

Age-related changes in oxidative stress markers and abscisic acid levels in a drought-tolerant shrub, *Cistus clusii* grown under Mediterranean field conditions

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Abstract Compared with our knowledge of senescence in annuals and biennials, little is known about age-related changes in perennials. To get new insights into the mechanisms underlying aging in perennials, we measured oxidative stress markers in leaves and organelles, together with abscisic acid levels in leaves of 2- and 7-year-old *Cistus clusii* dunal plants grown under Mediterranean field conditions. Recently emerged leaves, which either appeared during autumn or spring, were compared to evaluate the effects of environmental constraints on oxidative stress and abscisic acid accumulation as plants aged. Plant aging led to an enhanced oxidation of α -tocopherol and ascorbate, increased lipid peroxidation and reduced PSII efficiency in leaves during the more stressful conditions of spring and summer, but not during autumn. Analyses of lipid peroxidation in organelles isolated from the same leaves revealed that oxidative stress occurred both in chloroplasts and mitochondria. Although both plant groups showed similar leaf water and nitrogen contents throughout the study, abscisic acid levels were markedly higher (up to 75%) in 7-year-old plants compared to 2-year-old plants throughout the study. It is concluded that (a) meristematic tissues of *C. clusii* maintain the capacity to make new leaves with no symptoms of oxidative stress for several years, unless these leaves are exposed to environmental constraints, (b) leaves of oldest plants show higher oxidative stress than those of young plants when exposed to adverse

climatic conditions, thus supporting the idea that the oxidative stress associated with aging is due at least partly to extrinsic factors, (c) at the subcellular level, age-induced oxidative stress occurs both in chloroplasts and mitochondria, and (d) even in the absence of environmental stress, newly emerged leaves accumulate higher amounts of ABA as plants age.

Keywords Abscisic acid · Aging · Antioxidants · Oxidative stress · Perennials · Senescence

Abbreviations

ABA	Abscisic acid
DW	Dry weight
F_v/F_m	Maximum efficiency of PSII
FW	Fresh weight
MDA	Malondialdehyde
PPFD	Photosynthetically-active photon flux density
PSII	Photosystem II
ROS	Reactive oxygen species
RWC	Relative water content

Introduction

Aging has received considerable attention in biomedicine, but little is known about the regulatory mechanisms responsible for the aging not associated with senescence in plants, particularly in perennials. In plant development, pre-reproductive plants are considered juveniles; the onset of flowering and seed production marks a transition phase, and fully reproductive plants are considered mature (Bond 2000). Perennials maintain the capacity to develop new leaves and grow throughout their life until they enter a developmentally

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controlled senescing process that inevitably leads to plant death. With increasing age and size, however, growth tends to slow and perennials are more likely to die. Aging has been classically defined as the accumulation of changes in plant development responsible for slow, progressive, and sequential alterations that accompany the plants as they age (Noodén and Guimé 1996; Dangl et al. 2000). Disentangling the mechanisms by which plants age is still a matter of debate today (Morgan 2003; Peñuelas 2005).

Recent studies indicate that physiological burdens, such as demands on water and nutrient supply, may be responsible for reduced growth as plants age (Mencuccini et al. 2005). Aside from biotic and abiotic stresses limiting plant growth as plant age, it is also possible that intrinsic changes in the shoot meristems could occur through repeated cell divisions and could be fixed during plant development, thereby affecting foliar gas characteristics, such as stomatal conductance and photosynthetic rates in the leaves and shoots that originated from these cells (Day et al. 2002). In both cases, characterization of oxidative stress markers may help better understanding the mechanisms underlying this phenomenon. Aside from measurements on the extent of lipid peroxidation, estimations of the redox state of low-molecular-weight antioxidants may allow better understanding age-related oxidative processes in plants. Although levels of antioxidants indicate the potential extent of antioxidative protection and the balance between their synthesis, oxidation and regeneration, their redox state indicates the extent of oxidative load toward these compounds and provides us with a reliable estimation of oxidative stress in the cell (Munné-Bosch and Alegre 2003).

In animals, age-associated disorders in the cell are believed to be connected with the time-dependent shift in the antioxidant/prooxidant balance in favour of oxidative stress, and it has been suggested that mitochondria are the target organelles for oxidative stress in age-associated changes observed in animals (Ashok and Ali 1999; Rustin et al. 2000). Photosynthesis generally decreases in leaves as plants age (Bond 2000, and references therein), and chloroplasts have been proposed as one of the target organelles of oxidative stress as plants age (Munné-Bosch and Alegre 2002a). However, the relative contribution of mitochondria and chloroplasts in the oxidative stress associated with aging in perennials is still unknown.

The aim of the present study was to characterize (a) the oxidative stress associated with aging in a drought-tolerant shrub, *C. clusii* grown under Mediterranean field conditions, (b) the contribution of chloroplasts and mitochondria in the aging process,

and (c) the mechanism(s) underlying the aging process in perennials. To address these questions, we measured simultaneously oxidative stress markers (levels of photosynthetic pigments, reduced and oxidized forms of α -tocopherol and ascorbate, the F_v/F_m ratio and the extent of lipid peroxidation in leaves, chloroplasts and mitochondria) in parallel with markers of water and nutrient stress, and abscisic acid (ABA) and sucrose levels in recently emerged leaves of 2- and 7-year-old plants of *C. clusii* grown under Mediterranean field conditions.

Materials and methods

Plant material, growth conditions and sampling

The study was conducted using two groups of *Cistus clusii* Dunal plants of different ages: (a) 2-year-old plants, which can be considered young since the formation of flowers was observed once; and (b) 7-year-old plants, which can be considered mature non-senescing plants (the formation of flowers has been observed several times and the plants continue flowering; Bond 2000). Two-year-old plants were obtained from seeds collected from the other plant group as described below, thus studies were conducted on the same population of *C. clusii*.

Plants were obtained as follows. *C. clusii* seeds were germinated and grown in 0.5-l pots containing a mixture of soil:peat:perlite (1:1:1, by vol). Plants were maintained in a greenhouse with a controlled temperature (24/18°C, day/night) and were watered twice a week, once with tap water and once with Hoagland's solution. After 1 year of growth, plants were transplanted during the spring of 1999 to the experimental fields of the University of Barcelona (Barcelona, Spain) and 16 plants per plot were distributed homogeneously in a square, 1 m apart, so all plants had the same orientation to sun. After 5 years of growth, seeds were obtained from these plants and grown as described before. After 1 year of growth, plants were transplanted during the spring of 2004 to the same experimental fields and 16 plants per plot were distributed as described for the other plant group. The experimental area consisted of two plots of 4.5 m² each of calcic Luvisol (FAO) separated by 2 m only. Before the plants were transferred, the soil was ploughed and treated with N: P: K (1:1:1) fertilizer at the rate of 100 kg ha⁻¹ and an analysis of mineral composition of soil was carried out annually to avoid mineral deficiency in plants.

Lipid peroxidation in leaves, chloroplasts and mitochondria, chlorophylls and carotenoids, the levels of

reduced and oxidized α -tocopherol and ascorbate, the F_v/F_m ratio, RWC, C and N contents, and ABA and sucrose contents were analyzed simultaneously in the two age groups during October and November of 2004, and during April, May and June of 2005 from samples collected at midday (at maximum incident diurnal PPF) in the field. The leaves collected during October–November appeared during mid September, while the leaves collected during April–June appeared during the beginnings of March. Recently emerged leaves (situated up to 15 cm from the apex of branches) from each group of plants were selected for all the measurements. For analyses of MDA, photosynthetic pigments and antioxidants, ABA and sucrose contents in leaves, samples were collected at midday, immediately frozen in liquid nitrogen and stored at -80°C until analyses. For analyses of MDA in chloroplasts and mitochondria, leaves were collected at midday and were immediately subjected to cell fractionation.

Plants received water exclusively from rainfall during the growth and study period. Environmental conditions were monitored at 5-min intervals throughout the day with a weather station (Delta-T Devices, Newmarket, UK). Photosynthetically active photon flux density (PPFD) was measured with a quantum sensor (Li-Cor, Lincoln, Nebraska, USA). Air temperature and relative humidity were measured with a Vaisala thermohygrometer (Vaisala, Helsinki, Finland). The vapor pressure deficit was determined from relative humidity and air temperature data following Nobel (1991). Precipitation was measured with a standard rain gauge. The environmental conditions during the experiment were typical of the Mediterranean climate but characterized by an unexpected dry spring, with very low precipitation (3.2 mm) during April (Table 1).

Estimation of lipid peroxidation in leaves and organelles

The extent of lipid peroxidation was estimated by measuring the amount of MDA in leaves, chloroplasts and

mitochondria by the method described by Hodges et al. (1999), which takes into account the possible influence of interfering compounds in the thiobarbituric acid-reactive substances (TBARS) assay.

Chloroplasts were isolated according to Munné-Bosch and Alegre (2001). After grinding the sample in isolation buffer [0.5–1.5 M sorbitol depending on the plant water status, 50 mM tricine, 1 mM dithiothreitol, 1 mM MgCl_2 , 1 mM citric acid, 1 mM butylated hydroxytoluene (BHT), 0.1% (w/v) bovine serum albumin, pH 7.8], the homogenate was filtered through four layers of cheese cloth and centrifuged at 2,500g for 4 min. The pellet was resuspended in isolation buffer and then centrifuged at 200g for 1 min. The chloroplasts in the supernatant were sedimented by centrifugation at 2,500g for 4 min. Chloroplasts were purified by resuspending the pellets in isolation buffer, layering onto 10 ml of 25% (v/v) percoll (in isolation buffer), and centrifuging at 15,800g for 20 min. The chloroplast pellets were resuspended in isolation buffer, centrifuged at 2,500g for 4 min, and used immediately.

Mitochondria were isolated according to Fan et al. (1999). Leaves were homogenized with a mortar and pestle in prechilled grinding medium containing 0.3 M mannitol, 50 mM Mes, pH 7.2, 1 mM EDTA, 1 mM MgCl_2 , 0.2% (w/v) defatted BSA, 0.5% (w/v) PVP-400, 4 mM Cys, and 10 mM β -mercaptoethanol. The homogenates were filtered through four layers of cheesecloth and centrifuged at 3,300g for 20 min. The pellet was suspended in resuspension medium I containing 0.3 M mannitol, 20 mM Mes, pH 7.2, 2 mM potassium phosphate, 1 mM EDTA, 0.1% (w/v) defatted BSA, 2 mM MgCl_2 , and 14 mM β -mercaptoethanol. The suspension was loaded onto a discontinuous gradient composed of 5 ml of 47%, 6 ml of 26%, and 3 ml of 21% (v/v) percoll. The gradients were centrifuged at 58,500g for 45 min in a swinging-bucket rotor. The mitochondrial band, located at the interface between the 26 and 47% percoll layers, was removed and diluted with an equal volume of resuspension medium II containing 0.3 M mannitol, 20 mM

Table 1 Accumulated rainfall (during the preceding 30 days) and climatological conditions (maximum diurnal vapor pressure deficit [VPD], photosynthetically-active photon flux density [PPFD] and air temperature [T_{air}]), during the measurement days of autumn of 2004, and spring–summer of 2005

Year	Sampling date	Rainfall (mm)	VPD (KPa)	PPFD ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	T_{air} ($^\circ\text{C}$)
2004	5 October	49.8	1.2	1476	25.8
	12 November	22.0	1.1	1048	17.0
2005	29 April	3.2	2.6	1841	25.0
	26 May	39.1	2.3	1940	25.6
	30 June	3.1	1.4	1853	28.5

Plants grew under Mediterranean field conditions and received water exclusively from rainfall. Accumulated rainfall during March was of 32.3 mm

Mes, pH 7.2, 1 mM EDTA, 0.1% (w/v) defatted BSA, 2 mM MgCl_2 , and 2 mM DTT. The diluted mitochondria were loaded onto a second percoll gradient composed of 7.5 ml of 47% and 7.5 ml of 26% (v/v) percoll prepared as in the first gradient. The gradient was centrifuged at 58,500g for 30 min. The mitochondrial band, located at the interface between the 26 and 47% (v/v) percoll layers, was collected and diluted with 10 vol of medium II. The mitochondria were pelleted by centrifugation at 18,800g for 5 min and resuspended in 1 ml of medium II. The purity of chloroplasts and mitochondria was determined by assaying amounts and activities of appropriate markers and confirmed further by microscopic observation as described (Munné-Bosch and Alegre 2001; Keech et al. 2005). Chloroplasts- and mitochondria-enriched fractions did not show detectable activities of NADPH-cytochrome *c* reductase and vanadate-sensitive ATPase, which are markers of endoplasmic reticulum and plasma membrane, respectively. Furthermore, chloroplasts-enriched fractions did not show detectable activities of cytochrome *c* oxidase, which is a marker of mitochondrion. Mitochondria showed very small thylakoid contamination as shown by the chlorophyll contents, which averaged 3.2 mg ml^{-1} for the crude extract and $4.3 \text{ } \mu\text{g ml}^{-1}$ for the purified mitochondria. To give MDA values per dry weight (DW) of organelles, a separate aliquot of chloroplasts- and mitochondria-enriched fractions was used to determine DW by oven-drying the samples to constant weight at 80°C .

For MDA analyses, leaf samples (500 mg FW), chloroplasts- and mitochondria-enriched fractions (50 mg DW) were extracted with 80:20 (v/v) ethanol/water using ultrasonication (Vibra-Cell Ultrasonic Processor, Sonics & Materials Inc., Canbury, CT, USA). After centrifuging at 3000g for 10 min at 4°C , the pellet was re-extracted twice with the same solvent. Supernatants were pooled and an aliquot of appropriately diluted sample was added to a test tube with an equal volume of either (a) –TBA solution comprised of 20% (w/v) trichloroacetic acid and 0.01% (w/v) BHT, or (b) +TBA solution containing the above plus 0.65% (w/v) TBA. Samples were heated at 95°C for 25 min, and after cooling; absorbance was read at 440, 532, and 600 nm. MDA equivalents (nmol ml^{-1}) were calculated as $10^6 \times [(A-B)/15,7000]$, where $A = [(Abs\ 532_{+TBA}) - (Abs\ 600_{+TBA}) - (Abs\ 532_{-TBA} - Abs\ 600_{-TBA})]$, and $B = [(Abs\ 440_{+TBA} - Abs\ 600_{+TBA}) \times 0.0571]$ (Hodges et al. 1999).

Chlorophyll fluorescence measurements

The maximum efficiency of PSII photochemistry (F_v/F_m) was calculated from chlorophyll fluorescence data

obtained from leaves exposed to darkness for 2 h with a portable fluorimeter mini-PAM (Walz, Effeltrich, Germany), by using the equations described by Van Kooten and Snel (1990).

Pigment determination

For determination of photosynthetic pigments, leaf samples (100 mg FW) were ground in a mortar and extracted repeatedly with 80% (w/w) acetone using ultrasonication (Vibra-Cell Ultrasonic Processor). The resulting extracts were immediately assayed spectrophotometrically. Specific absorption coefficients of chlorophylls and total carotenoids reported by Lichtenthaler and Wellburn (1983) were used.

Determination of reduced and oxidized forms of antioxidants

The extraction and analysis of α -tocopherol and its oxidation product, α -tocopherol quinone, was carried out as described (Munné-Bosch and Alegre 2003). In short, leaf samples (300 mg FW) were ground in liquid nitrogen and repeatedly extracted with n-hexane containing $1 \text{ } \mu\text{g ml}^{-1}$ BHT using ultrasonication (Vibra-Cell Ultrasonic Processor). α -Tocopherol and α -tocopherol quinone were analyzed by HPLC using a Partisil-10 ODS column (Sharlau, St Cugat, Spain). The solvents consisted of (a) methanol/water (95:5, v/v) and (b) methanol at a flow rate of 1 ml min^{-1} . The gradient used was: 0–10 min 100% A, 10–15 min decreasing to 0% A, 15–20 min 0% A, 20–23 min increasing to 100% A, and 23–28 min 100% A. α -Tocopherol and α -tocopherol quinone were quantified through their absorbance at 283 and 256 nm, respectively (diode array detector 1000S, Applied Biosystems, Foster City, CA, USA). Compounds were identified by their characteristic spectra and by coelution with authentic standards, which were obtained from Sigma (St Cugat, Spain) and Prof. Kazimierz Strzalka (Jagellonian University, Krakow, Poland).

The extraction and HPLC analysis of reduced and oxidized ascorbate was performed as described (Munné-Bosch and Alegre 2003). In short, leaf samples (100 mg FW) were ground in liquid nitrogen and repeatedly extracted with ice-cold extraction buffer (40% [v/v] methanol, 0.75% [w/v] *m*-phosphoric acid, 16.7 mM oxalic acid, 0.127 mM diethylenetriaminepentaacetic acid) using ultrasonication (Vibra-Cell Ultrasonic Processor). After centrifugation, 0.1 ml of the supernatant was transferred to 0.9 ml of the mobile phase (24.25 mM Na-acetate/ acetic acid, pH 4.8; 0.1 mM diethylenetriaminepentaacetic acid; 0.015%

[w/v] *m*-phosphoric acid; 0.04% [w/v] octylamine; 15% [v/v] methanol) for determination of reduced ascorbate. For determination of total ascorbate (reduced plus oxidized) 0.1 ml of the supernatant was incubated for 10 min at room temperature in darkness with 0.25 ml of 2% (w/v) dithiothreitol and 0.5 ml of 200 mM NaHCO₃. The reaction was stopped by adding 0.25 ml of 2% (v/v) sulfuric acid and 0.8 ml of the mobile phase. Ascorbic acid was isocratically separated on a Spherisorb ODS C₈ column (Teknokroma, St Cugat, Spain) at a flow rate of 0.8 ml min⁻¹. Detection was carried out at 255 nm (Diode array detector 1000S, Applied Biosystems). Ascorbic acid was identified by its characteristic spectrum and by coelution with an authentic standard from Sigma (Steinheim, Germany).

Plant water status and elemental analyses

Relative leaf water content (RWC) was determined as $100 \times (\text{FW}-\text{DW})/(\text{TW}-\text{DW})$, where FW is the fresh matter, TW is the turgid matter after re-hydrating the leaves for 24 h at 4°C in darkness, and DW is the dry matter after oven-drying the leaves for 24 h at 80°C. Total C and N concentrations in leaves were measured by the Dumas elemental analysis method by using a protein nitrogen analyzer NA2100 (Thermo, Milan, Italy).

ABA and sucrose levels

The extraction and analysis of ABA was carried out essentially as described (López-Carbonell and Jáuregui 2005). Leaf samples (100 mg FW) were ground in liquid nitrogen and repeatedly extracted with acetone/water/acetic acid (80:19:1, by vol) using ultrasonication (Vibra-Cell Ultrasonic Processor). The supernatants were combined and dried in a rotavapor until the aqueous fractions were obtained; these fractions were dried completely under a nitrogen stream. The extracts were immediately reconstituted in solvent A, filtered through a 0.45 μm PTFE filter (Waters, Milford, MA, USA) and injected into the LC-MS/MS system. The HPLC system consisted of a Perkin Elmer Series 200 (Norwalk, CT, USA) quaternary pump equipped with an autosampler and an UV detector. For the analysis of the extracts, a Luna C₁₈ Phenomenex (Torrance, CA, USA) column (50 × 2.1 mm, 3.5 μm) equipped with a Securityguard C₁₈ Phenomenex (4 × 3 mm, i.d.) was used. Gradient elution was done with water with 0.05% acetic acid (solvent A) and acetonitrile (solvent B) at a constant flow rate of 0.4 ml min⁻¹. A linear gradient profile with the following proportions of solvent A was applied (*t* (min), %A): (0, 85), (5, 0), (5.2, 0), (6,

85), (10, 85). MS/MS experiments were performed on an API 3000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ont, Canada). All the analyses were performed using the Turbo Ionspray source in negative ion mode with the following settings: capillary voltage -3500 V, nebulizer gas (N₂) 10 (arbitrary units), curtain gas (N₂) 12 (arbitrary units), collision gas (N₂) 4 (arbitrary units), declustering potential (DP) -55 V, focusing potential -250 V, entrance potential 10 V, collision energy (CE) -30 V; drying gas (N₂) was heated to 400°C and introduced at a flow-rate of 5,000 cm³ min⁻¹. In negative mode, the spectrum for ABA gave the deprotonated molecule [M-H]⁻. MRM acquisition was done monitoring the 263/153 transition with a dwell time of 2,000 ms. Quantification used MS/MS (MRM method, MRM). The MRM mode was required because many compounds could present the same nominal molecular mass, but the combination of the parent mass and unique fragment ions was used to selectively monitor ABA (López-Carbonell and Jáuregui 2005).

The extraction and HPLC analysis of sucrose was essentially performed as described (Gerrits et al. 2001). Leaf samples (50 mg FW) were ground in liquid nitrogen and repeatedly extracted with 80% [v/v] ethanol at 80°C. After centrifugation, the supernatants were pooled and dried completely under a nitrogen stream. The extracts were immediately reconstituted in 1 ml of water and passed through a Sep-Pak Plus cartridge (Waters, Milford, MA, USA). Sucrose was isocratically separated on an Aminex HPX-87C column (Bio-Rad, Hercules, CA, USA) using water as a solvent at a flow rate of 0.6 ml min⁻¹. Detection was carried out with a differential refractometer (Knauer, Berlin, Germany) with the cell at 40°C. Sucrose from Sigma (Steinheim, Germany) was used for quantification.

Statistical analyses

Statistical differences between age groups were analyzed by the Student *t*-test using the SPSS package (Chicago, IL). Differences were considered significant when *P* < 0.05.

Results

Plant aging increases oxidative stress under adverse climatic conditions

Plants were exposed to adverse climatic conditions during spring and summer, including exposure to drought and high light stress, as indicated by the low

precipitation and high diurnal PPFD values during this period (Table 1). Chlorophyll and carotenoid levels changed in parallel throughout the study and were always significantly smaller in 7-year-old plants than in 2-year-old plants, both during autumn and the more stressful period of spring and summer (Fig. 1). 7-year-old plants displayed significantly smaller α -tocopherol levels in leaves throughout the study, which were between 22 and 34% smaller than those observed in 2-year-old plants (Fig. 2). This age-related reduction in α -tocopherol was associated with an enhanced oxidation of this antioxidant, but only during spring and summer. The amount of α -tocopherol quinone per total tocopherol (reduced plus oxidized forms) was higher in 7-year-old plants than in 2-year-old plants, the former showing values 31–97% higher than the latter between April and June. Seven-year-old plants also showed significantly increased proportion of oxidized ascorbate in leaves during this period, since the amount of dehydroascorbate per total ascorbate, was between 1.7 and 3-fold, higher in 7-year-old plants than in 2-year-old plants. In contrast, no consistent differences in the amounts of reduced ascorbate were observed throughout the study between both plant groups (Fig. 3).

The maximum efficiency of PSII photochemistry, estimated as the F_v/F_m ratio, was also slightly smaller during spring and summer in 7-year-old plants, which displayed reductions in this ratio up to 6% compared to 2-year-old plants (Fig. 4). Seven-year-old plants showed enhanced lipid peroxidation, estimated as malondialdehyde (MDA) accumulation, in leaves during spring and summer, which were between 10 and 50 % higher than those observed in 2-year-old plants during the same period. No significant differences in the F_v/F_m ratio and the extent of lipid peroxidation were observed between both age groups during autumn (Fig. 4).

Plant aging increases lipid peroxidation in chloroplasts and mitochondria

To evaluate the effects of plant aging on lipid peroxidation at the subcellular level, MDA levels were measured in isolated organelles obtained from leaves collected between April and June (Fig. 5). These studies revealed that enhanced age-dependent lipid peroxidation in leaves was, at least partly, due to increased lipid peroxidation in chloroplasts and mitochondria. The amount of MDA in chloroplasts and mitochondria increased with plant aging, showing 7-year-old plants levels between 2 and 2.8-fold and between 1.6 and 3.6-fold higher, respectively, than those of 2-year-old plants (Fig. 5).

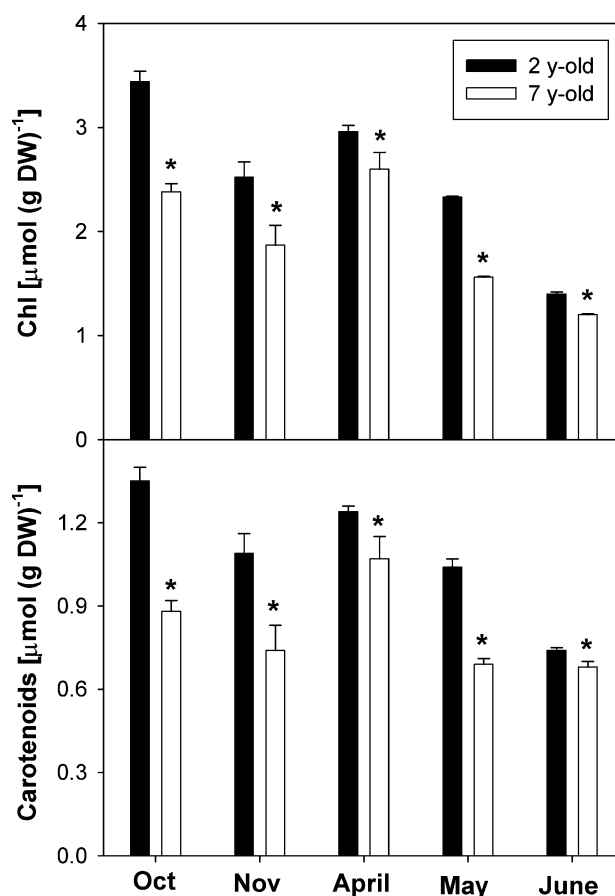


Fig. 1 Chlorophyll a + b (Chl) and carotenoid contents in leaves of 2 (black bars) and 7-year-old (white bars) *C. clusii* plants grown under Mediterranean field conditions. Data correspond to the mean \pm SE of four measurements. Statistical differences ($P \leq 0.05$, Student's *t*-test) between 2- and 7-year-old plants are indicated by an asterisk

Plant aging increases ABA levels in leaves

Plants kept midday relative leaf water content (RWC) above 74% during autumn, while they decreased to *ca.* 55% when plants were subject to drought during spring and summer (Fig. 6). Besides drought, plants were exposed to high light stress, as indicated by the maximum diurnal PPFD values ranging between 1,841 and 1,940 $\mu\text{mol m}^{-2}\text{s}^{-1}$ during the measurement days of spring and summer. April was unexpectedly dry (Table 1), which explains the low RWC values obtained during this period. No differences in RWC between 2- and 7-year-old plants were observed throughout the experiment. Neither carbon nor nitrogen contents differed in leaves of both plant groups (Fig. 1). Although no differences were observed in these parameters, ABA levels were markedly higher (up to 75%) in 7-year-old plants compared to 2-year-old plants. Interestingly, age-induced differences in

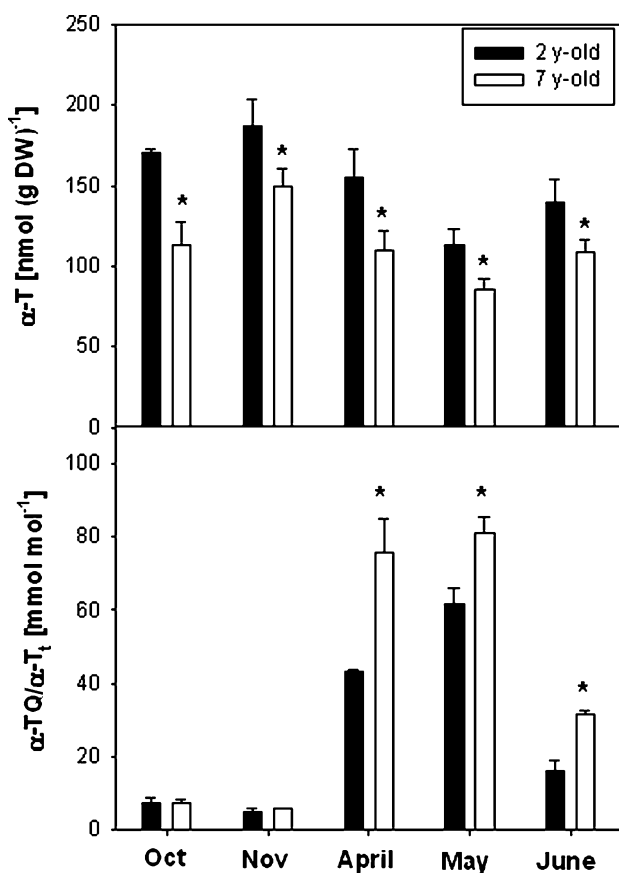


Fig. 2 α -Tocopherol (α -T) contents and α -TQ/ α -T_i ratio, where α -TQ is α -tocopherol quinone and α -T_i = α -T + α -TQ, in leaves of 2- (black bars) and 7-year-old (white bars) *C. clusii* plants grown under Mediterranean field conditions. Data correspond to the mean \pm SE of four measurements. Statistical differences ($P \leq 0.05$, Student's *t*-test) between 2- and 7-year-old plants are indicated by an asterisk

ABA levels were observed both during autumn and during the more stressful period of spring and summer (Fig. 7). Furthermore, ABA levels were significantly higher (up to 30 pmol [g dry wt]⁻¹) during spring and summer than during autumn in both plant groups. Sucrose contents were also (up to four-fold) higher during spring and summer compared to autumn, but although this increase, both plant groups showed similar sucrose levels per DW throughout the study. However, when given per unit of chlorophyll, sucrose contents were significantly higher (up to 57%) in the oldest plants, and this was consistent throughout the study (Fig. 8).

Discussion

Although studies on aging in perennials have been limited in part by the challenges of working with long-lived organisms, such studies are essential if we are to

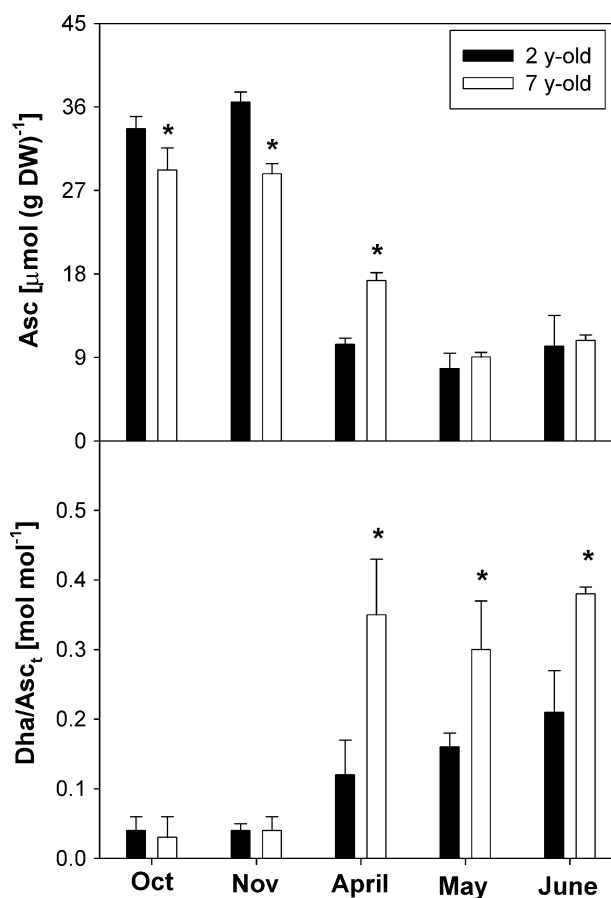


Fig. 3 Ascorbate (Asc) contents and Dha/Asc_i ratio, where Dha is dehydroascorbate and Asc_i = Asc + Dha, in leaves of 2- (black bars) and 7-year-old (white bars) *C. clusii* plants grown under Mediterranean field conditions. Data correspond to the mean \pm SE of four measurements. Statistical differences ($P \leq 0.05$, Student's *t*-test) between 2- and 7-year-old plants are indicated by an asterisk

understand the phenomenon of aging in plants. In the present study, the use of *C. clusii* as an experimental model allowed studying the effects of environmental stress on the aging process in a drought-tolerant shrub. This species is a branched sclerophyllous shrub with a lifespan of 15–20 years that is naturally found in littoral brushwood growing in calcareous and dry soils of the Mediterranean coast, but it can also inhabit sandy and perturbed areas, being therefore characterized by its resistance to drought stress. The study was performed by comparing the physiology of recently emerged leaves of plants of different ages, the youngest ones being obtained from seeds of the oldest ones, so both plant groups had the same genetic background, and they grew under the same environmental conditions.

In the present study, plant aging increased oxidative stress during the more stressful period of spring and summer, as indicated by an enhanced oxidation of α -tocopherol and ascorbate. α -Tocopherol, in

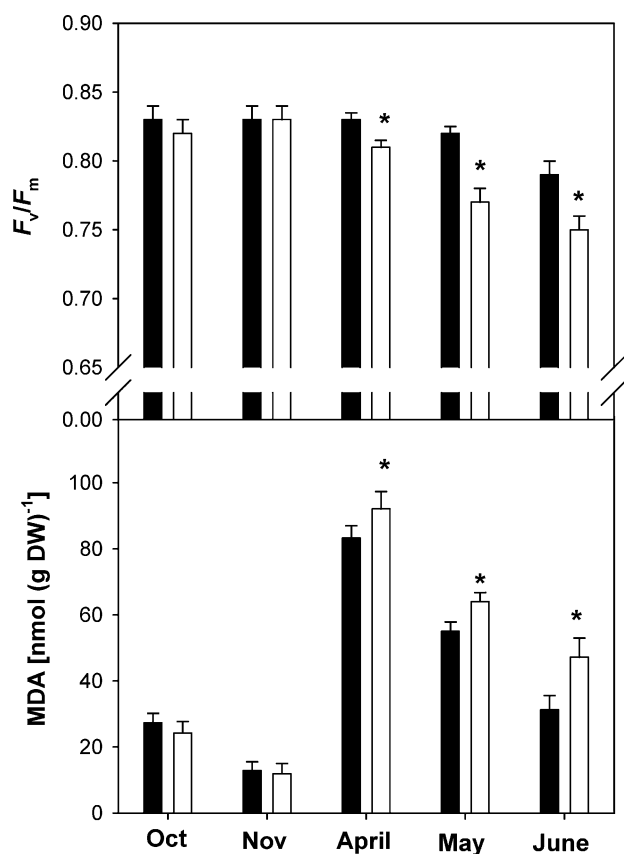


Fig. 4 Maximum efficiency of PSII photochemistry (F_v/F_m ratio) and malondialdehyde (MDA) levels, an indicator of lipid peroxidation, in leaves of 2 (black bars) and 7-year-old (white bars) *C. clusii* plants grown under Mediterranean field conditions. Data correspond to the mean \pm SE of eight measurements. Statistical differences ($P \leq 0.05$, Student's *t*-test) between 2- and 7-year-old plants are indicated by an asterisk

co-operation with other antioxidants, contributes to preserve an adequate cellular redox state in chloroplasts and to maintain thylakoid membrane structure and function. α -Tocopherol can physically quench, and therefore deactivate singlet oxygen in photosynthetic membranes (Trebst et al. 2002; Trebst 2003). In addition, α -tocopherol can chemically scavenge singlet oxygen and lipid peroxy radicals. The chemical scavenging of the former by α -tocopherol irreversibly leads to their quinones and epoxides, while the scavenging of lipid peroxy radicals results in the formation of tocopheroxyl radicals, which can be recycled back to α -tocopherol by the ascorbate-glutathione cycle (Munné-Bosch and Alegre 2002b). Thus, age-dependent enhanced oxidation of α -tocopherol to α -tocopherol quinone indicates that enhanced lipid peroxidation in chloroplasts in 7-year-old plants was caused at least in part by an enhanced production of reactive oxygen species (ROS) in photosynthetic membranes. Ascorbate is also essential for photosynthetic

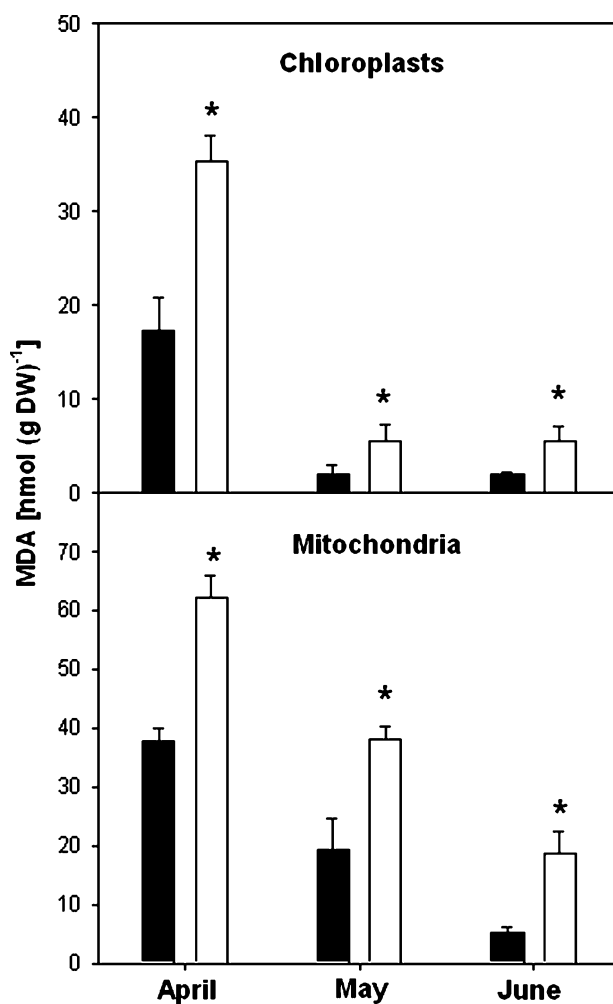


Fig. 5 Malondialdehyde (MDA) levels in chloroplasts and mitochondria obtained from leaves of 2- (black bars) and 7-year-old (white bars) *C. clusii* plants grown under Mediterranean field conditions. Data correspond to the mean \pm SE of four measurements. Statistical differences ($P \leq 0.05$, Student's *t*-test) between 2- and 7-year-old plants are indicated by an asterisk

activity in the chloroplasts and for a variety of processes in other cellular compartments via the detoxification of hydrogen peroxide (Smirnoff 1996; Asada 1999; Smirnoff and Wheeler 2000), thus age-dependent enhanced oxidation of this antioxidant indicates an overall enhanced production of ROS within the cell.

Age-induced oxidation of antioxidants was accompanied by reductions in the F_v/F_m ratio and increases in the extent of lipid peroxidation, which is indicative of increased inhibition of PSII and oxidation to lipids in the oldest plants. It is interesting to note that age-induced differences in these oxidative stress markers were only noticeable during spring and summer, when plants were exposed to periods of drought and high light stress, which can lead to excess energy in chloroplasts, and therefore to the formation of ROS by

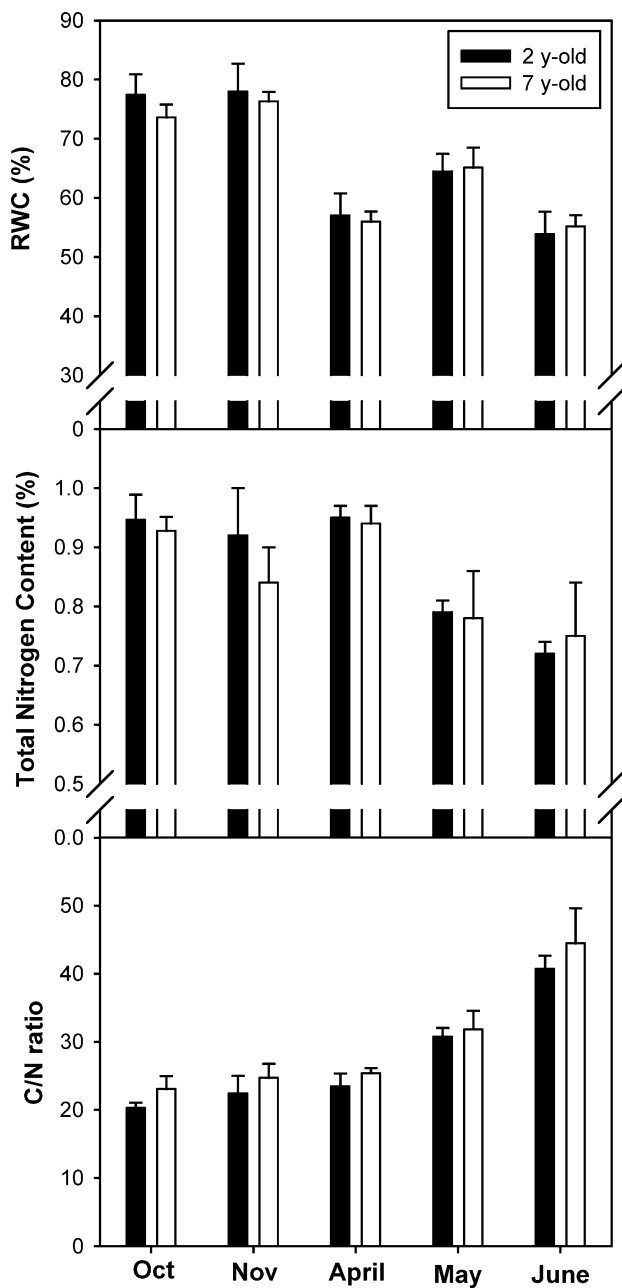


Fig. 6 Relative leaf water content (RWC), total N content and the C/N ratio in leaves of 2 (black bars) and 7-year-old (white bars) *C. clusii* plants grown under Mediterranean field conditions. Data correspond to the mean \pm SEM of eight measurements. No statistical differences ($P \leq 0.05$, Student's *t*-test) were observed between 2- and 7-year-old plants

increasing stomatal closure and consequently decreasing the activity of the Calvin cycle as a sink for ATP and reducing equivalents produced in photosynthetic electron transport. This contention is supported by the results, which demonstrate an enhanced accumulation of endogenous ABA levels in leaves as plants age. ABA functions primarily in plant responses to drought stress by inducing stomatal closure, but it also regulates

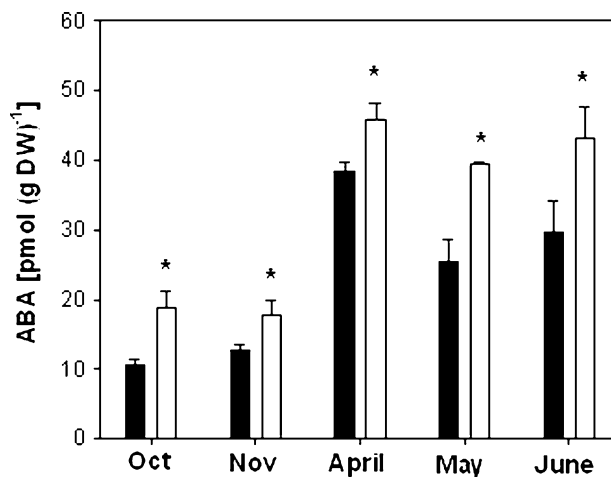


Fig. 7 ABA levels in leaves of 2- (black bars) and 7-year-old (white bars) *C. clusii* plants grown under Mediterranean field conditions. Data correspond to the mean \pm SE of four measurements. Statistical differences ($P \leq 0.05$, Student's *t*-test) between 2- and 7-year-old plants are indicated by an asterisk

the production of desiccation protectants and limits cell division and expansion (reviewed by Filkelstein et al. 2002; Himmelbach et al. 2003). Age-induced reductions in photosynthesis due to stomatal closure have already been reported in a previous study using the same species (Munné-Bosch and Alegre 2002a), as it has been reported in many other woody perennials (reviewed by Bond 2000). It is therefore likely that an age-related enhanced production of ABA levels in leaves favour the photo-oxidative stress associated with aging in plants.

What is, in turn, inducing an enhanced production of ABA in recently emerged leaves as plants age? Size is probably the determining factor responsible for an age-induced production of ABA in leaves. As plants age and increase in size, plants tend to increase their total biomass and leaf area. It is therefore essential that plants increase ABA levels to reduce water loss by transpiration. ABA reduces transpiration of the plant by inducing stomatal closure, but also by decreasing leaf area (through reductions in cell division and expansion), while maintaining leaf turgor. In the present study, RWC values were not affected by aging, while ABA levels increased, which supports this contention.

Although not statistically significant, 7-year-old plants showed slightly higher C/N ratios than 2-year-old plants throughout the experiment, thus indicating a possible feed back inhibition of photosynthesis by sugars. While sucrose contents per DW were similar in both plant groups, sucrose levels per unit of chlorophyll were higher in 7-year-old plants compared to 2-year-old plants throughout the study. Sucrose accumulation per

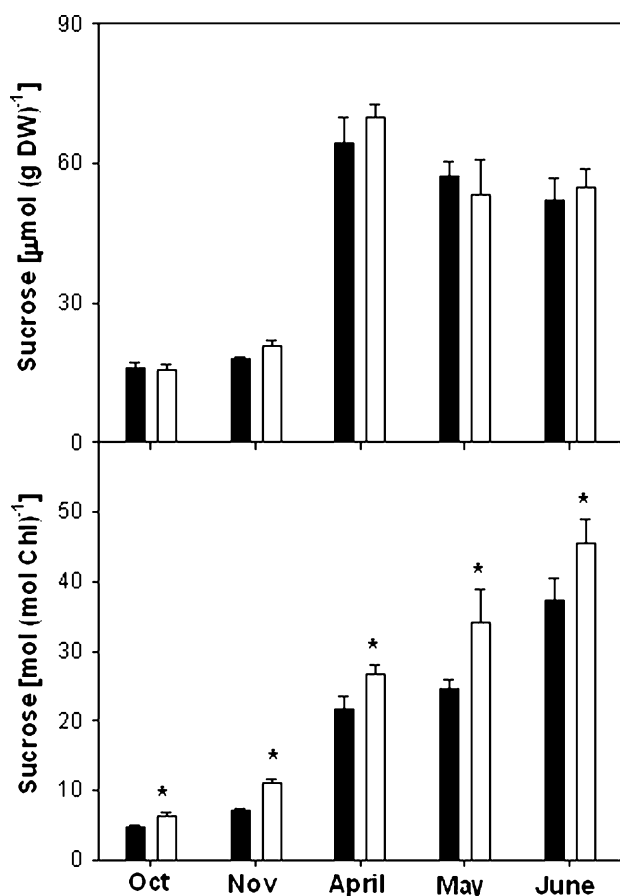


Fig. 8 Sucrose levels, given per DW and per chlorophyll unit, in leaves of 2- (black bars) and 7-year-old (white bars) *C. clusii* plants grown under Mediterranean field conditions. Data correspond to the mean \pm SE of four measurements. Statistical differences ($P \leq 0.05$, Student's *t*-test) between 2- and 7-year-old plants are indicated by an asterisk

photosynthetic unit appeared therefore to be higher in the oldest plants. Increased sucrose contents could indicate a higher general availability of sugars, including hexoses, although hexose contents were not determined in the present study. Sugars, in a process that appears to be hexose-dependent, repress the transcription of photosynthetic genes, thus leading to a feed back inhibition of photosynthesis (Sheen 1990; Wingler et al. 1998). It is therefore likely that age-induced reductions in photosynthesis are not only caused by ABA-induced stomatal closure, but also by a feed back inhibition of photosynthesis by sugars. Further research is however needed to support this contention.

In plants, chloroplasts are one the organelles most exposed to oxygen toxicity because they function at high oxygen and in the light (Halliwell and Gutteridge 1999) and it has been shown that they may be responsible for the oxidative stress associated with aging in plants (Munné-Bosch and Alegre 2002a). Furthermore, the metabolism of chloroplasts and mitochondria is inter-

connected and mitochondria play a crucial role in protecting chloroplasts from photoinhibition (Igamberdiev et al. 2001; Padmasree et al. 2002), thus we wondered about the relative contribution chloroplasts and mitochondria play in plant age-induced oxidative stress. Plant aging not only increased lipid peroxidation in chloroplasts, as previously shown (Munné-Bosch and Alegre 2002a), but also in mitochondria. Mitochondria showed between 1.8 and 6.8-fold higher MDA levels than chloroplasts in 7-year-old plants, thus it appears that age-induced oxidative stress in leaves during periods of environmental stress may result from an increased production of ROS not only within chloroplasts, but also within mitochondria. In fact, oxidation of lipids may also contribute to decreasing leaf growth rates during periods of drought as plants age. Moreover, we could see during the experiment that the leaves emerged in autumn senesced and dropped during the next summer in 7-year-old plants, but not in 2-year-old plants. This observation supports further the role of ABA and oxidative stress in the regulation of aging in perennials, since both ABA and oxidative stress are known to accelerate leaf senescence in water-stressed plants (Munné-Bosch et al. 2001; Yang et al. 2003).

First, it is concluded that leaves of oldest plants showed higher oxidative stress than those of young plants when exposed to adverse climatic conditions, thus supporting the idea that the oxidative stress associated with aging is due to extrinsic factors. Second, at the subcellular level, age-induced oxidative stress occurred both in chloroplasts and mitochondria, thus indicating that not only chloroplasts but also the overall cellular oxidative metabolism may be affected by plant aging. Third, meristematic tissues of *C. clusii* maintained the capacity to make new leaves with no symptoms of oxidative stress for several years, unless these leaves were exposed to environmental constraints. However, even in the absence of environmental stress, newly emerged leaves accumulated higher amounts of ABA as plants aged. Thus, it appears that intrinsic factors, such as plant size (and probably others), may also influence plant response to the environment by inducing ABA increases, thus modulating plant responses to extrinsic factors. Enhanced ABA levels may contribute to maintain leaf turgor by inducing stomatal closure and by reducing leaf area, but they may also accelerate leaf senescence, thus leading to a shortening of the lifespan of leaves as plants age.

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