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Enhanced Formation of α-Tocopherol and Highly Oxidized Abietane Diterpenes in Water-Stressed Rosemary Plants[1]

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The lipid-soluble antioxidants α-tocopherol and carnosic acid were studied in field-grown rosemary (*Rosmarinus officinalis* L.) plants subjected to drought. During summer in the Mediterranean region, the predawn water potential decreased to −3 MPa and the relative water content to 42%, which caused a depletion of the maximum diurnal CO_2 assimilation rate by 80%. Meanwhile, the maximum efficiency of photosystem II photochemistry and the chlorophyll content of leaves remained unaltered, indicative of the absence of photooxidative damage. The concentration of α-tocopherol increased by 15-fold and that of carotenoids by approximately 26% in response to water stress. Enhanced formation of the highly oxidized abietane diterpenes isorosmanol (by 25%) and dimethyl isorosmanol (by 40%) was observed during the summer as result of the oxidation of carnosic acid, which decreased by 22%. The large amounts of carnosic acid, α-tocopherol, and carotenoids present in rosemary leaves might contribute to the prevention of oxidative damage in plants exposed to drought.

Plants grown in Mediterranean field conditions cope with the interaction of several stresses, especially during the summer when water deficit, high light, and high temperature limit CO_2 fixation (Munné-Bosch and Alegre, 1999). Under such environmental stress conditions, limitations of carbon assimilation within the plant cell result in exposure to excess excitation energy. Although dissipation of excitation energy in the photosystem II (PSII) antennae by non-radiative decay processes and other electron sinks such as nitrogen metabolism and oxygen reduction via photorespiration confer photoprotection to some extent, it has been suggested that electron flux to oxygen via the Mehler reaction increases (Fryer et al., 1998). This results in the formation of activated oxygen species, which can lead to chlorophyll degradation, photodamage, and lipid photooxidation (Smirnoff, 1993; Foyer et al., 1994a; Asada, 1996; Osmond et al., 1997).

To cope with water stress and to avoid photooxidative damage, plants have evolved a series of enzymatic and nonenzymatic antioxidant systems. From the latter, tocopherols and carotenes protect lipid membranes from oxidative stress because they deactivate singlet oxygen, reduce superoxide radicals, and terminate lipid peroxidation by reducing fatty acyl peroxy radicals (Burton and Ingold, 1984; Fryer, 1992; Polle and Rennenberg, 1994). Although α-tocopherol is found throughout the plant kingdom, abietane diterpenes such as carnosic acid, methoxycarnosic acid, carnosol, rosmanol, and isorosmanol have only been found in the genus *Salvia* and in rosemary (*Rosmarinus officinalis* L.). Carnosic acid and carnosol are present in large amounts within the plant and possess the highest antioxidant activity of the abietane diterpenes (Nakatani, 1992; Schwarz and Ternes, 1992). It has been demonstrated that these abietane diterpenes are able to inhibit lipid peroxidation and superoxide generation in isolated chloroplasts and microsomes, protecting biological membranes against chemically induced oxidative stresses (Haraguchi et al., 1995; Haraguchi, 1998).

Some authors have proposed an in vivo oxidative pathway for carnosic acid in which enzymatic dehydrogenation and activated oxygen play a key role (Luis et., 1994b; González et al., 1995). In these reactions, formation of highly oxidized abietane diterpenes such as rosmanol, isorosmanol, and dimethyl isorosmanol occurs after the oxidation of carnosic acid (Fig. 1). In contrast to what happens with α-tocopherol (Winston, 1990; Kruk and Strzalka, 1995), carnosic acid is not regenerated once it is oxidized (González et al., 1995).

The aim of this work was to study the effects of drought on photosynthesis and the role of the antioxidants α-tocopherol and abietane diterpenes in drought-induced oxidative stress in rosemary plants growing in Mediterranean field conditions.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Cuttings of rosemary (*Rosmarinus officinalis* L.) were rooted and grown in 0.5-L pots containing a mixture of soil:peat:perlite (1:1:1, v/v). The plants were maintained in a greenhouse with controlled temperature (24°C/18°C, day/night) and watered twice a week, once with tap water and once with Hoagland's solution. After 1 year of growth, plants of the same height (35 cm) were transplanted to the experimental fields of the University of Barcelona (Spain).

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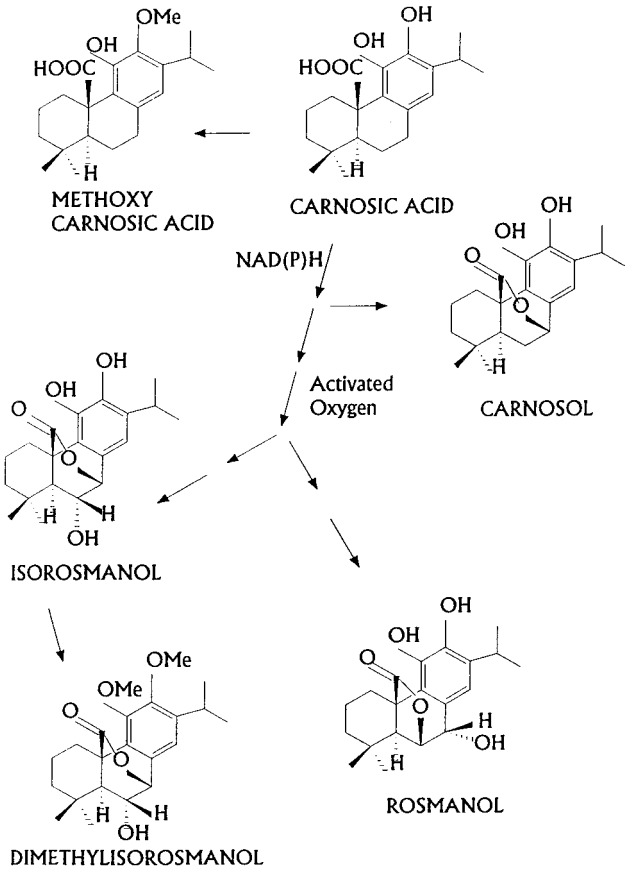

Figure 1. Formation of abietane diterpenes in rosemary plants. The highly oxidized abietane diterpenes rosmanol, isorosmanol, and dimethyl isorosmanol are formed from carnosic acid by enzymatic dehydrogenation and the participation of activated oxygen (adapted from Luis et al., 1994b).

Before the plants were transferred, the soil was treated with N:P:K (1:1:1, nutrient ratio) fertilizer at the rate of 100 kg N ha^{-1}. Plants were transplanted on April 10, 1996, and until October 1996 were watered twice a week with 15 mm of water. From November 1996 to August 1997 plants received only natural rainfall. Sixteen plants of approximately the same size were chosen for this study. The experiment was carried out from April 1997 to August 1997, and during this period 5 sunny days were selected for the measurements (April 30, May 28, July 1, July 26, and August 22).

Environmental conditions were monitored by a weather station (Delta-T Devices, Newmarket, UK). Measurements of photosynthetic photon flux density, air temperature, and relative humidity were taken at 1-min intervals, and 5-min means were logged. The photosynthetic photon flux density was measured with a sensor (Quantum Sensor, LI-COR, Lincoln, NE), air temperature and relative humidity were measured with a thermocouple (Vaisala, Helsinki), and the precipitation (in millimeters) was measured with a standard rain gauge. The vapor pressure deficit was determined from relative humidity and air temperature data according to the method of Nobel (1991).

Abietane Diterpenes and α-Tocopherol Determination

Leaves were collected at predawn and midday over the 5-d period, immediately frozen in liquid nitrogen, and stored at −80°C until analysis. Leaves were freeze-dried, and after grinding, leaf samples (1 g) were extracted with 5 mL of methanol containing 5 mg of citric acid and isoascorbic acid per 100 mL and sonicated for 20 s in a sonicator (Sonoplus HD 200, Bandelin, Berlin) equipped with a probe (MS 73, Bandelin). The extract was centrifuged at 1,500*g* for 3 min at 3°C, and the supernatant was transferred into a volumetric flask. The extraction procedure was repeated four times with 5 mL of methanol each time. The collected supernatants were put through a 110-μm-pore-size cellulose nitrate filter (Schleicher & Schuell, Dassel, Germany). For the measurement of abietane diterpenes, the supernatant was resuspended in an appropriate volume of methanol (1:10, v/v) prior to injection. For α-tocopherol determination the extract was passed through vacuum distillation to eliminate the methanol, de-gassed with nitrogen, and stored at −20°C. Prior to injection, the extract was dissolved in 4 mL of acetonitrile and centrifuged at 1,500*g* for 3 min at 3°C.

For analysis of α-tocopherol and abietane diterpenes a HPLC method similar to that described by Schwarz and Ternes (1992) was used. α-Tocopherol was separated at room temperature on a 5-μm column (250 × 4 mm; ODS Hypersil, Knauer, Berlin) using acetonitrile:distilled water:2 M citric acid (98:2:0.2, v/v) as an eluant at a flow rate of 1.1 mL min^{-1}. UV detection was carried out at 295 nm (Spectralphotometer, Knauer) and fluorescence detection was carried out at an excitation wavelength of 295 nm and emission at 340 nm (FS 970, Kratos, Ramsey, NJ). One-hundred microliters of sample was injected, and duplicates were run for each extract. α-Tocopherol (98.4% purity, Merck, Rahway, NJ) was used for calibration. Diterpenes were separated on the 5-μm column for 52 min at a flow rate of 0.6 mL min^{-1}. Eluant A consisted of acetonitrile: distilled water:2 M citric acid (51:49:0.83, v/v) and eluant B was acetonitrile:distilled water:2 M citric acid (97:3:0.5, v/v). The UV detection was carried out at 230 nm and the electrochemical detection at +800 mV with a range of 10 nA (model 656/641 VA, Metrohm, Filderstadt, Germany). The injection volume was 20 μL and duplicates were run for each extract. Carnosic acid (98% purity) was used for calibration. All abietane diterpenes were quantified relative to carnosic acid at 230 nm, because the UV spectra of other abietane diterpenes are similar to that of carnosic acid.

Physiological Parameters

The water potential of 10-cm apical shoots was measured using a Scholander-type pressure chamber (ARIMAD-2, ARI Far Charuv-Water Supply Accessories, Ramat Haqolan, Israel). The relative leaf water content was determined as RWC (%) = (fresh weight − dry weight)/(turgid weight − dry weight) × 100. Diurnal cycles of CO_2 assimilation rates were performed using a portable measuring system (model 6200, LI-COR) in the field. Net CO_2 assimilation rates were calculated from changes in the gas con-

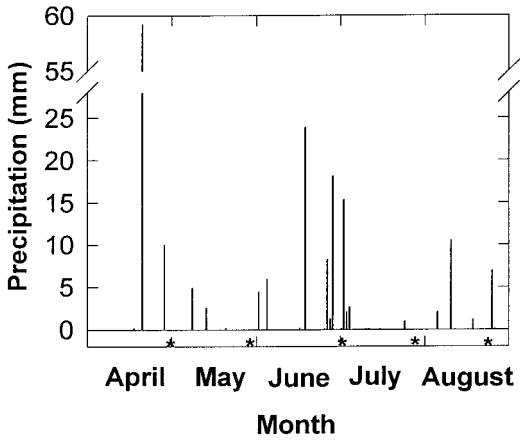

Figure 2. Rainfall pattern at the experimental fields of the University of Barcelona during the period of study. Measurement days are indicated by asterisks.

centration over a 20-s period using the equations developed by von Caemmerer and Farquhar (1981). Six measurements were made at 1.5-h intervals from predawn to sunset. Steady-state modulated chlorophyll fluorescence of leaves was measured using a portable fluorimeter (mini-PAM, Walz, Effeltrich, Germany).

The maximum and relative quantum efficiency of PSII photochemistry (ϕ_{PSII}) was estimated according to the method of Genty et al. (1989) as: $\phi_{PSII} = (F_m' - F_s)/F_m'$ and $F_v/F_m = (F_m - F_o)/F_m$ respectively, where F_m and F_m' are the maximum fluorescence yields obtained in the dark- and light-adapted state, respectively, F_s is the fluorescence yield at steady-state photosynthesis, F_v is the variable fluorescence yield, and F_o is the basal fluorescence yield obtained in the dark-adapted state. Chlorophyll $a + b$ and the total carotenoid content of leaves were determined spectrophotometrically in 80% (v/v) acetone extracts using the equations described by Lichtenthaler (1987).

RESULTS

Environmental Conditions

The pattern of precipitation during the measurement period is shown in Figure 2. It was characterized by two rainfalls of 59 and 10 mm just before the experiment began, followed by a very dry period from the beginning of May until the end of August, disturbed only by some rainfalls concentrated at the end of June and beginning of July. During the period of the study, the maximum photosynthetic photon flux density moved around 1,800 μmol m^{-2} s^{-1}, midday air temperature increased from 19°C to 30°C as the season progressed, and the vapor pressure deficit was very high on April 30 and May 28 at midday, with values around 4 kPa (Table I).

Plant Water Status and Photosynthesis

Rosemary plants grown in natural field conditions were subjected to different degrees of water stress during the spring and summer of 1997. After the major rainfalls of April, plants had a water potential of −0.3 MPa and a RWC of 80% at predawn (Table I, April 30). As water stress progressed during May, the water potential decreased to −2.14 MPa and RWC to 61% (May 28). Both parameters recovered again to similar pre-drought values after the rainfalls of the end of June (July 1). Nevertheless, there was a second dry period during July and August, which decreased the predawn water potential values from −0.3 to −3 MPa and the predawn RWC values from 75% to 42%, indicating severe stress (Table I, July 26 and August 22).

Maximum diurnal CO_2 assimilation rates moved from 10 to 2 μmol m^{-2} s^{-1} during this period, depending on plant water status (Table I). Summer drought caused a depletion of 80% in the maximum diurnal CO_2 assimilation rate and an almost complete depletion of photosynthesis at midday. Rosemary plants displayed midday depression of photosynthesis during the whole period of the study (except on July 1) and this was not only associated with improved plant water status but also with low vapor pressure deficit during that day (Table I).

Despite the large depletion of photosynthesis at midday in water-stressed plants, the ϕ_{PSII} was maintained unaltered (ANOVA, $P < 0.05$ probability level) during the whole period of study, with midday ϕ_{PSII} values around 0.30 (Table I). The maximum efficiency of PSII photochemistry and the chlorophyll content of the leaves remained significantly unaltered (ANOVA, $P < 0.05$ probability level) throughout the experiment at around 0.78 and 30 μg cm^{-2}, respectively (Fig. 3, A and B). The total carotenoid content of the leaves increased by 26% at midday, when RWC values below 50% were reached (Fig. 3C).

Table I. *Environmental conditions, water relations, and photosynthetic parameters of rosemary plants grown in Mediterranean field conditions*

Values of photosynthetically active photon flux density (PPFD), vapor pressure deficit (VPD), and air temperature (T) at midday are given. Water potential (ψ) and RWC correspond to predawn values. A_{max} values correspond to measurements taken in the morning, and A and ϕ_{PSII} are for measurements made at midday. Data are the means ± SE of six independent replicates.

Day	PPFD	VPD	T	ψ	RWC	A_{max}	A	ϕ_{PSII}
	μmol m^{-2} s^{-1}	KPa	°C	MPa	%		μmol m^{-2} s^{-1}	
30 April	1,783	3.8	19.0	−0.29 ± 0.07	80.18 ± 1.90	10.06 ± 0.61	5.72 ± 0.67	0.26 ± 0.03
28 May	1,885	4.4	24.6	−2.14 ± 0.14	61.45 ± 3.63	3.37 ± 0.23	1.60 ± 0.16	0.27 ± 0.06
1 July	1,816	1.6	21.3	−0.32 ± 0.07	75.35 ± 2.50	9.98 ± 0.72	8.62 ± 1.07	0.34 ± 0.04
26 July	1,884	2.7	26.3	−3.00 ± 0.12	43.48 ± 0.99	2.63 ± 0.28	0.85 ± 0.10	0.33 ± 0.05
22 August	1,654	2.8	30.6	−3.10 ± 0.08	42.67 ± 2.16	1.50 ± 0.15	0.21 ± 0.07	0.32 ± 0.04

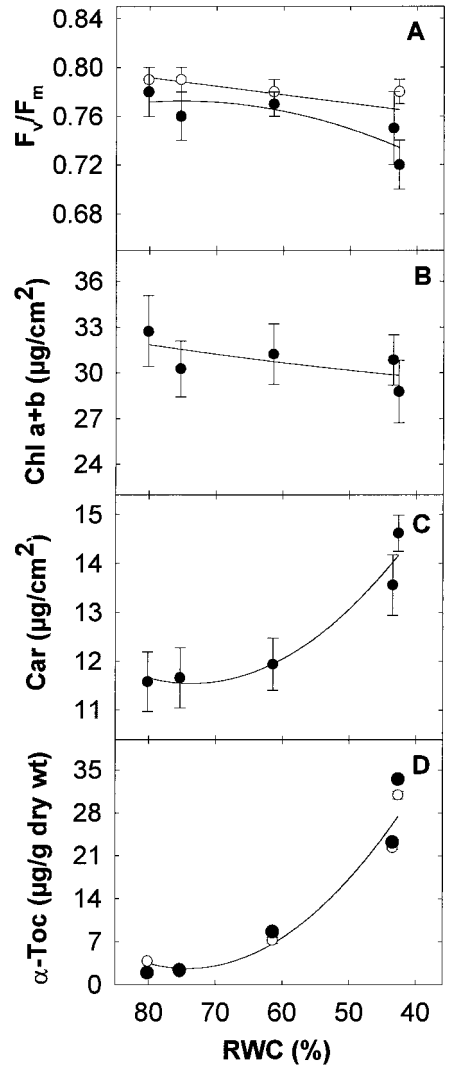

Figure 3. Relationship between the RWC at predawn and the ratio of variable to maximum fluorescence (F_v/F_m) (A), chlorophyll $a + b$ (B), carotenoid (C), and α-tocopherol (D) content in rosemary plants. Values at predawn (○) and midday (●) are given. Data are the means of six replicates ± SE.

α-Tocopherol and Drought

Figure 3D shows the relationship between the RWC and the α-tocopherol content of leaves. Rosemary plants showed a concentration ranging from 2 to 35 μg of α-tocopherol g^{-1} dry weight depending on plant water status but irrespective of time of day, and a 15-fold increase in the α-tocopherol content of leaves, when predawn RWC values below 50% were reached on July 26 and August 22. Although the α-tocopherol content of leaves was highly sensitive to RWC decreases imposed by drought, no significant differences were observed between predawn and midday during any of the measurement days.

Abietane Diterpenes and Drought

The relationship between the RWC and the abietane diterpene concentration in plants at predawn and midday is shown in Figure 4. Carnosic acid, the main constituent of abietane diterpenes with a concentration ranging from 2.8 to 4.2 mg g^{-1} dry weight, followed a completely different pattern from that observed for α-tocopherol in response to water stress. Figure 4A shows that carnosic acid decreased both in response to water stress and during the day. The concentration of carnosic acid at predawn had a strong correlation (r^2 =0.97) with RWC decreases in plants, decreasing by 22% when the RWC reached values below 50%. The concentration of carnosic acid also decreased by 12% during the day, although the differences between predawn and midday were smaller when RWC values below 50% were reached.

The abietane diterpenes carnosol (Fig. 4B), present in concentrations ranging from 0.5 to 1.3 mg g^{-1} dry weight, and methoxycarnosic acid (Fig. 4C), present in concentrations ranging from 0.3 to 0.5 mg g^{-1} dry weight, followed a trend similar to that observed for carnosic acid. In contrast, the highly oxidized abietane diterpenes isorosmanol (Fig. 4D) and dimethyl isorosmanol (Fig. 4E), present in concentrations ranging from 0.28 to 0.36 mg g^{-1} dry weight and from 0.50 to 0.72 mg g^{-1} dry weight, respectively, followed a different trend. The differences during the day disappeared in water-stressed plants for these compounds and the concentrations increased at midday by 25% and 40%, respectively, in response to water stress. Rosmanol, another highly oxidized abietane diterpene, was also found in rosemary leaves at low concentrations ranging from 0.10 to 0.12 mg g^{-1} dry weight throughout the experiment.

DISCUSSION

Rosemary plants maintained constant midday ϕ_{PSII} values throughout the experiment, whereas the net CO_2 assimilation rates decreased by 80% when RWC values below 50% were attained. Plants suffered from severe water stress during the summer, but the maintenance of the chlorophyll content of leaves and the constant ratio of variable to maximum fluorescence throughout the experiment show that the photosynthetic machinery was unlikely to be damaged by dehydration (Cornic and Massacci, 1996). Efficient means for protection from photoinhibitory damage may be provided by the thermal dissipation of excess excitation energy, photorespiration that may serve as a sink for the consumption of excess reducing equivalents, and scavenger systems for the removal of reactive oxygen species (Asada, 1994; Foyer et al., 1994b).

A decrease in carnosic acid and carnosol concentration, the most active antioxidants and abundant abietane diterpene compounds, was observed under drought conditions. The concentration of these abietane diterpenes also decreased during the day. By contrast, the concentration of the highly oxidized abietane diterpenes isorosmanol and dimethyl isorosmanol increased in response to water stress, and the differences between the concentrations at predawn and midday completely disappeared in water-stressed plants for these compounds, indicating that the formation of highly oxidized abietane diterpenes increased when plants were subjected to the interaction of water

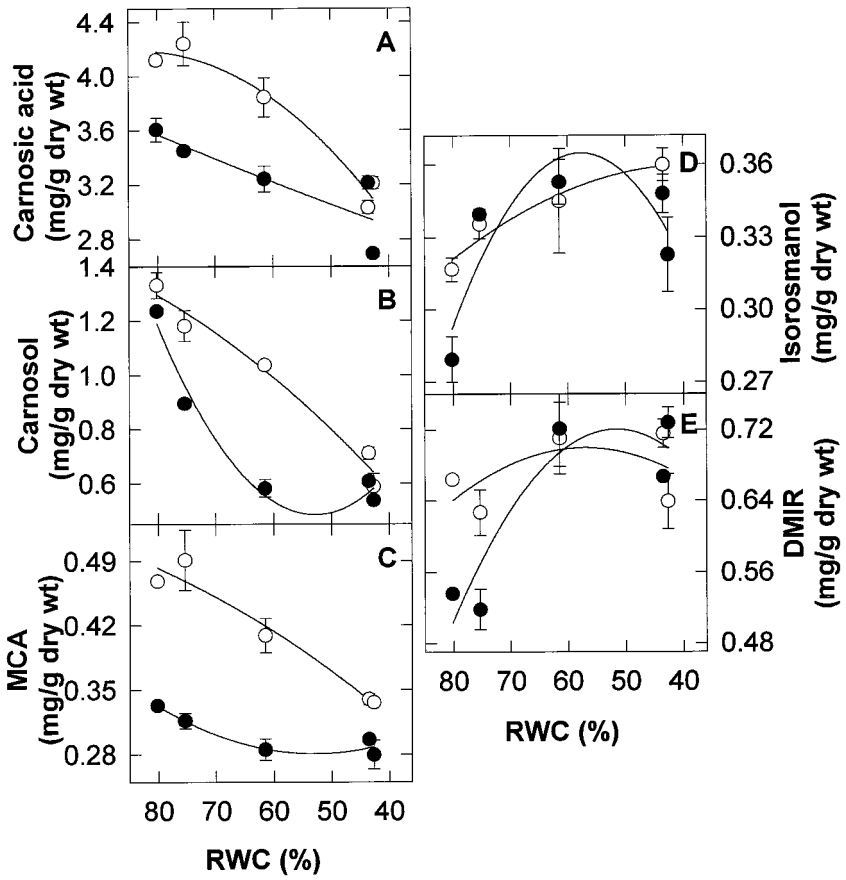

Figure 4. Relationship between the RWC at predawn and the abietane diterpene content in rosemary plants. A, B, and C show decreases in the concentration of the abietane diterpenes carnosic acid, carnosol, and methoxycarnosic acid in response to water stress and during the day. D and E show enhanced formation of the highly oxidized abietane diterpenes isorosmanol and dimethyl isorosmanol in response to water stress. This behavior is observed very clearly in water-stressed plants at midday. Values at predawn (○) and midday (●) are given. DMIR, Dimethyl isorosmanol. Data are the means of four replicates ± SE.

stress and high light during Mediterranean summer. Carnosic acid gives rise to highly oxidized diterpenes such as isorosmanol, dimethyl isorosmanol, and other related compounds by enzymatic dehydrogenation and scavenging of activated oxygen (Luis et al., 1994a; Luis et al., 1994b; González et al., 1995). Therefore, the enhanced formation of highly oxidized abietane diterpenes was most likely to be caused by the antioxidant activity of carnosic acid when plants were subjected to water stress and high light during Mediterranean summer, when photosynthesis was limited and activated oxygen formation may have increased. The decrease observed in the concentration of carnosic acid during the summer was therefore due to its consumption during the summer drought, as well as to the lack of regeneration once oxidized (Luis et al., 1994b; González et al., 1995). Levinsohn et al. (1993) and McGarvey and Croteau (1995) demonstrated that the diterpene cyclase activity decreases in coniferous plants in response to light and water stresses. Thus, the possibility that part of the decrease observed might also be caused by a decrease in the synthesis of carnosic acid should not be ruled out. Although the concentration of carnosic acid decreased during the summer, the large amounts found in rosemary leaves might allow continuous antioxidant activity within the plant cell.

The 15-fold increases in α-tocopherol observed in water-stressed plants might prevent chlorophyll photooxidation, as has been shown previously (Wise and Naylor, 1987; Simontacchi et al., 1993). No differences were observed in the α-tocopherol content of leaves between predawn and midday throughout the experiment, suggesting that although the α-tocopherol concentration of leaves increased in response to water stress, this species was able to efficiently regenerate α-tocopherol during the day. The increase observed in the carotenoid content of leaves could also contribute to prevent chlorophyll degradation and photodamage in rosemary plants.

In summary, our results prove that enhanced formation of highly oxidized abietane diterpenes occurred in rosemary plants as a result of the antioxidant activity of carnosic acid when photosynthesis was limited and activated oxygen formation may have increased. The increases in α-tocopherol and carotenoid concentration, together with the large amounts of carnosic acid found in rosemary leaves, contribute to help the plant withstand water stress and high light during Mediterranean summer.

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