmore and Ball (2000). This would result in an underestimation of NPQ, which should be named "apparent NPQ." This phenomenon has been described previously (Adams et al. 1995*a*, 1995*b*, 2001, Adams and Demmig-Adams 1995).

We were able to detect lutein-epoxide in *Quercus ilex* subsp. *ballota* leaves by HPLC, but it accounted for only a small proportion of the total lutein pool (data not shown). We suggest that lutein-epoxide is equivalent to violaxanthin in the V + A + Z cycle, and zeaxanthin is equivalent to lutein. Therefore, if pigments of this "second" xanthophyll cycle participate in dissipation (cf. García-Plazaola et al. 2003), we suggest that lutein and not lutein-epoxide would have a function similar to zeaxanthin. This suggestion is supported by our observations of the effects of other stress treatments where we found high correlations between lutein concentrations and energy dissipation (cf. Larbi et al. 2004).

In summary, photoinhibition in *Q. ilex* subsp. *ballota* was greater in winter than in summer. We found that *Q. ilex* subsp. *ballota* was able to avoid damage to the photosynthetic apparatus during periods of low temperature in two ways. First, photo-oxidative damage, in the form of bleaching of photsynthetic pigments (especially chlorophylls), was not detected. Second, the low F_v/F_m ratios found at predawn did not recover during the day in winter, but recovered when temperature increased in spring. Thus, leaves subjected to two different episodes of stress, summer stress and winter stress, were able to photosynthesize when temperatures increased in spring.

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