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Research paper

Climatic origins predict variation in photoprotective leaf pigments in response to drought and low temperatures in live oaks (*Quercus* series *Virentes*)

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Climate is a major selective force in nature. Exploring patterns of inter- and intraspecific genetic variation in functional traits may explain how species have evolved and may continue evolving under future climate change. Photoprotective pigments play an important role in short-term responses to climate stress in plants but knowledge of their long-term role in adaptive processes is lacking. In this study, our goal was to determine how photoprotective mechanisms, morphological traits and their plasticity have evolved in live oaks (*Quercus* series *Virentes*) in response to different climatic conditions. For this purpose, seedlings originating from 11 populations from four live oak species (*Quercus virginiana*, *Q. geminata*, *Q. fusiformis* and *Q. oleoides*) were grown under contrasting common environmental conditions of temperature (tropical vs temperate) and water availability (droughted vs well-watered). Xanthophyll cycle pigments, anthocyanin accumulation, chlorophyll fluorescence parameters and leaf anatomical traits were measured. Seedlings originating from more mesic source populations of *Q. oleoides* and *Q. fusiformis* increased the xanthophyll de-epoxidation state under water-limiting conditions and showed higher phenotypic plasticity for this trait, suggesting adaptation to local climate. Likewise, seedlings originating from warmer climates had higher anthocyanin concentration in leaves under cold winter conditions but not higher de-epoxidation state. Overall, our findings suggest that (i) climate has been a key factor in shaping species and population differences in stress tolerance for live oaks, (ii) anthocyanins are used under cold stress in species with limited freezing tolerance and (iii) xanthophyll cycle pigments are used when photoprotection under drought conditions is needed.

Keywords: anthocyanins, climatic gradient, clinal variation, cold response, drought response, physiological adaptation, red leaves, xanthophyll cycle.

Introduction

Climate is considered one of the main selective forces in nature (Etterson 2004, Jump et al. 2006, Ramírez-Valiente et al. 2010). Spatial variation in climate exerts differential selective pressures, which can drive local adaptation to the environment (Cregg and Zhang 2001, Allen et al. 2006, Marchin et al. 2008, Ramírez-Valiente et al. 2009). This phenomenon, called 'divergent or diversifying selection', promotes genetic differentiation within species and can lead to allopatric speciation even in the presence of gene flow (Allen et al. 2006, Allen and Gillooly 2006). Exploring patterns of inter- and intraspecific genetic variation in functional traits may explain how species have evolved and may continue evolving under future climate change (Hoffmann and Sgrò 2011). In sessile organisms like plants, response to climate change is ultimately related to traits involved in avoidance and tolerance of environmental stresses (Brodribb and Hill 1999, Cavender-Bares et al. 2004).

Photoprotective pigments play an important role in short-term responses to climate stress in plants (Logan et al. 1998, García-Plazaola et al. 1999). Under unfavorable conditions such as drought or low temperatures, plants down-regulate photosynthesis, limiting the metabolic use of the absorbed light (Price et al. 1989, Moran et al. 1994). Photoprotective pigments dissipate excess excitation energy in the form of heat via de-epoxidation reactions (xanthophyll cycle pigments) (Demmig et al. 1987, Adams and Demmig-Adams 1994, Demmig-Adams and Adams 1996) or operate as an optical filter absorbing visible light and therefore reducing the amount of photons captured by chlorophylls (anthocyanins) (Choinski and Johnson 1993, Neill and Gould 2003, Archetti et al. 2009, Zeliou et al. 2009, Nikiforou et al. 2010, Hughes et al. 2012). Differences in de-epoxidation states and anthocyanin accumulation among species have been observed in many different ecosystems (Johnson et al. 1993, Veres et al. 2002, Archetti et al. 2009, Lev-Yadun and Holopainen 2009, Peguero-Pina et al. 2009, Savage et al. 2009).

In a meta-analyses, Wujeska et al. (2013) found higher zeaxanthin concentration in leaves (indicative of higher de-epoxidation rates) in species from arid habitats suggesting an evolution towards the accumulation of de-epoxidated forms in species that inhabit dry environments. However, it has also been reported in some species that ecotypes from xeric sites have low photoprotective capacity compared with those from mesic sites when subjected to similar drought conditions (García-Plazaola and Becerril 2000*a*). The latter has been explained in terms of the acquisition of morphofunctional traits such as reduced specific leaf area (SLA) in stress-tolerant ecotypes, which help these plants maintain positive photosynthetic rates and thus reduce excess absorbed light (García-Plazaola and Becerril 2000*a*, 2000*b*). To our knowledge, there are currently no studies that examine the relationship between inter- or intraspecific differences in anthocyanin content in leaves in relation to climate. Overall, only a limited number of studies have examined inter- and intraspecific variation in photoprotective pigments and their plasticity, and little is known about their role of local adaptation to climate (Wujeska et al. 2013).

In this study, our goal was to determine how photoprotective pigment accumulation, de-epoxidation state and their plasticity have evolved in live oaks in response to different climatic conditions. The live oaks (*Quercus* series *Virentes*) are a small monophyletic lineage consisting of a group of seven species distributed along the tropical–temperate divide through the southern USA, Mexico and Central America (Muller 1961, Nixon 1985, Nixon and Muller 1997). They are a useful study system to explore inter- and intraspecific genetic differentiation and phenotypic plasticity of photoprotective mechanisms for several reasons. First, these species span a large latitudinal gradient of 24° and ranging from –0.5 to 19.6 °C average minimum temperatures of the coldest month (see Table S1 available as Supplementary Data at *Tree Physiology* Online). High rainfall variation also occurs across the distribution ranges of these species. Second, live oaks are evergreen (leaves turnover on an annual basis (Cavender-Bares and Holbrook 2001) and their photosynthetic apparatus must have evolved in response to a wide variety of environmental stress factors, including water deficit and high/low temperatures (Verhoeven 2014). Third, previous studies have shown that species and populations from colder climates have an increased ability to cold acclimate and have higher freezing tolerance than those from more tropical latitudes (Cavender-Bares and Holbrook 2001, Cavender-Bares 2007, Koehler et al. 2012) and species also differ in drought response (Cavender-Bares et al. 2004, 2007, Cavender-Bares and Pahlich 2009), indicating the presence of phenotypic plasticity and genetic divergence caused by adaptation to different climates.

Our specific objectives were (i) to test for genetic differences and phenotypic plasticity in xanthophyll cycle activity (xanthophyll pool size and de-epoxidation states), chlorophyll fluorescence parameters [electron transport rate (ETR), non-photochemical quenching] and anthocyanin accumulation in 11 populations of four live oaks (*Quercus virginiana*, *Q. oleoides*, *Q. geminata* and *Q. fusiformis*) established under contrasting treatments of temperature (tropical vs temperate) and water (droughted vs well-watered), (ii) to explore inter- and intraspecific differences in two morphological traits, SLA (leaf area/leaf dry mass) and stomatal pore index [stomatal density × (pore length)^2, SPI; Sack et al. 2003], (iii) to study the extent to which leaf morphology acts as a complementary photoprotective mechanism based on the hypothesis stated by García-Plazaola and Becerril (2000*a*, 2000*b*) and (iv) to examine the relationship between photoprotective mechanisms and climate of origin of the populations. We expected inter- and intraspecific differences in the activation of photoprotective xanthophyll cycle pigments and accumulation of anthocyanins under stressful conditions for live oaks as a result of a long-term adaptation to local climatic conditions. We further expected inter- and intraspecific variation in phenotypic plasticity of these photoprotective mechanisms in response to stressful conditions.

Materials and methods

Seed collection and sowing

Acorns were collected from one to five populations within four live oak species: *Q. virginiana* (North Carolina, northern Florida, southern Florida, Louisiana and Texas); *Q. geminata* (northern Florida and North Carolina); *Q. fusiformis* (Texas); and *Q. oleoides* (Mexico, Belize and Costa Rica) (see Table S1 available as Supplementary Data at *Tree Physiology* Online). Seeds were collected from five to nine mother trees randomly selected within each population in 2004. Mother trees were separated by more than 150 m in order to avoid familial structures. The number of

maternal families per population was relatively low and these families may not adequately represent the entire population. Acorns were stored at 4 °C until synchronous sowing in glasshouse facilities at the University of Minnesota in January 2006.

A common garden experiment was established in greenhouse facilities at the University of Minnesota. Seeds were germinated in deepots with Promix potting soil and then transplanted to 9.6 dm^3 pots with a 50% mix of sand and Bio-Comp Professional growing mix (BC-5s). Potted seedlings were then installed in experimental treatments following a randomized incomplete block design. Four rooms were established with five benches (blocks) per room, where all studied populations (11) from the four species were represented by a variable number of maternal families (from one to nine) ($N = 4$ rooms × 5 blocks × 4 species × 1–5 populations × 1–9 maternal families × 1–4 individuals = 3511 plants). A summary of the timeline of each of the experiments and the treatments imposed are included in Figure S1 available as Supplementary Data at *Tree Physiology* Online.

Climate treatments

As part of the Experiment 'a' (see Figure S1 available as Supplementary Data at *Tree Physiology* Online), two climate treatments (temperate and tropical) were implemented during the winter months (November–April) in 2006; a tropical treatment, in which daytime temperature was maintained between 30 and 35 °C, and night-time temperatures were between 22 and 26 °C, and a temperate treatment, in which winter growth temperatures reached a minimum night-time temperature of 5 °C with a daytime temperature of 15 °C, simulating the monthly average temperatures in Liberia, Costa Rica (southern range limit) and Wilmington, North Carolina (northern range limit). Each climate regime was replicated in two independently controlled glasshouse rooms. Climate treatments were repeated in the winter of 2007 and 2008. During summer months climate was equal across all rooms (tropical conditions). The photoperiod was extended to 12 h during the winter, similar to Costa Rica (southern range limit). Summer photoperiods were left at natural levels for Minnesota with a maximum of 15.5 h, which was up to 1 h longer than in North Carolina (northern range limit) and 3 h longer than in Costa Rica.

In addition, to test for possible differences in phenology between temperature treatments (see Materials and methods), we analyzed unpublished data of a previous common garden experiment conducted in a glasshouse in 2005 (Experiment 'b', Figure S1 available as Supplementary Data at *Tree Physiology* Online). This study followed a randomized complete block design with four populations and five maternal families within populations of *Q. oleoides* and *Q. virginiana* representing the extremes of the latitudinal gradient of live oaks. The experimental design included two climate treatments (temperate and tropical) with conditions equivalent to the climate treatments in the Experiment 'a'. In total, 10–11 plants per family were established in each climate treatment (10–11 plants/family × 5 families/population × 2 populations/species × 2 species × treatments = 409 plants) (see Cavender-Bares 2007 for other details).

Watering treatments

As part of the Experiment 'a' (see Figure S1 available as Supplementary Data at *Tree Physiology* Online), in the summer of 2007 two watering treatments were implemented in which soil moisture was maintained at ~15% in half of the plants (well-watered treatment) and reduced from 15 to 7% in 30 days and then maintained at 7% during other 60 days in the other half of the plants (droughted treatment). After summer, all plants were watered and kept in optimal conditions of moisture. Water treatments were repeated in summer 2008.

Climatic treatment measurements

Chlorophyll fluorescence and xanthophyll pigments in a short-term temperature treatment In winter 2008, after three months of climate treatments, a total of 166 plants were randomly selected from all the species and populations (83 per climate treatment) to investigate the effects of short-term warm and chilling temperature treatment in a growth chamber (called hereafter 'short-term treatments', Experiment 'c', Figure S1 available as Supplementary Data at *Tree Physiology* Online). First, dark-adapted chlorophyll fluorescence was measured in situ on plants grown in the glasshouse rooms under the climatic treatments. Second, branchlets with at least five leaves (including the measured leaf) of the same plants were cut under water, placed in water-filled rose tubes in the dark and then moved to a growth chamber at 25 °C for 2 h. A light treatment of 700 μmol m^{-2} s^{-1} was imposed for 1 h, after which light acclimated chlorophyll fluorescence was measured. Branchlets were allowed to recover in the dark for 24 h at 25 °C. Dark-adapted chlorophyll fluorescence was measured before the branchlets went into a chamber at 3 °C with a light treatment of 700 μmol m^{-2} s^{-1} where chlorophyll fluorescence was measured after 1 h. Dark and light adapted chlorophyll fluorescence was measured in the same leaves and positions using the same light level. For our analysis, we calculated: Stern-Volmer non-photochemical quenching, NPQ $[(F_m - F'_m)/F'_m)]$ and ETR (assuming a leaf absorbance of 0.8 and equal photon excitation of PSII and PSI).

At the time of light acclimated chlorophyll fluorescence measurements at both 25 and 3 °C, a 0.2 cm^2 leaf disc was removed from an opposite leaf at the same level on the branchlet and immediately frozen in liquid nitrogen for xanthophyll and chlorophyll analysis. Leaf discs were immediately frozen in liquid nitrogen and stored at –80 °C until analysis. Pigments were extracted in acetone following (Adams and Demmig-Adams 1992) and separated by HPLC (Agilent 1200 Series) on an Allosphere ODS-1 column. Solvents and methods were adapted from

Gilmore and Yamamoto (1991). Pigment were separated on a gradient from Solvent A (76 : 17 : 7, Acetonitrile : Methanol : 0.1 M TRIS pH 8.0) to Solvent B (4 : 1, Methanol : Hexane). Peaks were detected at 445 nm and pigment concentrations were calculated by peak area. Total chlorophyll and xanthophylls were measured in each sample. The total xanthophyll pool was calculated as violaxanthin + antheraxanthin + zeaxanthin (VAZ). We also calculated the total xanthophyll pool relative to chlorophyll content (VAZ/Chl) and de-epoxidation state of the xanthophylls (DPS) as (A + Z)/(V + A + Z), hereafter referred to as AZ/VAZ.

Anthocyanins In April 2008, after 4–5 months of cold temperature exposure in the temperate treatment anthocyanins were measured in all plants established in the glasshouse (Experiment 'a', Figure S1 available as Supplementary Data at *Tree Physiology* Online, $N = 3511$) using Dualex 3.3 ANTH leaf clip in both adaxial and abaxial sides of the leaves (Force-A, Orsay, France). Anthocyanins were measured in two leaves per plant: (i) the reddest leaf (a young leaf); and (ii) a randomly selected leaf (mature leaf) from the top part of the plant. The total amount of anthocyanins in each leaf was calculated using a calibration equation (see Appendix 1 available as Supplementary Data at *Tree Physiology* Online for details). Dualex and methanol extraction values were only associated in young leaves, so mature leaf values were not used for further analyses.

Phenology Phenology is a potential factor that could influence the association between anthocyanins accumulation and climate. If plants in the temperate treatment had started to break buds later than the plants in the tropical treatment and we measured anthocyanins concentrations at the same time in both treatments, then we might have collected anthocyanin data in young leaves under two different phenological periods (i.e., differences would be caused by phenological disparities). The same idea would apply to inter- and intraspecific differentiation. We did not measure vegetative phenology in the common garden experiment used for pigment analyses but we analyzed unpublished data of a previous common garden experiment conducted in a glasshouse in 2005 (Experiment 'b', Figure S1 available as Supplementary Data at *Tree Physiology* Online). We recorded the date of the bud break and monitored the lifespan of one of the new leaves that emerged for all the plants established in the experiment (10–11 plants/family × 5 families/population × 2 populations/species × 2 species × treatments = 409 plants). For our purpose, only the bud break date of the leaf was used for further analyses.

Water treatment measurements

Chlorophyll fluorescence In summer of 2008, 9–10 plants per population within each water treatment were randomly selected (196 plants in all) in Experiment 'a' in the tropical rooms during the third month of drought. Chlorophyll fluorescence measurements were performed on the selected plants. Recently, Peguero-Pina et al. (2013) have showed that the activation of photoprotective mechanisms is very variable among leaves within plants and it is very sensitive to light intensity and solar angle. In the present study, light acclimated measurements of chlorophyll fluorescence were taken at solar noon with a PAR of 500 μmol m^{-2} s^{-1} in sun leaves with the same orientation, which potentially would reduce the environmental noise according to this recent study (Peguero-Pina et al. 2013). A PAR value of 500 μmol m^{-2} s^{-1} was used to match daytime ambient light levels in the greenhouse. Dark-adapted chlorophyll fluorescence was measured 2 h before dawn on the day of light acclimated measurement. We calculated Stern-Volmer non-photochemical quenching, NPQ and ETR (see details above).

Water potential Predawn water potential (ψ) was measured in one leaf per plant using a Scholander pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA) 2 h before dawn each day of our chlorophyll fluorescence measurements.

Chlorophyll and xanthophylls During the same period, a 0.2 cm^2 leaf disc was collected in a sun leaf from each selected plant to analyze the amount of chlorophyll and xanthophyll pigments. The total xanthophyll pool (VAZ) and the de-epoxidation state (AZ/VAZ) were calculated. See details above for pigment extraction protocol.

Leaf morphology Within 2 days of chlorophyll fluorescence measurements during water treatment, two leaves were collected from each individual for stomatal analysis and SLA. For stomatal analysis, clear nail varnish was applied to the abaxial surface of the leaf between the mid-vein and the leaf margin to create an impression of the leaf epidermis. Stomatal density and aperture length of three stomates were measured in three areas on each leaf impression (ImageJ software). Stomatal pore index was calculated as the stomatal density × $(\text{pore length})^2$ (Sack et al. 2003). Leaves were scanned for leaf area (ImageJ software) and dried at 60 °C for 48 h to obtain leaf mass. Specific leaf area was calculated as leaf area/leaf mass. Averaged SPI and SLA values for both leaves were used for further analyses.

Statistical analyses

All traits were averaged across maternal families. ANOVAs were conducted with population nested within species to determine species- and population-level differentiation and interactions with climate and water treatments (see Koehler et al. 2012 for a similar procedure). Additional ANOVAs for traits measured in the drought experiment were performed using water potential as a covariate instead of estimating the fixed effects of water treatment. A mixed model analysis was performed for phenology, including treatment, species and population within species as fixed factors and maternal family as a random factor. In order to test whether photoprotective response is associated with climate

of origin, when differences among populations or species were found, we performed (i) linear regressions between minimum temperatures in the coldest month and means and plasticities measured in the temperature experiments and (ii) linear regressions between a moisture index and traits measured in the water experiments. We also performed linear regressions to explore for associations between traits. Index of moisture (I_m) was calculated as $I_m = 100 \times (P - \mathrm{PET})/\mathrm{PET}$, where P is annual precipitation and PET is potential evapotranspiration. Phenotypic plasticity for traits was calculated as plasticity index (PI):

$$\mathrm{PI} = \frac{\mathrm{mean(env1)} - \mathrm{mean(env2)}}{\mathrm{mean(env1)} + \mathrm{mean(env2)}}$$

We used PI instead of other published plasticity indices because it best reflects the reaction norms and it is not sensitive to variance between samples. The mixed models were performed in SAS 9.2. The rest of the analyses were conducted in STATISTICA 10.0 (Statsoft).

Results

Response to temperature

On average, exposure to short-term chilling temperatures (3 °C) in a growth chamber increased NPQ and AZ/VAZ for those seedlings grown in the temperate climate treatment (Figure 1). Electron transport rate was reduced under short-term chilling temperatures, particularly for plants grown in the tropical climate treatment. In fact, the hierarchical ANOVA showed a significant interaction of climate treatment and short-term treatment for NPQ, AZ/VAZ and ETR (Table 1). NPQ and AZ/VAZ were only correlated in the 25 °C treatment in plants grown in tropical temperate conditions (see Figure S2 available as Supplementary Data at *Tree Physiology* Online). Non-photochemical quenching varied among species, and ETR varied among populations within species (Table 1). However, neither NPQ nor ETR was associated with climate at the source of origin within or among species (data not shown). There were also differences in F_v/F_m among climate treatments, short-term treatments and populations within species (see Table S2 available as Supplementary Data at *Tree Physiology* Online). Cold acclimated *Q. fusiformis* seedlings showed the highest values of $\Delta F/F_m'$ under light at 3 °C but all plants had similar initial and final F_v/F_m values in response to light at 25 °C and at 3 °C, indicating a lack of damage for all the species (see Figure S3 available as Supplementary Data at *Tree Physiology* Online). The total pool of xanthophylls (VAZ), chlorophyll content and VAZ/Chl differed between climate treatments, but there was no effect of short-term exposure to chilling temperatures (see Table S2 available as Supplementary Data at *Tree Physiology* Online). There were also differences among species in VAZ/Chl and *Q. oleoides* showed the highest values (data not shown).

The hierarchical ANOVA showed that plants in the climate treatment had higher anthocyanin concentration in leaves than plants in the tropical treatment (Table 1, Figure 2). There were also differences among species and among populations within species in anthocyanins (Table 1, Figure 2). In fact, there was a relationship between minimum temperatures in the coldest month and anthocyanin concentration in the reddest leaf (Figure 3). ANOVA also showed a significant interaction between population and climate treatment indicating differences in plasticity for anthocyanin accumulation (Table 1). However, PI was not associated with minimum temperatures in the coldest month ($R^2 = 0.01$, $P = 0.488$).

The results of the mixed model analysis for phenology showed that on average, plants in the temperate treatment delayed the onset of the vegetative growth season by 36.2 days (Table 2, Figure S4 available as Supplementary Data at *Tree Physiology* Online). There were also differences among species and populations within species (Table 2). The southern population of *Q. oleoides* (Costa Rica) showed the earliest onset of growth, whereas the northern population of *Q. virginiana* had the latest (Table 2, Figure S4 available as Supplementary Data at *Tree Physiology* Online). There were no differences among maternal families in phenology (Table 2).

Response to water

The hierarchical ANOVA showed differences between water treatments in NPQ, de-epoxidation state AZ/VAZ, ETR and SPI (Table 3). On average, plants established under drought conditions exhibited higher values of AZ/VAZ and NPQ, and lower ETR, F_v/F_m and SPI (Figures 4 and 5). There were also differences among species and populations in total xanthophyll pool (VAZ) and VAZ/Chl, but an effect of watering treatment was not observed (Table 3). *Quercus oleoides* showed significantly lower VAZ/Chl and marginally lower VAZ compared with the other species (data not shown).

Non-photochemical quenching and AZ/VAZ were positively correlated in both water treatments although R^2 values were relatively low (~0.17, Figure S5 available as Supplementary Data at *Tree Physiology* Online). There were differences among species in SPI and differences among populations within species in AZ/VAZ and SLA (Table 3, Figure 4). Linear regressions showed that SPI was positively associated with the index of moisture at the source of origin (I_m) in both treatments, although R^2 values were relatively low (0.09–0.10, Figure S6 available as Supplementary Data at *Tree Physiology* Online). The same pattern was found between AZ/VAZ and I_m in *Q. fusiformis* and *Q. oleoides* but only in the droughted treatment (Figure 6). There was also a significant interaction between population and water treatment for AZ/VAZ indicating differences in plasticity among maternal families (Table 3). In fact, linear regressions showed a positive association between index of moisture and PI for AZ/VAZ in both *Q. fusiformis* ($R^2 = 0.92$, $P = 0.009$) and *Q. oleoides*

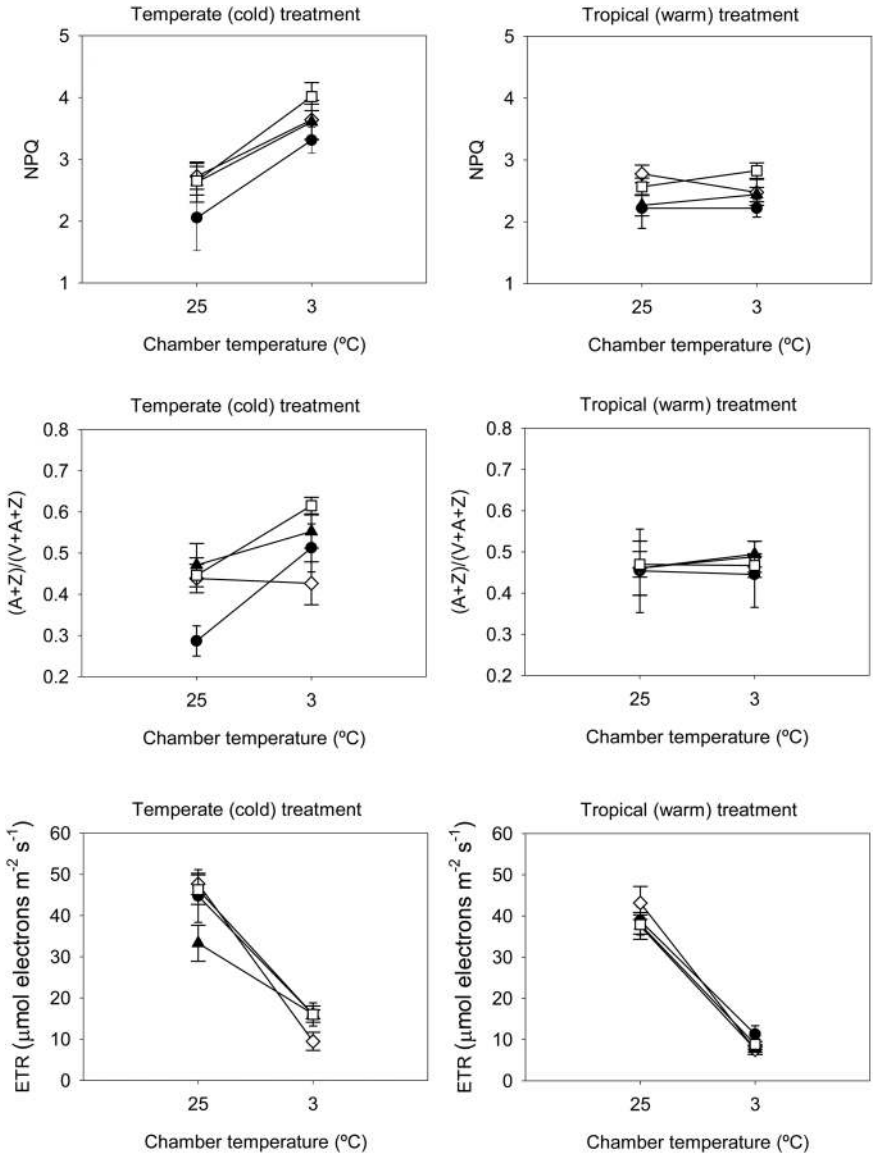

Figure 1. Effects of short-term exposure to warm (25 °C) and chilling (3 °C) temperatures in seedlings grown under temperate (left panels) and tropical (right panels) conditions for NPQ, de-epoxidation state [(A + Z)/(V + A + Z)] and electron transport rate (ETR). *Quercus fusiformis* (circles), *Q. geminata* (open diamonds), *Q. oleoides* (triangles) and *Q. virginiana* (open squares).

($R^2 = 0.44$, $P = 0.013$) (Figure 7). In addition, AZ/VAZ was positively associated with SPI in *Q. oleoides*, such that families that increased de-epoxidation state in response to drought tended to increase stomatal pore area (Figure 8). There was also a significant population by water treatment interaction for SLA (Table 3). However, neither metric of plasticity for SLA showed a relationship with I_m.

Discussion

Response to temperature

Contrary to expectation, our results showed no inter- or intraspecific differences in AZ/VAZ responses to chilling despite differences among species in NPQ and among populations in ETR. Therefore, interspecific differences in NPQ could not be explained by xanthophyll cycle activity, and in fact, the association between NPQ and AZ/VAZ was very weak, contrary to observations in other studies (see Figure S2 available as Supplementary Data at *Tree Physiology* Online) (e.g., Savage et al. 2009). Together, these results suggest that other carotenoids such as lutein could be involved in the variation in NPQ among live oaks under cold conditions (Niyogi et al. 1997, García-Plazaola et al. 2007, Esteban et al. 2010, Förster et al. 2011). Additionally, the method used to measure NPQ that does not use F_o' might not be as predictive of de-epoxidation state. Interestingly, without longer-term exposure to cold, all species and populations showed a limited de-epoxidation capacity to respond to short-term cold stress (significant climate treatment by short-term treatment

Table 1. Results of ANOVA for the effects of species, population (species), climate treatment, short-term treatment and their interactions on NPQ, de-epoxidation state of the xanthophyll cycle [(A + Z)/(V + A + Z)], ETR and anthocyanin leaf content. Significant values ($P < 0.05$) are shown in bold and marginally significant values ($P < 0.1$) are shown in italics. SS, sum of squares.

Effect	df	SS	*F* ratio	*P* value
NPQ				
Species	**3**	**9.710**	**3.18**	**0.0247**
Population (species)	7	11.699	1.64	0.1242
Climate treatment	**1**	**14.401**	**14.17**	**0.0002**
Short-term treatment	**1**	**11.750**	**11.56**	**0.0008**
Climate treatment × short-term treatment	**1**	**17.318**	**17.04**	**<0.0001**
Species × climate treatment	3	0.439	0.14	0.9335
Species × short-term treatment	3	8.517	1.20	0.3055
Population (species) × climate treatment	7	0.849	0.28	0.8409
Population (species) × short-term treatment	7	8.104	1.14	0.3397
(A + Z)/(V + A + Z)				
Species	3	0.111	1.23	0.3011
Population (species)	7	0.058	0.28	0.9631
Climate treatment	1	0.008	0.26	0.6123
Short-term treatment	*1*	*0.096*	*3.18*	*0.0764*
Climate treatment × short-term treatment	**1**	**0.180**	**5.97**	**0.0156**
Species × climate treatment	3	0.058	0.65	0.5867
Species × short-term treatment	3	0.067	0.32	0.9455
Population (species) × climate treatment	7	0.025	0.28	0.8399
Population (species) × short-term treatment	7	0.122	0.58	0.7745
ETR				
Species	*3*	*0.244*	*2.42*	*0.0669*
Population (species)	**7**	**0.497**	**2.11**	**0.0433**
Climate treatment	**1**	**0.580**	**17.26**	**<0.0001**
Short-term treatment	**1**	**10.249**	**305.18**	**<0.0001**
Climate treatment × short-term treatment	**1**	**0.915**	**27.24**	**<0.0001**
Species × climate treatment	3	0.068	0.68	0.5673
Species × short-term treatment	**3**	**0.829**	**3.53**	**0.0013**
Population (species) × climate treatment	7	0.101	1.00	0.3931
Population (species) × short-term treatment	7	0.361	1.54	0.1561
Anthocyanins				
Species	3	0.099	15.37	**<0.0001**
Population (species)	8	0.063	3.68	**0.0007**
Climate treatment	1	0.063	29.33	**<0.0001**
Species × climate treatment	3	0.005	0.75	0.5256
Population (species) × climate treatment	8	0.039	2.25	**0.0276**

interaction). In other words, only with cold acclimation live oaks had capacity to increase NPQ and de-epoxidation state in response to short-term chilling (Figure 1). In warm temperatures, they were not able to increase NPQ and de-epoxidation state (Figure 1).

The total xanthophyll pool (VAZ and VAZ/Chl) did not show any response to short-term chilling temperatures but did show differences among climatic treatments. *Quercus oleoides* showed the highest accumulation of xanthophylls as well as the highest anthocyanin concentration in the temperate treatment (Figures 2 and 3), suggesting that long-term exposure to cold temperatures triggers the synthesis of photoprotective pigments in leaves of cold-sensitive species.

We found marked inter- and intraspecific differences in anthocyanin concentration in leaves. A positive association between minimum temperatures in the coldest month in the source of origin and the level of anthocyanin concentration was observed in both treatments (Figure 3). In a previous study on the same species and populations, Koehler et al. (2012) found that maternal families from climates with colder winters had lower growth rates and greater freezing tolerance than those from milder winters. In fact, those maternal families exhibiting higher freezing tolerance in Koehler et al. (2012) had lower anthocyanin accumulation in this study. These results are consistent with the hypothesis that maternal families with lower freezing tolerance use anthocyanins by both intercepting green light (light attenuation) and/or neutralizing reactive oxygen species to diminish the risk of photodamage under low temperatures (Pietrini et al. 2002, Gould 2004, Hughes et al. 2012).

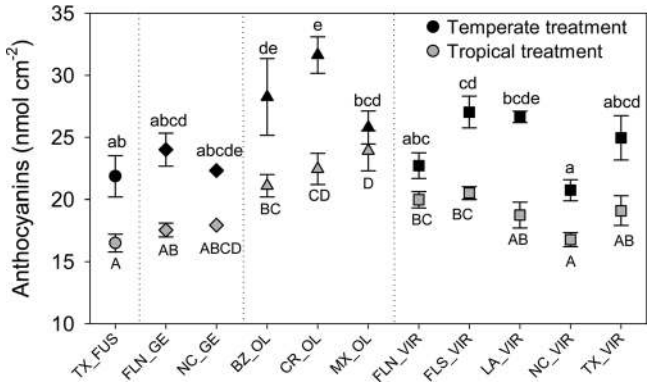

Figure 2. Means for populations (within species) ±SE under temperate (black) and tropical (gray) treatments for anthocyanin concentration in the reddest leaf. Different letters indicate significant differentiation ($P < 0.05$) within the low water and well-watered treatments. Populations: *Q. fusiformis* (circles), TX_FUS; *Q. geminata* (diamonds) FLN_GE, northern Florida; NC_GE, North Carolina Texas; *Q. oleoides* (triangles), CR_OL, Costa Rica; BZ_OL, Belize; MX_OL, Mexico; *Q. virginiana* (squares), FLS_VIR, southern Florida; FLN_VI, northern Florida; LA_VIR, Louisiana; TX_VIR, Texas; NC_VIR, North Carolina.

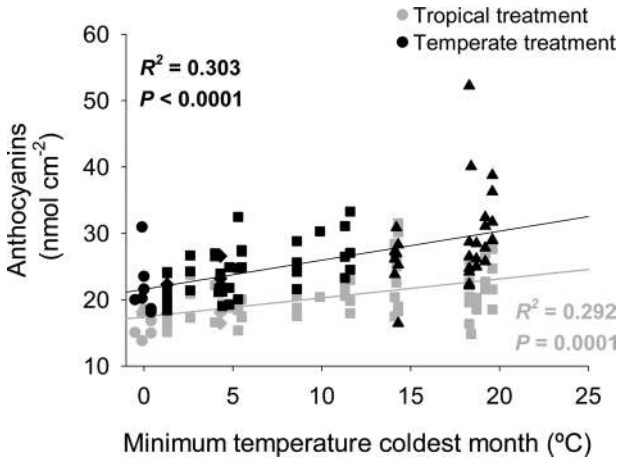

Figure 3. Relationship between anthocyanins and minimum temperatures of the coldest month in the source of origin of the maternal families under cold-winter temperate (black) and warm-winter (gray) treatments. *Quercus fusiformis* (circles), *Q. geminata* (diamonds), *Q. oleoides* (triangles) and *Q. virginiana* (squares). Points represent mean maternal-family values.

However, anthocyanins seem to be multifunctional pigments and alternative mechanisms need to be considered in explaining the observed pattern (Gould 2004).

Phenology is a factor that potentially could have influenced the observed association between anthocyanins accumulation and climate. Anthocyanin concentration, obtained by methanol extraction, was reduced in mature leaves relative to young leaves for each single plant. Phenology could be a potential factor explaining the observed patterns if plants in the temperate treatment broke bud later than the ones in the tropical treatment. We measured anthocyanin concentrations at the same time in both treatments and may have thus collected anthocyanin data in young leaves under two different phenological periods such that

Table 2. Results of the mixed model for the effects of species, population (species), maternal family (population), climate treatment and their interactions on vegetative phenology. Significant values ($P < 0.05$) are shown in bold and marginally significant values ($P < 0.1$) are shown in italics.

Effect	Test	P value
Species	$F_{1,356} = 8.190$	**0.0111**
Population (species)	$F_{2,356} = 5.654$	**0.0145**
Family (population)	$Z = 1.169$	0.2424
Climate treatment	$F_{1,356} = 461.806$	**<0.0001**
Species × climate treatment	$F_{1,356} = 0.073$	0.7900
Population (species) × climate treatment	$F_{2,356} = 2.959$	*0.0821*
Family (population) × climate treatment	$Z = 0.340$	0.7340

differences would be caused by phenological disparities. However, we have evidence to suggest that phenology had little influence on the anthocyanin pattern. We found that the vegetative period of growth started ~15 days later in northern populations compared with southern populations in the temperate treatment (see Figure S4 available as Supplementary Data at *Tree Physiology* Online). Northern populations presented the lowest anthocyanin concentrations, contrary to what would be expected if phenological variation was driving variation in anthocyanin concentration. In other words, our results indicate that differences in anthocyanins among live oak species and populations are unlikely to be explained in terms of phenological disparities.

Increased defense investment in younger leaves could provide an alternative explanation for the variation in anthocyanin content in our study (Karageorgou and Manetas 2006). Biogeographical theories have traditionally considered that more intense herbivory occurs in young leaves at lower latitudes. As a consequence, higher investment in defense strategies and compounds is expected in the more palatable young leaves of plants growing in lower, rather than higher, latitudes (Dobzhansky 1950, MacArthur 1972, Coley and Aide 1991, Van Alstyne et al. 2001). Since minimum temperatures in the coldest month are highly correlated to latitude in live oaks (Koehler et al. 2012), our relationship between anthocyanin accumulation and climate at the place of origin could be due to a genetically based latitudinal gradient in defense investment rather than a response to light stress. Nevertheless, in a recent review, Moles et al. (2011), found that empirical data did not support either the widespread idea that herbivory is generally more intense at lower latitudes or that plants from low latitudes are generally better defended than plants from higher latitudes are. Indeed, they even found that chemical defenses were significantly higher in plants from higher, rather than lower, latitudes.

Another possible explanation for the increase in anthocyanins in low latitude populations is that fast growing plants accumulate more phenolic compounds, including anthocyanins, in leaves because of their higher photosynthetic rates and rapid carbon accumulation necessary for phenolic synthesis (Hernández and

Table 3. Results of ANOVA for the effects of species, population (species), water treatment and their interactions on NPQ, total xanthophyll pool (V + A + Z), (V + A + Z)/Chl content, de-epoxidation state of the xanthophyll cycle. [(A + Z)/(V + A + Z)], F_v/F_m, chlorophyll content, ETR, SLA and SPI for plants grown under tropical conditions. Significant values ($P < 0.05$) are shown in bold and marginally significant values ($P < 0.1$) are shown in italics. SS, sum of squares.

Effect	DF	SS	*F* ratio	*P* value
NPQ				
Species	3	7.514	0.78	0.5070
Population (species)	7	26.607	1.18	0.3165
Water treatment	**1**	**54.512**	**16.98**	**0.0001**
Species × treatment	*3*	*23.019*	*2.39*	*0.0718*
Population (species) × treatment	7	32.992	1.47	0.1843
V + A + Z				
Species	**3**	**117.401**	**13.89**	**<0.0001**
Population (species)	*7*	*37.893*	*1.92*	*0.0746*
Water treatment	1	0.144	0.05	0.8217
Species × treatment	3	6.461	0.76	0.5167
Population (species) × treatment	7	17.908	0.91	0.5037
(V + A + Z)/Chl				
Species	**3**	**0.018**	**3.62**	**0.0159**
Population (species)	**7**	**0.026**	**2.15**	**0.0452**
Water treatment	1	0.005	2.71	0.1031
Species × treatment	3	0.006	1.22	0.3065
Population (species) × treatment	7	0.017	1.39	0.2174
(A + Z)/(V + A + Z)				
Species	3	0.0065	0.05	0.9854
Population (species)	**7**	**0.7428**	**2.41**	**0.0261**
Water treatment	**1**	**0.7824**	**17.74**	**<0.0001**
Species × treatment	3	0.1744	1.32	0.2732
Population (species) × treatment	**7**	**0.7964**	**2.58**	**0.0177**
F_v/F_m				
Species	3	0.0072	0.18	0.9123
Population (species)	7	0.0926	0.97	0.4546
Water treatment	**1**	**0.3169**	**23.25**	**<0.0001**
Species × treatment	3	0.0056	0.14	0.9373
Population (species) × treatment	7	0.0603	0.63	0.7289
Chlorophyll				
Species	**3**	**55,353.9**	**7.63**	**0.0001**
Population (species)	7	7053.8	0.42	0.8904
Water treatment	*1*	**12,517.5**	**5.18**	**0.0247**
Species × treatment	3	14,960.1	2.06	0.1089
Population (species) × treatment	7	14,505.8	0.86	0.5429
ETR				
Species	3	0.2962	0.57	0.6380
Population (species)	7	1.3744	1.13	0.3504
Water treatment	**1**	**4.4746**	**25.68**	**<0.0001**
Species × treatment	3	0.4489	0.86	0.4644
Population (species) × treatment	7	0.6986	0.57	0.7769
SLA				
Species	3	2103.3	0.85	0.4685
Population (species)	7	**13,504.2**	**2.34**	**0.0280**
Water treatment	1	20.1	0.02	0.8760
Species × treatment	3	437.8	0.18	0.9117
Population (species) × treatment	*7*	**13,653.6**	**2.37**	**0.0264**
SPI				
Species	**3**	**0.0052**	**3.05**	**0.0320**
Population (species)	7	0.0040	0.99	0.4428
Water treatment	**1**	**0.0118**	**31.74**	**<0.0001**
Species × treatment	3	0.0013	0.77	0.5108
Population (species) × treatment	7	0.0005	0.13	0.9957

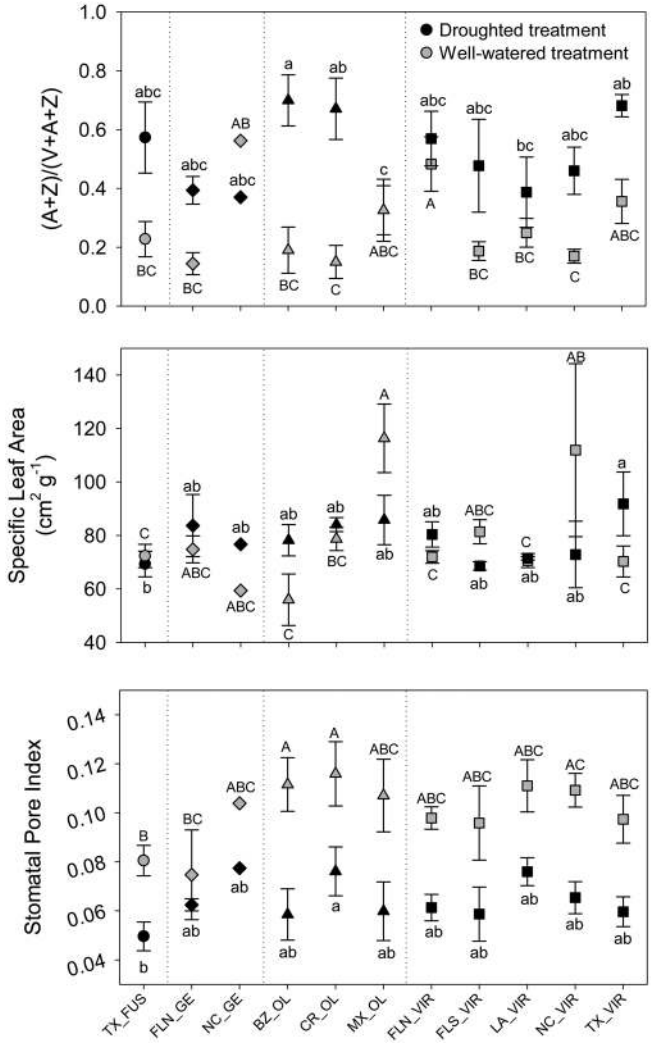

Figure 4. Means for populations (within species) ±SE under droughted (black) and well-watered (gray) treatments for de-epoxidation state in the xanthophyll cycle [(A + Z)/(V + A + Z)], SLA and SPI. *Quercus fusiformis* (circles), *Q. geminata* (diamonds), *Q. oleoides* (triangles) and *Q. virginiana* (squares). See Figure 2 legend for definitions of place of origin abbreviations.

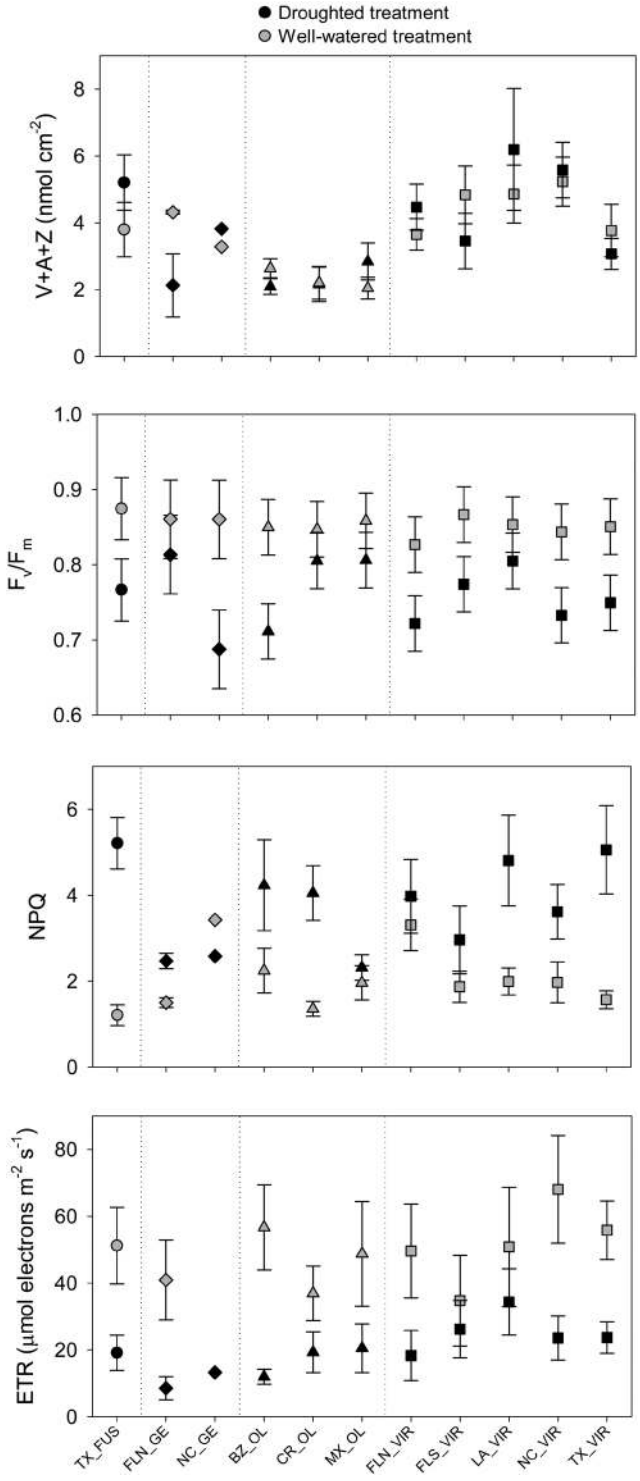

Figure 5. Means for populations (within species) ±SE under droughted (black) and well-watered (gray) treatments for total xanthophyll pool (V + A + Z), F_v/F_m, NPQ and electron transport rate (ETR). *Quercus fusiformis* (circles), *Q. geminata* (diamonds), *Q. oleoides* (triangles) and *Q. virginiana* (squares). See Figure 2 legend for definitions of place of origin abbreviations.

Van Breusegem 2010). However, while carbohydrate accumulation could potentially explain the accumulation of anthocyanins in low latitudinal populations that have higher growth rates in the tropical treatment, it fails to explain the significantly increased anthocyanin content observed under temperate conditions where growth and carbon accumulation were reduced.

Response to drought

We investigated inter- and intraspecific differences in xanthophyll cycle activity and its phenotypic plasticity in response to drought conditions in four live oak species. Our results showed that the de-epoxidation state (AZ/VAZ) varied markedly within species. Only a few studies have sought to examine inter- or intraspecific variation in xanthophyll cycle activity under contrasting water conditions. Previous findings do not show a general pattern for the observed variation based on habitats or climates of origin. For example, Savage et al. (2009) in a study with six willow (*Salix*) species found that habitat generalists had

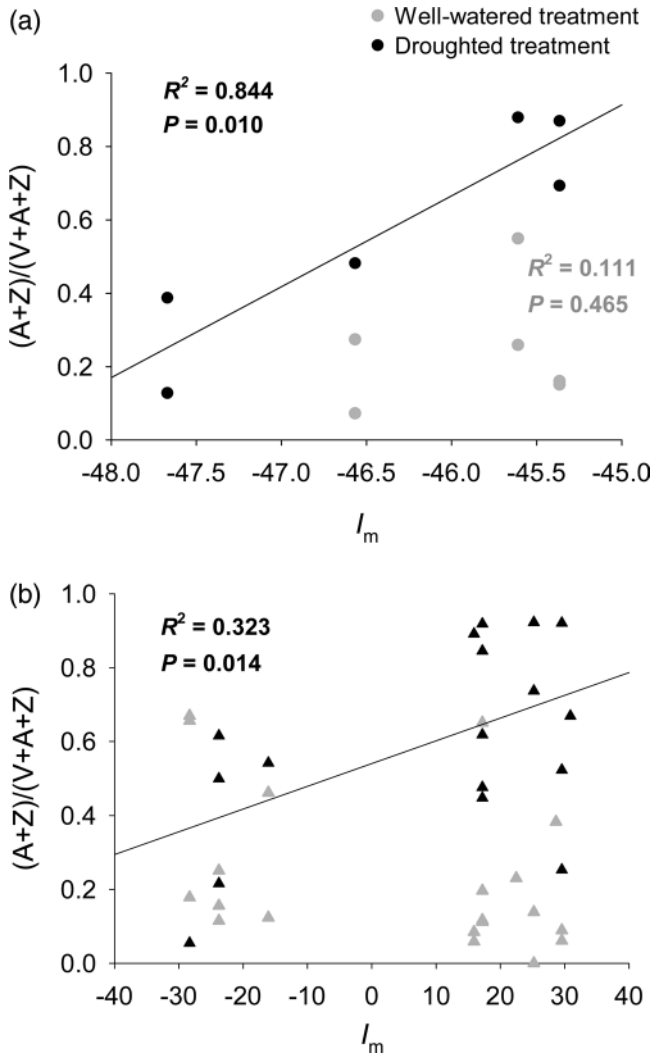

Figure 6. Relationship between de-epoxidation state of the xanthophyll cycle [(A + Z)/(V + A + Z)] under droughted (black) and well-watered (gray) treatments and index of moisture (I_m) in the source of origin of the maternal families of *Q. fusiformis* (a) and *Q. oleoides* (b). Points represent mean maternal-family values.

lower photosynthetic rates and stomatal conductance and greater photoprotective responses in terms of de-epoxidation state than wetland specialists in a dry-down experiment. Peguero-Pina et al. (2009) in a study with three evergreen oak species also found interspecific differences in de-epoxidation state. Specifically, they observed that the two drought-tolerant species, *Q. ilex* and *Q. coccifera*, noticeably increased the pool of de-epoxidated xanthophylls at lower water potentials. In contrast, the de-epoxidated xanthophyll pool barely changed in *Q. suber*, a species more sensitive to drought.

At the intraspecific level, Camarero et al. (2012) observed an increase in xanthophyll pool in xeric to mesic populations. Interestingly, photoprotection was suggested to be mediated by an increase in reflectance and shading through higher trichome density and lower SLA. García-Plazaola and Becerril (2000*a*) found differences in the de-epoxidation state (AZ/VAZ) among

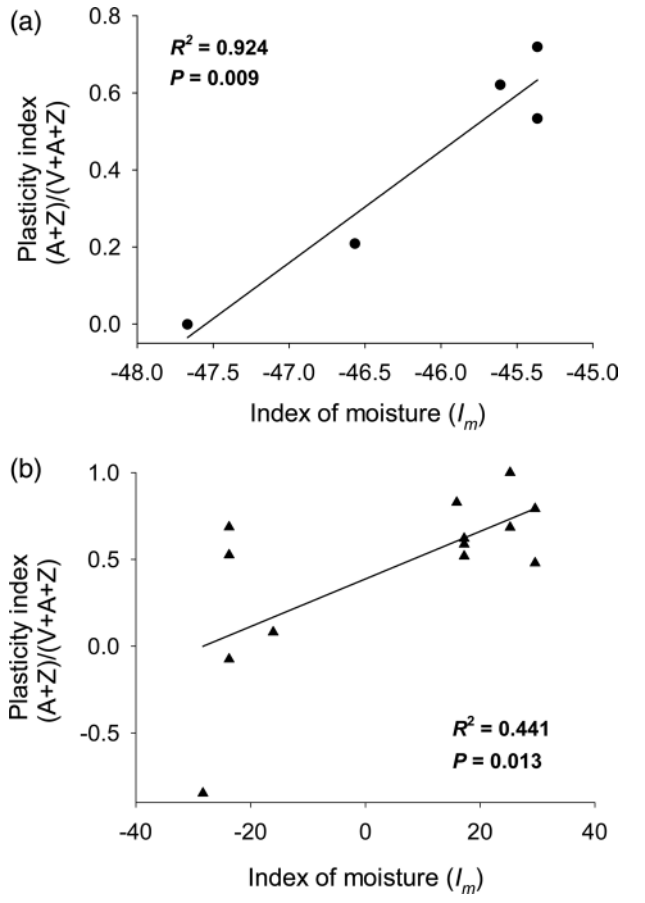

Figure 7. Relationship between index of moisture (I_m) in the source of origin of the maternal families of *Q. fusiformis* (a) and *Q. oleoides* (b) and the plasticity in de-epoxidation state of the xanthophylls across treatments (droughted—well-watered) measured by PI. Points represent mean maternal-family values.

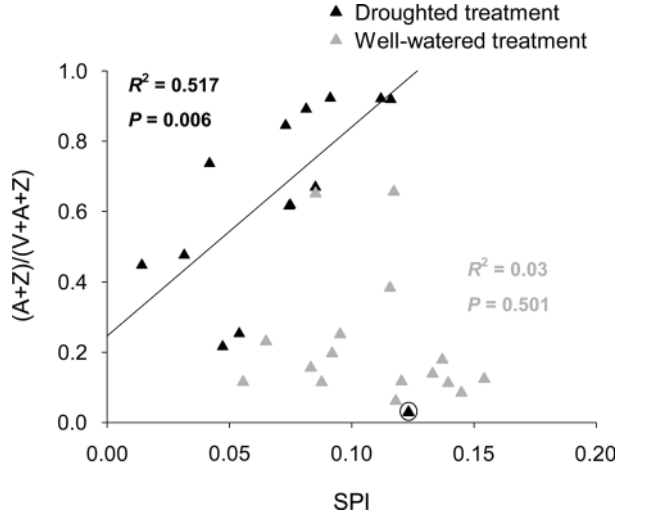

Figure 8. Relationship between SPI and de-epoxidation state [(A + Z)/(V + A + Z)] under droughted (black) and well-watered conditions for the maternal families of *Q. oleoides* (b). Linear fits (R^2) and significance levels were obtained excluding the outlier maternal family. Points represent mean maternal-family values.

Fagus sylvatica populations, with the xeric ecotype showing the lowest values. To explain this finding, García-Plazaola and Becerril (2000*a*, 2000*b*) maintained that drought tolerance in the xeric ecotype was based on morphological characteristics such as lower SLA to a greater extent than on xanthophyll-mediated photoprotective responses. In our study, AZ/VAZ values measured in the droughted treatment were positively associated with moisture of the maternal source for *Q. oleoides* and *Q. fusiformis*, similar to the findings of García-Plazaola and Becerril (2000*a*) (Figure 4). We also observed a positive correlation between PI of AZ/VAZ across treatments and index of moisture at the place of origin, indicating that maternal families from places with higher water availability had higher phenotypic plasticity in this photoprotective mechanism in response to variation in water availability (Figure 5). When exposed to drought stress, the more mesic ecotypes invoked photoprotective mechanisms to a greater extent than the xeric ecotypes.

In order to test whether leaf morphology is related to photoprotective traits as García-Plazaola and Becerril (2000*a*, 2000*b*) suggested, we explored the relationship between AZ/VAZ and two structural/morphological traits: SLA and SPI. Contrary to our expectation, we did not find a relationship between AZ/VAZ and SLA. However, we did find a strong positive association between AZ/VAZ and SPI in one of the species, *Q. oleoides*, in the drought treatment (Figure 6). Stomatal pore index is related to leaf hydraulic conductance, and higher SPI values are consistent with lower leaf resistance to water loss and less conservative water use (Sack et al. 2003). However, our preliminary results in a different common garden show that mesic populations of *Q. oleoides* reduce water potential at turgor loss point more than populations originating from dry sites (J.A. Ramírez-Valiente and J. Cavender-Bares, unpublished results) whereas the latter ones drop a substantial amount of leaves under drought conditions (drought avoidance). Together, our results suggest that drought tolerant populations activate photoprotection in response to limiting water conditions.

In conclusion, our results indicate that there are strong associations between climate of origin, climatic stress tolerance and accumulation of photoprotective pigments in live oaks. Specifically, species and populations from mesic climates, activated xanthophyll cycle pigments under drought conditions which preliminary results suggest that increased drought tolerance, whereas species and populations originating from climates with warmer winters, which have lower freezing tolerance, accumulated anthocyanins under cold conditions. Overall, our findings suggest that (i) climate plays an important role in shaping species and population genetic differences in stress tolerance for live oaks, (ii) anthocyanins are used under cold stress in species with limited freezing tolerance and (iii) xanthophyll cycle pigments are used when photoprotection under drought conditions is needed.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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