RESEARCH PAPER

Hydrogen peroxide is involved in the acclimation of the Mediterranean shrub, *Cistus albidus* L., to summer drought

Tana Jubany-Mari*, Sergi Munne-Bosch, Marta López-Carbonell and Leonor Alegre

Departament de Biologia Vegetal, Facultat de Biologia, Universitat de Barcelona, Avinguda Diagonal 645, 08028 Barcelona, Spain

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Abstract

This study evaluated the possible role of hydrogen peroxide ($H_2O_2$) in the acclimation of a Mediterranean shrub, *Cistus albidus* L., to summer drought growing under Mediterranean field conditions. For this purpose, changes in $H_2O_2$ concentrations and localization throughout a year were analysed. $H_2O_2$ changes in response to environmental conditions in parallel with changes in abscisic acid (ABA) and oxidative stress markers, together with lignin accumulation, xylem and sclerenchyma differentiation, and leaf area were also investigated. During the summer drought, leaf $H_2O_2$ concentrations increased 11-fold, reaching values of $10 \mu mol g^{-1}$ dry weight (DW). This increase occurred mainly in mesophyll cell walls, xylem vessels, and sclerenchyma cells in the differentiation stage. An increase in ABA levels preceded that of $H_2O_2$, but both peaked at the same time in conditions of prolonged stress. *C. albidus* plants tolerated high concentrations of $H_2O_2$ because of its localization in the apoplast of mesophyll cells, xylem vessels, and in differentiating sclerenchyma cells. The increase in ABA, and consequently of $H_2O_2$, in plants subjected to drought stress might induce a 3.5-fold increase in ascorbic acid (AA), which maintained and even decreased its oxidative status, thus protecting plants from oxidative damage. After recovery from drought following late-summer and autumn rainfall, a decrease in ABA, $H_2O_2$, and AA to their basal levels ($\leq 60$ pmol $g^{-1}$ DW, $\leq 1 \mu mol g^{-1}$ DW, and $\leq 20 \mu mol g^{-1}$ DW) was observed.

Key words: Abscisic acid, ascorbate, ascorbate oxidative status, *Cistus albidus*, hydrogen peroxide, leaf plasticity, lignin, Mediterranean shrubs, oxidative markers, summer drought.

Introduction

Environmental stresses, both biotic and abiotic (Apel and Hirt., 2004), promote enhanced production of hydrogen peroxide ($H_2O_2$) and its consequent accumulation in plants. Abiotic stress factors known to increase $H_2O_2$ production include exposure to ozone (Oksanen et al., 2003; Chaparzadeh et al., 2004), drought (Kocsy et al., 2005), or, in general, when metabolic activity is limited by stress conditions. Most studies of $H_2O_2$ accumulation have been performed on plants growing in a growth chamber or have applied drought conditions to detached leaves (Yesbergenova et al., 2004). Few studies have been conducted on plants growing under natural climatic conditions (Munné-Bosch et al., 2001, 2003; Merquiol et al., 2002; Cheeseman, 2006). To elucidate the role of $H_2O_2$ in plant responses to a combination of stress factors, research is required on plants growing under their environmental conditions (Cheeseman, 2006; Mittler, 2006).

Chloroplasts, mitochondria, and peroxisomes are often included among the major sites of intracellular $H_2O_2$ production. However, under stress conditions much attention is focused on plasmalemma and apoplast compartments, in which increases in $H_2O_2$ could be associated with the activities of NADPH oxidases and peroxidases (Frahry and Schopfer, 1998; Bolwell et al., 2002; Bindscheler et al., 2006). The reactive oxygen species (ROS) $H_2O_2$ is a harmful cellular metabolite; however, it also serves as a signalling molecule that mediates responses to various stimuli in both plant and animal cells. Apart from its role in plant responses to abiotic and biotic stresses (Neill et al., 2002), this ROS is emerging as a key regulator of plant...
was any relationship established between drought, \( \text{H}_2\text{O}_2 \), and habitat diversity could be attributed to leaf plasticity (Gapper and Dolan, 2006; Kwak et al., 2006). Furthermore, \( \text{H}_2\text{O}_2 \) is involved in making cell walls stiff as growth ceases and cells differentiate (Hohl et al., 1995; Ros-Barceló et al., 2002; Ros-Barceló 2005). Plants tolerate much higher concentrations of endogenous \( \text{H}_2\text{O}_2 \) than other organisms (Queval et al., 2008). This increased tolerance could be attributed to the fact that accumulation preferentially occurs in the apoplast and that intracellular concentrations are much lower. Therefore, tissue localization is essential to determine the function of \( \text{H}_2\text{O}_2 \).

Abscisic acid (ABA) and \( \text{H}_2\text{O}_2 \) are produced and accumulated in plants under adverse environmental conditions such as drought, and are crucial in signalling adaptive responses, including stomatal closure (Pei et al., 2000; Zhang et al., 2001) and antioxidant defence (Jiang and Zhang, 2002; Hu et al., 2005, Zhang et al., 2006). In addition, \( \text{H}_2\text{O}_2 \) generated in response to ABA treatment was detected in the major veins of leaves. In mesophyll and bundle sheath cells, ABA-induced \( \text{H}_2\text{O}_2 \) was observed only in the apoplast, and the greatest accumulation occurred in the walls of mesophyll cells facing large intercellular spaces (Hu et al., 2005). Furthermore, ABA stimulates \( \text{H}_2\text{O}_2 \) production by NADPH oxidases (Kwak et al., 2003; Hu et al., 2005; Hirayama and Shinozaki, 2007).

The genus *Cistus* is a shrub, the dominant plant of ecosystems in the Mediterranean area (Correia et al., 1992). *Cistus* species are found in a wide range of habitats. Such habitat diversity could be attributed to leaf plasticity (Núñez-Olivera et al., 1996) and various aspects of growth that confer advantages under drought (Gibson, 1996). *Cistus albidus*, a semi-deciduous species (Grant and Incoll, 2005), responds to summer drought by gradually losing some of its older leaves, and showing vertically oriented (erectophilic) leaves. In previous studies, where cytochemical localization of \( \text{H}_2\text{O}_2 \) was carried out, \( \text{H}_2\text{O}_2 \) accumulation was reported in the apoplastic mesophyll cells of drought-stressed leaves of *C. clusii* and *C. albidus* (Munne-Bosch et al., 2003). However, quantitative analyses of total \( \text{H}_2\text{O}_2 \) in leaves throughout the year was not performed, nor was any relationship established between drought, \( \text{H}_2\text{O}_2 \), and ABA accumulation or drought-ABA-\( \text{H}_2\text{O}_2 \)-inducing antioxidant defence. Given that summer drought is the most characteristic aspect of the Mediterranean climate, in terms of its effect on vegetation, and that water resources are expected to become increasingly limited in the Mediterranean Basin as a result of climate change, the relationship between drought, ABA, \( \text{H}_2\text{O}_2 \), and oxidative status of a Mediterranean plant is of special interest.

Here, the endogenous \( \text{H}_2\text{O}_2 \) concentrations and intracellular localization over 1 year in the leaves of *C. albidus* plants growing under Mediterranean field conditions was studied, and the putative contribution of \( \text{H}_2\text{O}_2 \) to plant response to summer drought was examined. To this end, simultaneous measurements of plant water status, ABA, and \( \text{H}_2\text{O}_2 \) contents; ascorbate oxidative status and other oxidative stress markers such as the extent of lipid peroxidation and the \( \text{F}_{v}/\text{F}_{m} \) ratio; as well as leaf area and lignin levels were performed in the leaves of these plants.

**Materials and methods**

**Plant material and growth conditions**

Experiments were conducted on a Mediterranean shrub, *C. albidus* L., grown in the experimental fields of the Faculty of Biology at the University of Barcelona (Barcelona, NE Spain), as described previously by Munné Bosch et al. (2003). The experimental area had a calcic Luvisol soil (FAO) and 16 two-year-old *C. albidus* plants were uniformly distributed in a 16 m² square, 1 m apart so, all plants had the same orientation to the sun. Plants received water exclusively from rainfall throughout the experiment.

Environmental conditions were monitored throughout the study by means of a weather station situated 300 m from the experimental plot. The weather station consisted of a photon flux density (PFD) pyranometer sensor CM11 (Kipp and ZONEN, Delft, The Netherlands) and a HMP35AC thermohygrometer (Vaiśala, Finland). Vapour pressure deficit (VPD) was calculated from air temperature and relative humidity data, following Nobel (1991). Precipitation was measured with a standard rain gauge. Soil water content and temperature measurements were taken every hour from three ECHO sensors (Decagon Devices, Inc., Pullman, WA, USA), one measuring at a depth of 0–20 cm, another at 40–60 cm, and the last one measuring temperature at 0–20 cm.

During the study, plants were subjected to Mediterranean climate conditions. During the measurement days, plants were exposed to a maximum diurnal PFD ranging between 896 µmol m⁻² s⁻¹ (December) and 1902 µmol m⁻² s⁻¹ (July).

Air temperature (Tair) in June and July reached maximum values of 30 °C (Fig. 1), and was maintained higher than 27 °C from early morning (~7 am) to evening (~7 pm); minimum Tair values, usually registered before dawn (4–5 am), in these 2 months were of ~23 °C. In August and September, Tair max and min ranged from 26 °C and 20 °C, and decreased to a Tair max and a Tair min of 23 °C and 18 °C, respectively, in October. Lowest registered Tair measurements were attained in February (Tair max 7.8 °C, and Tair min 1.7 °C). Also maximum soil temperature values were observed from June to July, reaching values of 35 °C. Accumulated precipitation in winter was 160 mm and in an unusually dry spring was lower (53.4 mm). From the beginning of June to the end of July, precipitation was scarce (5.4 mm) even though rainfall was registered on August 1 and 2 (10.9 mm and 19.8 mm, respectively). Thereafter, rain was scarce (3.2 mm in total) until the beginning of September. In autumn, precipitation was more abundant (231.2 mm) over 2 months. Soil water content at a depth of 0–20 cm during winter and early spring oscillated between 5% and 12%, depending greatly on rainfall patterns, decreased from May to the end July (from
11% to 1.5%), and increased again to 13% after autumn rainfall. Soil water content at a depth of 40–60 cm followed a similar pattern to that recorded at a depth of 0–20 cm, but was more constant and higher than the superficial layer. After autumn rainfall, soil water content increased to a maximum of 24% in December, and decreased from April to August, reaching a minimum of 10%, to increase again in September and reach a maximum of 32% at the end of the experiment.

There were clear seasonal differences in VPD, which decreased from October 2004 to February 2005 (from 1.73 kPa to 0.57 kPa), increased from March to August...
(1.43, 1.95, and a maximum of 2.59 kPa in June) and decreased again after autumn rainfall to 1.43 kPa and 1 kPa in September and October 2005, respectively (Fig. 1).

Plant water status, \( \text{H}_2\text{O}_2 \), ABA content, ascorbic acid (AA), ascorbate oxidative status, lipid peroxidation, chlorophyll fluorescence, ultrastructural studies, leaf area, and lignin contents were analysed in leaves situated 5–15 cm from the apex. Leaves were collected at midday (at maximum diurnal incident PFD) on a clear sunny day every month from November 2004 to October 2005. For biochemical studies, samples were immediately frozen in liquid nitrogen and stored at \(-80 \)°C until analyses.

**Plant water status and leaf area**

Plant water status was determined by measuring relative water content (RWC) and hydration (H) of leaves at midday. The RWC (%) was determined as \( 100(\text{FW–DW})/ (\text{TW–DW}) \), and H (g \( \text{H}_2\text{O} \) g \( ^{-1} \) DW) as \((\text{FW–DW})/\text{DW}\); where FW is fresh mass, DW is dry mass after drying the sample at 80 °C to constant mass, and TW is turgid mass after re-hydrating the leaves for 24 h at 4 °C. Leaf area (A) was calculated using a flatbed scanner (Model GT-5000, Epson, Nagano, Japan) and an image-processing program (Leaf Area measurement 1.3, Askew, UK).

**ABA determination**

ABA measurements were performed as described by López-Carbonell and Jáuregui (2005). Briefly, leaves were ground with a pre-chilled mortar and pestle in liquid nitrogen, extracted with acetone/water/acetic acid (80:19:1, v/v/v) at \(-20 \) °C, and centrifuged at 40 000 \( g \) at 4 °C for 15 min. The supernatants were collected and the pellets were re-extracted twice with 3 ml each of the extraction solvent. The resulting supernatants were collected and the pellets were re-extracted from the apex. Leaves were collected at midday (at maximum diurnal incident PFD) on a clear sunny day every month from November 2004 to October 2005. For biochemical studies, samples were immediately frozen in liquid nitrogen and stored at \(-80 \)°C until analyses.

**\( \text{H}_2\text{O}_2 \) determination and cellular localization**

Extraction and quantitative analyses of \( \text{H}_2\text{O}_2 \) were done as described by Rao et al. (2000) and Orozco-Cárdenas and Ryan (2002), with some modifications. Leaves (100 mg) were ground with a pre-chilled mortar and pestle in liquid nitrogen and extracted with 0.2 M perchloric acid (11 ml) by ultrasonication (Vibra-Cell Ultrasonic Processor; Sonics & Materials Inc., Danbury, CT, USA). After centrifugation at 5000 \( g \) for 5 min at 4 °C, the pellet was re-extracted three times with the same solvent. The supernatants were pooled and a sample of 0.5 ml was applied to 2 ml columns of AG 1-X8 Resin 200–400 mesh chloride form, 0.8×4 cm (Bio-Rad Laboratories, Inc., Hercules, CA, USA) previously calibrated with \( \text{HClO}_4 \) and eluted with double-distilled water (3 ml). Acidic purified extracts were neutralized to pH 7 with 0.2 M \( \text{NH}_4\text{OH} \).

Quantification of \( \text{H}_2\text{O}_2 \) in cleared and neutralized extracts was carried out using an Amplex Red Hydrogen Peroxide Assay kit (Molecular Probes, Invitrogen Detection Technologies, Leiden, The Netherlands), following the manufacturer’s recommendations. Briefly, 50 \( \mu \)l of extract was mixed with 50 \( \mu \)l of solution containing 1 U \( \text{ml}^{-1} \) horseradish peroxidase in 50 mM sodium phosphate buffer, pH 7.4, and was incubated for 15 min at room temperature. Fluorescence was measured with a fluorescence microplate reader (Fluostar Optima, BMG Labtechnologies, Germany) equipped for excitation at 520 nm and emission at 590 nm. The concentration of \( \text{H}_2\text{O}_2 \) in each sample was calculated using a standard curve obtained with known concentrations of pure \( \text{H}_2\text{O}_2 \) (Molecular Probes) diluted in 0.2 M \( \text{HClO}_4 \).

Cytochemical localization of \( \text{H}_2\text{O}_2 \) was determined following Bestwick et al. (1997) and Munne-Bosch et al. (2001). Briefly, cross-sections, 1–2 mm wide, from the middle of fully expanded young leaves were vacuum infiltrated by a syringe for 30 min at 4 °C in freshly prepared 5 mM CeCl\(_3\) in 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS), at pH 6.5. Specimens were fixed under vacuum at 4 °C in a mixture of 2% (v/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 50 mM sodium cacodylate buffer, pH 7.2, overnight, and post-fixed with 1% (v/v) osmium tetroxide in the same buffer for 2 h at room temperature. Specimens were then dehydrated in a graded acetone series and embedded in Spurr’s resin. Ultrathin sections (50–60 nm) were double-stained with uranyl acetate and lead citrate. Electron micrographs were obtained with a transmission electron microscope (Jeol JEM 1010, Japan) at an accelerating voltage of 80 kV with a Bioscan 812 Gatan camera to obtain the pictures. For each sampling, an average of 95 randomly chosen cells was examined. Samples incubated in MOPS devoid of CeCl\(_3\) were used as negative controls.

**Determination of reduced and oxidized ascorbate**

AA and dehydroascorbate (DHA) contents in leaves were measured by an adaptation of the method described by Kampfenkel et al. (1995). In brief, leaves (100 mg) were
ground with a pre-chilled mortar and pestle in liquid nitrogen, and extracted with 6% (w/v) trichloroacetic acid (10 ml) by ultrasonication (Vibra-Cell Ultrasonic Processor; Sonics & Materials Inc.). After centrifugation at 10 000 g for 10 min at 4 °C, the pellet was re-extracted three times with the same solvent. The supernatants were pooled and a sample of 0.2 ml was mixed with 0.2 mM phosphate buffer pH 7.4 (0.6 ml), double-distilled water (0.2 ml), 10% (w/v) trichloroacetic acid (TCA; 1 ml), 42% (w/v), H₃PO₄ (0.8 ml), 4% (w/v) 2,2′-bipyridyl (0.8 ml), and 3% (w/v) FeCl₃ (0.8 ml), and incubated in a bath for 40 min at 42 °C. Total AA determination was carried out with a 15 min incubation of the extract at 42 °C with 10 mM dithiothreitol (DTT) in 0.2 mM phosphate buffer at pH 7.4 (0.2 ml). The reaction was stopped with 0.5% (w/v) N-ethylmaleimide (0.2 ml). DHA levels were estimated as DHA = (AA₅ – AA₆), where AA₅ is reduced plus oxidized ascorbate.

Estimation of lipid peroxidation

The extent of lipid peroxidation was estimated spectrophotometrically by measuring the amount of malondialdehyde (MDA) in leaves by the method described by Hodges et al. (1999), which takes into account the presence of compounds interfering in the thiobarbituric acid-reactive substances assay (TBARS). In short, leaves (100 mg) were ground in liquid nitrogen in a mortar and pestle and extracted three times with 80% (v/v) ethanol/water (8 ml final volume) by ultrasonication (Vibra-Cell Ultrasonic Processor). Supernatants were pooled and an aliquot (2 ml) was added to a test tube with 2 ml of either (i) –TBA solution comprised of 20% (w/v) TCA and 0.01% butylated hydroxytoluene, or (ii) +TBA solution containing the above plus 0.65% TBA. Samples were vortexed and heated at 95 °C for 25 min. They were then cooled and centrifuged at 5000 g for 10 min. Absorbance was read at 440, 532, and 600 nm.

\[ \text{MDA} = \frac{[(\text{Abs} \text{ 532} - \text{TBA}) - (\text{Abs} \text{ 600} + \text{TBA}) - (\text{Abs} \text{ 600} + \text{TBA})]}{[\text{Abs} \text{ 532} + \text{TBA}] - (\text{Abs} \text{ 600} + \text{TBA})]} \times 0.0571 \]

Chlorophyll fluorescence measurements

The maximum efficiency of photosystem II (PSII) photo-chemistry (Fᵥ/Fm) 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).

Lignin determination

Extraction and analyses of lignin were performed as described by Bruce and West (1989) and Hatzilazarou et al. (2006), with some modifications. Leaves (100 mg) were ground with a pre-chilled mortar and pestle in liquid nitrogen and homogenized with pure methanol by ultrasonication (Vibra-Cell Ultrasonic Processor) to remove all alcohol-soluble residues. Alcohol-insoluble residues were dried at 60 °C for 2 h. To 25 mg of dry alcohol-insoluble residues in screw-capped tubes, 3 ml of 2 N HCl and 300 μl of thioglycolic acid were added. Sealed tubes were placed in an aluminium block digestor for 4 h at 100 °C. After digestion, tubes were cooled and the contents were then transferred to glass tubes and centrifuged at 28 000 g for 15 min. The resulting pellet was washed once with water and resuspended in 4 ml of 0.5 N NaOH, sealed, and shaken gently overnight with a horizontal shaker at room temperature to extract lignin thioglycolates. The samples were then centrifuged at 25 000 g for 15 min, supernatants were collected, 1 ml of concentrated HCl was added, they were kept at 4 °C for 4 h, and centrifuged at 25 000 g during 15 min. The resulting brown pellets were re-suspended in 8 ml of 0.5 N NaOH, and absorbance was measured at 280 nm. The concentration of lignin in each sample was calculated using a standard curve obtained with known concentrations of commercial lignin alkali (Sigma) dissolved in 0.5 N NaOH.

Statistical analysis

Statistical procedures were done using SPSS for Windows v. 14.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to compare mean values at a range of sampling times for plant water status, H₂O₂ content, ABA, lignin, leaf area, Fᵥ/Fm, MDA, AA, DHA, and ascorbate oxidative status. The post hoc Duncan’s test was applied. Significance levels of 95% (P < 0.05) are indicated in the figure legends. At each sampling time, significantly different means are marked by different letters.

Results

Summer drought-induced ABA and H₂O₂ accumulation

During the experimental period, plants received water exclusively from rainfall and were subjected to Mediterranean environmental conditions. From October to December 2004 increased in plant RWC and hydration was observed as a result of the autumn rainfalls, reaching values of 80% for RWC and 1.53 g H₂O g⁻¹ DW for H in December (Fig. 2). The high values of plant hydration were concomitant with low values of endogenous ABA concentration (65.42 pmol g⁻¹ DW) and basal levels of H₂O₂ (0.96 μmol g⁻¹ DW). From December to March the RWC was maintained high at ~80%, showing that plants were not subjected to drought stress during this period. The scarce precipitations in April might explain the momentary increase in ABA observed during this month, but after rainfalls at the beginning of May RWC increased again. Even though they showed a slight but significant decrease, ABA levels were maintained at ~2-fold higher than those of well-hydrated plants.

From June and until the end of July, precipitation was scarce and RWC fell to its lowest value of 52% and H to 0.8 g H₂O g⁻¹ DW. Furthermore, the lowest levels of soil
water content were registered (2% between 0 cm and 20 cm and 10% between 40 cm and 60 cm deep) concomitant with the highest values of VPD (2.6 kPa) and PFD (1902 μmol m$^{-2}$ s$^{-1}$). Therefore, plants were subjected to drought during summer (Fig. 1). In June and coinciding with the onset of drought, ABA and H$_2$O$_2$ levels were 3-fold and 2-fold higher than those attained in well-hydrated plants, respectively (December). However, the highest and most significant levels of ABA and H$_2$O$_2$ were recorded in July, showing a peak of ~6-fold and 11-fold, respectively. These

Fig. 2. Variations in plant water status, ABA, and H$_2$O$_2$ in C. albidus plants. Top: relative water content (filled circles, RWC, %) and hydration (open circles, g H$_2$O g$^{-1}$ DW). Middle: endogenous ABA content (pmol g$^{-1}$ DW). Bottom: endogenous H$_2$O$_2$ content (μmol g$^{-1}$ DW). For the experiment, leaves situated at 5–15 cm from the apex were collected at midday (at maximum diurnal incident PFD) on a clear sunny day once a month. Values followed by the same letter in the same column are not significantly different at $P<0.05$ according to Duncan’s multiple range test. Data are the means ± SE, $n=6$. 

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results showed that drought, ABA, and \( \text{H}_2\text{O}_2 \) accumulation were related. Indeed, ABA accumulation preceded that of \( \text{H}_2\text{O}_2 \) (Fig. 2).

Rainfall at the beginning of August was reflected by a sharp decrease in ABA of 82\%, \( \text{H}_2\text{O}_2 \) started to decrease slowly (22\%), although this decrease was not significant. The measurements given in the figure were a particular point, and values represented a sort of average, but they could be indicative of a relationship between drought and ABA and \( \text{H}_2\text{O}_2 \) contents in \( C.\) \textit{albidus} plants (Fig. 2). Late summer and autumn rainfall led to an increase in plant and soil water status, which reached their highest levels (86\% and 1.96 g \( \text{H}_2\text{O} \) g\(^{-1}\) DW of RWC and H, respectively, and 30\% of soil water content between a depth of 40 cm and 60 cm). Plants showed complete recovery and endogenous ABA and \( \text{H}_2\text{O}_2 \) concentrations decreased to basal levels (<60 pmol g\(^{-1}\) DW and 0.6 \( \mu \)mol g\(^{-1}\) DW, respectively (Fig. 2). Thereafter, \( C.\) \textit{albidus} plants sensed changes in environmental conditions, in particular water deficit during the summer, and responded by altering endogenous ABA and \( \text{H}_2\text{O}_2 \) concentrations.

Oxidative stress markers

Given that drought induced \( \text{H}_2\text{O}_2 \) production, experiments were carried out to examine whether this increase in \( \text{H}_2\text{O}_2 \) was related to oxidative damage in leaves. For this purpose, AA, DHA and ascorbate oxidative status; and MDA and \( \text{H}_2\text{O}_2 \) accumulation were related. Indeed, ABA accumulation preceded that of \( \text{H}_2\text{O}_2 \) (Fig. 2). Given that drought induced \( \text{H}_2\text{O}_2 \) production, experiments were carried out to examine whether this increase in \( \text{H}_2\text{O}_2 \) was related to oxidative damage in leaves. For this purpose, AA, DHA and ascorbate oxidative status; and MDA and \( \text{H}_2\text{O}_2 \) accumulation were related. Indeed, ABA accumulation preceded that of \( \text{H}_2\text{O}_2 \) (Fig. 2).

\( \text{H}_2\text{O}_2 \) localization studies

When studying stress-induced changes in \( \text{H}_2\text{O}_2 \), it is important to note that total \( \text{H}_2\text{O}_2 \) levels alone do not provide information about the cellular compartmentalization of this ROS. This information is essential to determine the role of \( \text{H}_2\text{O}_2 \), if any, in plant response to stress. Thus, in order to elucidate this role, the dynamics of \( \text{H}_2\text{O}_2 \) localization in diverse cell compartments were evaluated.

- **Cells with low levels of \( \text{H}_2\text{O}_2 \)** (coinciding with non-stressed plants, e.g. December) showed no accumulation either at the cell wall or in chloroplasts (Fig. 4A). Likewise, spiral thickening of xylem vessels did not accumulate \( \text{H}_2\text{O}_2 \) (Fig. 4B).

- **During the summer months**, \( \text{H}_2\text{O}_2 \) was also found to be involved in sclerenchyma differentiation (Fig. 5). Patchy spots of cerium perhydroxides (Fig. 4C) occurred in mesophyll cell walls facing the intercellular spaces (Fig. 4E). Despite high levels of \( \text{H}_2\text{O}_2 \) in the apoplast, mesophyll cells showed intact structures and organelles.

- **Lignin accumulation and leaf area**

The results showed that the main accumulation of \( \text{H}_2\text{O}_2 \) was in cell walls, xylem, and sclerenchyma. Thus, this accumulation could be related to processes such as cell wall tightening or lignification in which \( \text{H}_2\text{O}_2 \) may play a relevant role (Ros-Barceló, 2005). To test one of the possible roles of \( \text{H}_2\text{O}_2 \) in cell walls, lignin concentrations were measured.

- **Lignin levels** were low (~16 mg lignin g\(^{-1}\) DW) during autumn and winter. From May to August, a significant increase (69\%) was observed, reaching values of ~27 g lignin g\(^{-1}\) DW. Afterwards, a sharp and significant decrease was observed, and in September and October the lignin values recorded were similar to those of well-watered plants (December) (Fig. 6). The variations in lignin content were related to xylem and sclerenchyma differentiation which are differentiated through leaf growth. The experiment covered two periods of plant development. At the beginning of the experiment (November) and until the beginning of spring, measured leaves were those corresponding to the vegetative stage. This stage was soon followed by reproductive development (first flowering structures appeared during March) in which leaves were
19% smaller and showed a different shape (data not shown). It should be mentioned that, interestingly, lignin content increased before the appearance of the small leaves and, inversely, lignin content decreased before the increased leaf growth. After flowering, and at the onset of summer drought, smaller leaves appeared with an area 2.5-fold lower than at the beginning of spring (March). After late summer and autumn rainfalls, plants showed larger (4-fold) leaves, in which lignin levels were 2-fold lower than in drought-stressed leaves (Fig. 6).
Discussion

The present study provides new evidence for the physiological significance of the ABA–H$_2$O$_2$–AA interaction in the response of Mediterranean shrubs to summer drought.

Summer drought changes leaf water relations, given here as RWC (%) and H. However, following release from drought after late summer and autumn rainfall, plants recovered, showing that they can cope with stress. In summer, RWC was ~50% and H ~0.8 g H$_2$O g$^{-1}$ DW, Fig. 4. Ultrastructural localization of H$_2$O$_2$ in leaf cells of *C. albidus* with CeCl$_3$ staining and transmission electron microscopy. (A) Mesophyll cells of well-watered plants (December) did not show any H$_2$O$_2$ accumulation (bar = 1 μm), and (B) xylem vessels in the same period also did not show any accumulation (bar = 5 μm). (C) Mesophyll cells at the onset of drought (June) showed faint and patchy spots located at the outer part the cell walls, facing intercellular spaces (bar = 2 μm). (D, F) Xylem vessel spiral thickenings and sclerenchyma showed continuous deposits of cerium hydroperoxides at the onset of drought and after 1 month of stress (July, bar = 2 μm). (E) Mesophyll cells in July showed continuous deposits of H$_2$O$_2$, also at the outer part of cell walls and facing intercellular spaces, as well as an intact ultrastructure (bar = 2 μm). Arrowheads show electron-dense deposits of cerium perhydroxides. Chl, chloroplast; Cw, cell wall; Sc, sclerenchyma; St, spiral thickening; Xv, xylem vessel.

Fig. 5. Involvement of H$_2$O$_2$ in sclerenchyma formation. (A) Cell with thick walls surrounded by hydrogen peroxide localized at the plasmalemma (bar = 1 μm). (B) Cell death: hydrogen peroxide is located at the disrupted plasmalemma, and surrounding unstructured chloroplasts (bar = 1 μm). (C) Differentiated sclerenchyma (bar = 2 μm). Cw, cell wall; Sc, sclerenchyma.
a reduction of 37% in RWC and 57% in H with respect to well-hydrated plants.

Water deficit triggers an increase of ABA in plants (Zhu, 2002; Nambara and Marion-Poll, 2005). This increase is important for physiological and molecular responses of plants to water deficit, with stomatal closure and modulation of the expression of gene networks being the most studied responses. Furthermore, ABA induces the accumulation of H$_2$O$_2$. The relationship between ABA and H$_2$O$_2$ has been shown in Arabidopsis and Vicia faba guard cells (Zhang et al., 2001; Kwak et al., 2003; Desikan et al., 2004; She et al., 2004; Ann et al., 2008; Neill et al., 2008). In addition, ABA-induced H$_2$O$_2$ accumulation has been demonstrated in detached maize leaves in which water stress was induced by polyethylene glycol (Jiang and Zhang, 2002) and ABA treatment (Hu et al., 2005), and in tobacco BY-2 suspension-cultured cells (Hao et al., 2008). However, there is a lack of knowledge of what occurs in ABA induction of H$_2$O$_2$ in plants growing under natural climatic conditions and subjected to variations in environmental parameters, including summer drought.

In studying the time course of H$_2$O$_2$ and ABA content it is necessary to consider that both are very sensitive to brief changes in environmental conditions, and that the general trend of the time course is the most representative of their response to environmental conditions. Under drought

**Fig. 6.** Lignin accumulation and leaf area in C. albidus plants. Fluctuation on lignin formation (mg g$^{-1}$ DW). Leaf area (cm$^2$). For the experiment, leaves from the first 15 cm from the apex were collected at midday (at maximum diurnal incident PFD) on a clear sunny day once a month. Values followed by the same letter in the same column are not significantly different at $P <0.05$ according to Duncan’s multiple range test. Data are the means ±SE, n=4.
conditions, both ABA and H$_2$O$_2$ increased to a peak in July, when plants had been subjected to drought conditions for a long period. However, the increase in ABA preceded that of H$_2$O$_2$ and the time course for each process was very different: the leaf ABA content started to increase in April in conjunction with a significant 30% decrease in both RWC and H. After a momentary decrease related to an increase in plant water relations ABA continued increasing as drought progressed. However, a 2-fold increase in H$_2$O$_2$ (significantly different from that of well-watered plants, according to Student’s t-test, $P=0.0062$) was observed later in June, when ABA values were 2-fold higher than in December. These results suggest that in $C.$ albidus, drought induced ABA accumulation, which in turn induced an increase in H$_2$O$_2$, although a threshold of ABA had to be reached to induce a peak in the endogenous H$_2$O$_2$ concentrations. The water deficit imposed on plants during summer drought affected ABA and consequently H$_2$O$_2$ content, although the influence of solar radiation cannot be discarded (Chaparzadeh et al., 2004; Verslues et al., 2007). The removal of the drought stress factor led to a reduction in both ABA and H$_2$O$_2$ levels, as previously described (Nambara and Marion-Poll, 2005; Selote and Khanna-Chopra, 2006), but at a different rate, the response of H$_2$O$_2$ levels being much slower than that of ABA.

Therefore, in plants growing under natural conditions, the increase in ABA originating due to summer drought can induce H$_2$O$_2$ accumulation even though it cannot be discerned from the experiment which environmental factor is the determinant for ABA and H$_2$O$_2$ increases. However, the experiment is a realistic scenario because plants growing in their natural habitat need to cope with a combination of different abiotic stress conditions, rather than one particular factor, and the majority of abiotic stress studies performed under controlled conditions do not reflect the conditions that occur in the field (Mittler, 2006).

ABA-induced H$_2$O$_2$ enhanced AA as well as other enzymatic and non-enzymatic antioxidant systems in maize seedlings (Jiang and Zhang, 2001, 2002; Zhang et al., 2006). Furthermore, monodehydroascorbate reductase has been shown to be induced in Arabidopsis thaliana mutants lacking both AA peroxidase and catalase (Rizhsky et al., 2002). During summer drought, AA increased ~3.5-fold. This significant increase in AA could be triggered by drought stress-induced ABA and H$_2$O$_2$ accumulation. As a result of the increase in AA, a decrease in ascorbate oxidative status was observed during summer drought. Therefore, it appears to be the drought–ABA–H$_2$O$_2$ interaction that protects $C.$ albidus shrubs from oxidative damage and allows plant survival under adverse conditions.

Under summer drought stress, H$_2$O$_2$ reached values of 10 μmol g$^{-1}$ DW. This is a high concentration that can trigger oxidative damage to plants when located inside the cell (Queval et al., 2008). However, despite such high concentrations, $C.$ albidus plants did not show any oxidative damage. According to the results obtained in ultrastructural studies, plants tolerated such high concentrations of H$_2$O$_2$ inside their cells because its production preferentially occurred in apoplast-related compartments: mesophyll cell walls, xylem, and sclerenchyma. It has been proposed that the primary H$_2$O$_2$ accumulation in mesophyll cells is apoplastic, increasing in the plasma membrane and organelles only under conditions associated with visible damage or enhanced lipid peroxidation rates (Oksanen et al., 2003; Ranieri et al., 2003; Cheeseman, 2006). It is likely that H$_2$O$_2$ accumulates at cell walls because the apoplastic compartment only contains 5–10% of the total AA in plant cells (Sanmartín et al., 2003).

Therefore, concentrations of H$_2$O$_2$ inside $C.$ albidus mesophyll cells were much lower (under the CeCl$_3$ detection limit) because they were better protected than the apoplast. Only differentiating sclerenchyma cells showed H$_2$O$_2$ accumulation from the cell wall into the plasma membrane and chloroplast. The accumulation of H$_2$O$_2$ in undifferentiated sclerenchyma cells would trigger the signalling pathway that leads to programmed cell death in plants (Desikan et al., 1998; Mac-Carrone et al., 2000), and therefore sclerenchyma differentiation. The increase in sclerenchyma confers mechanical resistance to leaves, thus contributing to plant acclimation to summer drought (Gibson, 1996).

Furthermore, in leaves treated with ABA, H$_2$O$_2$ was generated in the apoplast (Hung and Kao, 2004), and induced cell wall peroxidase activity in roots (Lin and Kao, 2001). Drought stress is responsible for the increase in cell wall lignification (Lee et al., 2007) in xylem vessels and sclerenchyma. However, in this study, the increase in lignin preceded summer drought, even though it was after the decrease in leaf water status registered in April, which coincided with a slight increase in H$_2$O$_2$, and a significant increase in ABA. The mechanisms of drought-induced lignification could be mediated by H$_2$O$_2$, which is used by cell wall peroxidases to polymerize cinnamyl alcohols into lignin (Ros-Barceló et al., 2002; Ros-Barceló, 2005; Vreeburg and Fry, 2005). However, comparing the increase in lignin accumulation with H$_2$O$_2$ values, the highest values for lignin were observed from May to August (~27 mg g$^{-1}$ DW), which preceded the peak of H$_2$O$_2$ that occurred in July (10 μmol g$^{-1}$ DW). If H$_2$O$_2$ is a signal molecule for lignin increase, it acts at low levels. It is plausible that the slight rise in H$_2$O$_2$ observed in April, together with the significant increases in ABA levels and changes in environmental factors such as light (April PFD, 1856 μmol m$^{-2}$ s$^{-1}$), functions as a signal for the onset of lignin accumulation.

Leaf plasticity is one of the most striking aspects of $C.$ albidus, which showed significant differences in leaf dimensions throughout the experiment. A 60% reduction in leaf area was recorded from June to August in parallel with an increase in ABA and H$_2$O$_2$. This was not a result of increased lignin biosynthesis, which may be related to xylem and sclerenchyma differentiation but not to area reduction. The effect of drought on leaf area of several species of Cistus has been described (Núñez-Olivera et al., 1996), but here it is suggested that the decrease in leaf area preceded summer drought and that it was due to plant phenology and environmental conditions such as increased light and
temperature. The leaves that were measured throughout summer were those that emerged after plant flowering. These leaves were smaller and did not grow during the summer. This reduction in leaf area contributes to subsequent acclimation of the plant to summer drought (Munné-Bosch et al., 2003) and complements the shedding of older leaves (Schur et al., 2000). After rainfall, the leaves resumed growth and large leaves were attained by autumn. Leaf plasticity contributes to acclimation of plants to environmental conditions to a large extent. Furthermore, the plasticity of C. albidus leaves has been described (Grant and Incoll, 2005), but no studies have addressed the mechanisms that regulate leaf reduction under drought conditions in this Mediterranean shrub.

In conclusion, the results showed that H₂O₂ is involved in the acclimation of C. albidus to summer drought. No studies have been carried out so far on the role of drought stress in inducing ABA and triggering H₂O₂ accumulation in plants subjected to a combination of stresses. The drought stress-ABA-H₂O₂ interaction can induce an increase in AA, maintaining and even decreasing the ascorbate oxidative status under summer drought conditions, and thereby protecting plants from oxidative damage. Therefore, C. albidus plants tolerate high concentrations of H₂O₂ because of its localization in the apoplasm of mesophyll cells, xylem vessels, and in differentiating sclerenchyma cells. Xylem and sclerenchyma lignification and differentiation together with a reduction in leaf area contributed to drought acclimation.

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