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Enhanced Carnosic Acid Levels in Two Rosemary Accessions Exposed to Cold Stress Conditions

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Two rosemary accessions were subjected to chilling temperatures in control environmental cabins analyzing their variations in rosmarinic and carnosic acids together with their adaptability to these stress conditions. Cold stressed plants of both accessions showed increases in caffeic acid and carnosic acid concentration levels, while other secondary metabolites such as rosmarinic acid, naringin, cirsimaritin, hispidulin, and carnosol showed different responses in both accessions. In addition, cold stressed plants exhibited significant reductions in chlorophylls, β-carotene, and violaxanthin levels as well as the maximum quantum yield of PSII in both accessions. Hydrogen peroxide and lipid peroxidation levels showed similar responses in both accessions, which were positively and negatively correlated with rosmarinic and carnosic acids. From these results it is therefore suggested that carnosic acid biosynthesis in rosemary plants is induced by chilling periods. On the other hand, we demonstrate that not all rosemary accessions are equally well adapted to chilling temperatures. In fact, for (one) accession cold treated plants severe losses in chlorophyll, β-carotene, and even xanthophylls (including zeaxanthin and antheraxanthin) were observed, despite no visual symptoms of leaf injury. More research is needed to understand rosmarinic acid variations in rosemary plants under stress conditions.

KEYWORDS: Antioxidants; rosmarinic acid; carnosic acid; stress tolerance; photoinhibition; xanthophylls; chlorophylls

INTRODUCTION

It is widely demonstrated that herbs extracts have high antioxidant capacity becauase of the presence of phenolic type compounds when tested in vitro (*1–3*). For a long time rosemary has been an economically important herb for cosmetic companies, and nowadays the food and pharmaceutical industries are also interested in rosemary secondary metabolites, such as rosmarinic acid, carnosic acid, and carnosol, with demonstrated antioxidant capacities (*1–4*). In fact, food products with supplementary rosemary phenolic antioxidants have longer shelf life because of the inhibition of oxidative reactions (*5*). This is why several reports have been published analyzing not only the distribution of rosmarinic and/or carnosic acids during growth and vegetative development of rosemary plants but also their variability between different species and accessions (*2, 3, 6–8*). Additionally, several factors may have an influence on the concentration levels of these secondary metabolites during the collection, processing, and storage of rosemary plant material. However, not much attention has been paid to the effects of abiotic stress conditions on rosemary antioxidant compounds. Only a recent report demonstrates that carnosic acid variation levels in rosemary leaves during Mediterranean summers are due to its oxidation to carnosol, rosmanol, and isorosmanol, thus protecting the chloroplasts from oxidative damage (*9*).

Rosemary plants growing under southern U.K. weather conditions must cope with chilling temperatures during the autumn and winter months. Our previous studies analyzing the seasonal variations of rosmarinic acid carnosic acids showed an increase in their concentration during these months (*3*). These nonfreezing temperatures (0–12 °C) are common during the U.K. growing season and can substantially compromise plant productivity. Several reports described how chilling temperatures can disrupt all major components of photosynthesis including thylakoid electron transport and the carbon reduction cycle, as well as causing clear changes in secondary metabolites biosynthesis (*10–12*).

To address whether rosemary secondary metabolite variations are temperature dependent, two rosemary accessions with different carnosic acid contents were subjected to an imposed low temperature stress in environmental control cabins, simulating typical chilling periods of southern U.K. weather conditions. In addition, we will characterize the effects of cold stress on photosynthetic pigments, the maximum quantum yield of photosystem II (PSII) expressed as Fv/Fm (Fv, variable fluorescence; Fm, maximum fluorescence), the hydrogen peroxide content, and the lipid peroxidation process as malondialdehyde (MDA) to study their adaptation to these stress conditions.

MATERIAL AND METHODS

Chemicals. All solvents used in the experiments were HPLC grade and were purchased from Fisher Scientific (U.K.). The standard caffeic

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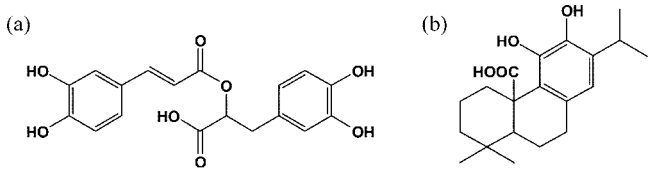

Figure 1. Rosmarinic acid (**a**) and carnosic acid (**b**) structures.

acid, trichloroacetic acid (TCA), potassium iodide (KI), hydrogen peroxide (H_2O_2), and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich Company Ltd. (U.K.). Rosmarinic acid and carnosic acids (**Figure 1**) were purchased from ICN Pharmaceuticals, Ltd. (U.K.), or obtained from the National Herb Centre (Banbury, U.K.). All other standards were obtained from the Phytochemistry Laboratory Department of Botany at The University of Reading.

Plants and Stress Treatments. Rosemary plants (cultivar Sissinghurst English, accessions 22 and 15) were selected and clonally propagated at the National Herb Centre (Banbury, U.K.). All plants were grown in pots of 2 L capacity with a mixture of soil/peat/sand (1:1:1, v/v) for 12 months before being used in any experiment. Both accessions were selected by their carnosic acid foliar concentration levels. No morphological or growth differences were found between the accessions. The plants were maintained in a glasshouse with ambient day temperatures of 17–25 °C during sunless days and 28–35 °C during sunny days, and they were daily irrigated with tap water and twice a week with a nutritive solution to avoid deficiencies (*3*). For the stress treatments, 24 plants from each accession were distributed in four different cabins where the temperature was set to 12 ± 1 °C during light (8 h) and 6 ± 2 °C during dark (16 h) periods. Control plants, 24 plants per accession, were distributed in two different cabins with temperatures of 22 and 18 ± 2 °C during light (8 h) and dark (16 h) periods, respectively. A constant photosynthetic photon flux density of 480 ± 20 μmol m^{-2} s^{-1} (taken from weather data) measured at the level of plant leaves, was used during the light period in all cabins.

Characterization of the Stress. Measurements were taken every 2 weeks during the course of the experiment. Six plants per accession and treatment were sampled after the maximum quantum yield of PSII (Fv/Fm) was measured on all the plants in the growth cabins. For Fv/Fm, the youngest and fully expanded leaf on an exposed branch was measured on each replicate plant. Different randomly chosen leaves were used on each occasion, and data were taken between 9:00 a.m. and 11:00 p.m. to minimize temporal artifacts. Leaves were dark-adapted by attaching light-exclusion clips to the leaf surface for a 45 min period. Chlorophyll fluorescence was then measured using a Plant Efficiency Analyzer Handy PEA (Hansatech Instruments Ltd., Norfolk, U.K.).

Hydrogen peroxide and lipid peroxidation (MDA levels) were measured spectrophotometrically (Ciba-Corning U.K., 2800 Spectrascan) following the method of Alexieva and co-workers (*13*). Hydrogen peroxide concentration was calculated using a calibration curve with a known concentration of hydrogen peroxide. The nmol ml^{-1} of the TBA–MDA complex was calculated following the method of Dhindsa and co-workers (*14*) by using MDA equivalents (nmol ml^{-1}) = [(A_{532}–A_{600})/155 mM cm^{-1}] × 10^6. A_{532} represented the maximum absorbance of the TBA-MDA complex at 532 nm, A_{600} the correction for nonspecific turbidity measured at 600 nm, and 155 mM cm^{-1} the molar extinction coefficient for MDA.

Chlorophylls a and b, together with β-carotene, zeaxanthin (Z), violaxanthin (V), and antheraxanthin (A), were analyzed in fully expanded young leaves (500 mg FW) which were immediately grounded with liquid nitrogen and extracted repeatedly with ice-cold acetone/water 85% (v/v) and 100% acetone in an ultrasonic bath. The extract was then bubbled for 2 min with N_2 gas, filtered through a 0.45 μm filter, capped, and kept at −20 °C until analysis. Pigments were separated on a Lichrosphere 100 RP18 5 μm column (250 × 4.6 mm, 21.6 C Hickron, U.K.) at 30 °C for 42 min at a flow rate of 1 mL min^{-1}. The solvents consisted of (A) acetonitrile/methanol (85:15, v/v) and (B) methanol/ethyl acetate (68:32, v/v). The gradient used was as follows: 0 min, 100% A; 14.6 min, 100% A; 16.6 min, 0% A; 30 min; 0% A; 32 min, 100% A. After 32 min the gradient was recycled to the initial conditions and held for 10 min before making a new injection. Detection was set at 445 nm. Compounds were identified by their characteristic spectra and by coelution with chlorophyll and carotenoid standards obtained from Fluka and Extrasynthasée, respectively.

Extraction and HPLC Identification of Phenolic Compounds. Fresh plant material (1 g) was grounded in liquid nitrogen and extracted three times with 15 mL of methanol for 15, 10, and 5 min at room temperature, in a sonic bath. The combined extracts were evaporated to dryness under reduced pressure at 30 °C. The residues were dissolved in 1 mL of methanol and kept at −20 °C for no longer than 24 h before the analysis. Before the HPLC analysis all the samples were filtered through a 0.45 μm filter. Aliquots of 20 μL were injected into a reverse phase Hypersil H5 ODS column (250 × 4.6 mm i.d.). A Waters 600 System controller coupled with a photodiode array detector Waters 994 series was used. Separation and quantification were achieved at 25 °C by using the gradient acetonitrile (solvent A) and acidified water containing 2.5% of acetic acid (solvent B). The gradient was as follows: 0 min, 10% A; 10 min, 20% A; 30 min, 30% A; 35 min, 50% A; 50 min, 60% A; 55 min, 90% A; 57 min, 100% A; 67 min, 100% A; 68 min, 10% A. The flow rate was 1 mL min^{-1}, and the detection was set at 280 nm. Identification of individual compounds was based on the comparison of the actual retention time to those of reference authentic standards. The total phenolic content was calculated as Luis and Johnson (*3*).

Statistical Analysis of the Data. All the data were statistically analyzed, and values for all variables are given as the mean. Student's *t* tests and regression analyses were conducted using SPSS (Chicago, IL, U.S.A.). Differences were considered significant at a probability level of $P < 0.05$.

RESULTS

Cold Stress Effects on Photosynthetic Pigments, Fv/Fm, H_2O_2, and Lipid Peroxidation. Cold treated plants exhibited significant reductions in both chlorophylls and β-carotene as well as the maximum quantum yield of PSII expressed as Fv/Fm in both accessions (**Table 1**). Values after 6 weeks of treatment seem to be stable, in accession 15 plants, around 1074 μg g^{-1} FW for total chlorophyll content, 30.3 μg g^{-1} FW for β-carotene levels, and 0.32 for the Fv/Fm value. A similar trend was observed in accession 22 plants; however, the total chlorophyll content and β-carotene levels showed a dramatic reduction (40% and 88%, respectively) in the last week of the experiment. Xanthophylls, expressed as zeaxanthin + antheraxanthin (Z+A) or violaxanthin (V), exhibited in accession 15 cold treated plants a 16% increase and 26% decrease, respectively, while in accession 22 plants zeaxanthin + antheraxanthin showed no significant changes and violaxanthin experimented a 46% decrease in the last week of the experiment.

Hydrogen peroxide content in cold treated accession 15 plants showed significant differences from control plants throughout the experiment, showing a 70% increase at the end, while accession 22 exhibited a 30% increase only in the last 2 weeks. Lipid peroxidation levels showed a similar response in both accessions. Nevertheless, levels found in accession 22 were higher than in accession 15, with 58% and 35% increases, respectively (**Table 2**).

Cold Stress Effects on Rosemary Secondary Metabolites. Results from the HPLC analysis (**Table 3**) showed rosmarinic acid (accession 15, 4.40 mg g^{-1} FW; accession 22, 4.40 mg g^{-1} FW) and carnosic acid (accession 15, 24.5 mg g^{-1} FW; accession 22, 6.25 mg g^{-1} FW) as the predominant compounds in both rosemary accessions. In addition, accession 15 cold treated plants showed an increase in carnosic and rosmarinic acid concentration levels, with values stabilized around 27 mg g^{-1} FW and 6.65 mg g^{-1} FW, respectively, in the last 2 weeks of the experiment. This represents a 3% and a 50% increase for carnosic and rosmarinic acids, respectively. In contrast, accession 22 cold treated plants showed a 50% decrease in

Table 1. Chlorophyll a and b (μg g^{-1} FW), β-Carotene, Xanthophylls (μg g^{-1} FW) Content, and Maximum Quantum Yield of PSII (expressed as Fv/Fm) of Control (C) and Stressed (S) Plants Subjected to Cold Stress Conditions[a]

		Chl. a		Chl. b		Chl. a/Chl. b		Z+A		V		β-CAR		Fv/Fm	
	weeks	C	S	C	S	C	S	C	S	C	S	C	S	C	S
accession 15	0	1216.5	1209.5a	200.1	196.0a	6.1	6.2a	53.6	54.6a	7.72	7.70a	37.8	37.8a	0.77	0.77a
	2	1193.5	1042.4b	210.3	176.3b	5.7	5.9a	56.2	71.8b	8.36	6.48b	37.2	36.1a	0.76	0.50b
	4	1210.6	890.5b	187.6	129.7b	6.5	6.9b	58.6	68.5b	8.32	3.20b	40.9	28.0b	0.75	0.38b
	6	1239.1	905.7b	189.8	168.6b	6.5	5.4b	64.1	78.2b	8.05	5.29b	39.3	30.3b	0.77	0.32b
accession 22	0	894.5	896.1a	152.7	152.7a	5.9	5.9a	59.4	58.5a	7.87	7.80a	21.3	20.5a	0.77	0.77a
	2	895.6	438.3b	150.7	126.9b	5.9	3.5b	58.4	56.4a	7.90	6.18b	22.4	14.6b	0.76	0.47b
	4	778.5	399.7b	147.7	110.2b	5.3	3.6b	57.7	47.4b	7.92	2.99b	18.9	8.7b	0.75	0.34b
	6	792.4	447.2b	143.3	119.0b	5.5	3.8b	55.4	54.1a	8.05	4.39b	17.3	3.9b	0.74	0.30b

[a] Data are the mean of three independent replicates for $n = 6$ samples. Means with different letters were found statistically significant from control plants. Data were analyzed by a Student's t test ($P < 0.05$). Chl. a, chlorophyll a; Chl. b, chlorophyll b; Z, zeaxanthin; A, antheraxanthin; V, violaxanthin; β-CAR, β-carotene.

Table 2. Hydrogen Peroxide Content (μmol g^{-1} FW) and MDA Levels (nmol TBARS g^{-1} FW) of Control (C) and Stressed (S) Plants Subjected to Cold Stress Conditions[a]

		H_2O_2		MDA	
	weeks	C	S	C	S
accession 15	0	0.65	0.67a	3.63	3.45a
	2	0.66	0.91b	3.58	5.18b
	4	0.67	1.17b	3.67	6.09b
	6	0.72	1.20b	3.59	4.88b
accession 22	0	0.85	0.89a	4.39	3.95a
	2	0.95	1.04b	4.58	8.01b
	4	0.90	0.99b	4.67	5.98b
	6	0.95	1.22b	4.59	7.28b

[a] Data are the mean of three independent replicates. Means associated with different letters were found statistically significant. Data were analyzed by a Student's t test ($P < 0.05$).

rosmarinic acid concentration levels at the end of the experiment, while carnosic acid exhibited a 40% increase. Other secondary metabolites such as caffeic acid showed an extraordinary increase in both accessions (from μg to mg g^{-1} FW) while carnosol exhibited an extraordinary reduction (94%) in accession 22 plants. Flavonoids such as Naringin, Cirsimaritin, and Hispidulin followed different patterns in both accessions, as well as the total phenolic content.

Carnosic Acid versus Photosynthetic Pigments, β-Carotene, and Xanthophylls. The relationship between carnosic acid concentration levels and photosynthetic pigments and carotenoids is represented in **Figure 2** for both accessions. Cold treated plants of both accessions showed an increase in their carnosic contents, which were negatively correlated with the total chlorophyll, β-carotene, and violaxanthine levels. However, zeaxanthin and antheraxanthin showed a positive correlation with carnosic acid content in accession 15 cold treated plants while for accession 22 stressed plants no significant results were found (data not shown).

Rosmarinic and Carnosic Acids versus H_2O_2 and Lipid Peroxidation. The relationship between rosmarinic and carnosic acids with the hydrogen peroxide and lipid peroxidation levels is represented in **Figure 3**. Increasing carnosic acid concentration levels to the rise of H_2O_2 and lipid peroxidation was both accessions' response, even when accession 15 plants have 4 times higher levels of carnosic acid than accession 22 plants. However, rosmarinic acid exhibited an opposite response in both accessions, even though both accessions have equal levels. Accession 22 cold treated plants showed the lowest levels of rosmarinic acid, while accession 15 exhibited the higher levels with the increase of H_2O_2 and lipid peroxididdation in rosemary cold stressed plants.

DISCUSSION

For this study, the physiological response of two different accessions of rosemary plants to cold temperatures was evaluated by analyzing different parameters. Two weeks after moving rosemary plants to the new growing conditions, the total chlorophyll content and the Fv/Fm values followed a similar pattern in cold stressed plants, despite the different chlorophyll content found in both accessions. In fact, chlorophyll loss and low Fv/Fm values typically occur in plants when cold stress conditions are imposed during short periods (*15, 16*). The decline in Fv/Fm values indicates a reduction in the potential PSII efficiency by the inactivation of part of the PSII reaction centers (*17, 18*).

The decrease in total chlorophyll content in rosemary cold stressed plants was concomitant with the decrease of β-carotene and violaxanthin levels in both accessions. However, zeaxanthin and antheraxanthin showed an increase in accession 15 cold stressed plants with no significant changes in accession 22 cold treated plants at the end of the experiment. This chlorophyll loss is a phenomenon previously described in rosemary but under drought stress conditions (*9*). A reduction in chlorophyll levels might limit the potentially damaging effects of high light intensities in stressed plants, because it decreases leaf light absorption and therefore increases the photoprotective and antioxidative capacity of leaves (*19*). Moreover, rosemary increases in xanthophylls such as zeaxanthine and antherxanthine contribute to dissipation of the excess of energy absorbed as heat, thus getting higher resistance to the imposed stress conditions (*20*).

The increase of H_2O_2 and MDA indicated that the oxidative metabolism of rosemary accessions was enhanced by cold treatment. However, such increase also plays an important signaling role in plants; in fact, recent identification of reactive oxygen species (ROS) generating enzymes has confirmed that plant cells can initiate and amplify ROS production for the purpose of signaling (*21*), thus controlling processes such as growth and development (*22*) and the response to biotic and abiotic environmental stimuli (*23*). In addition, previous reports in which the cold stress conditions were studied showed a pronounced increase in PAL activity, which was associated with a high accumulation of phenolic compounds in the plants leaves (*24, 25*).

Under these study experimental conditions cold treated plants showed an important increase in caffeic acid concentrations levels, while other secondary metabolites such as naringin, cirsimaritin, hispidulin, and carnosol showed proportional reductions or diverse responses in both accessions. Moreover, rosmarinic acid showed a notable increase in accession 15 cold treated plants, which was positively correlated with hydrogen

Table 3. Secondary Metabolites Changes (mg g^{-1} FW) during the 6 Weeks of the Experiment[a]

		CAF		ROS		NAR		HIS		CIR		CAR		CA		total phenolics	
	weeks	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S
accession 15	0	0.04	0.05a	4.40	4.57a	0.39	0.40a	0.02	0.02a	0.01	0.02a	4.83	4.79a	24.5	24.6a	34.19	34.45a
	2	0.05	0.41b	4.20	5.97b	0.40	0.90b	0.02	0.01a	0.01	0.01a	4.85	1.39b	23.5	22.8a	33.03	31.49a
	4	0.05	1.74b	4.50	6.68b	0.42	1.22b	0.03	0.02a	0.01	0.03b	4.86	2.38b	24.6	27.6b	34.47	39.67b
	6	0.06	1.83b	4.98	6.65b	0.44	1.30b	0.03	0.04a	0.01	0.03b	4.90	2.50b	25.8	26.6b	36.22	38.95a
accession 22	0	0.03	0.04a	4.40	4.57a	0.54	0.52a	0.01	0.01a	0.02	0.03a	6.61	6.59a	6.25	6.45a	17.88	18.21a
	2	0.04	0.08b	4.20	4.19a	0.56	0.41b	0.02	0.02a	0.01	0.06b	6.51	0.66b	6.36	8.19b	17.70	13.61b
	4	0.05	0.90b	4.50	3.50b	0.57	0.27b	0.03	0.12b	0.02	0.07b	6.56	0.48b	6.23	8.98b	17.96	14.32b
	6	0.06	1.38b	4.98	2.53b	0.60	0.48b	0.04	0.22b	0.03	0.07b	6.65	0.48b	6.61	9.36b	18.97	14.52b

[a] Data are the mean of three independent replicates for $n = 6$ samples. Means with different letters were found statistically significant from control plants. Data were analyzed by a Student's t test ($P < 0.05$). C, control plants; S, stressed plants; CAF, caffeic acid; ROS, rosmarinic acid; NAR, naringin; HIS, hispidulin, CIR, cirsimaritin; CAR, carnosol; CA, carnosic acid.

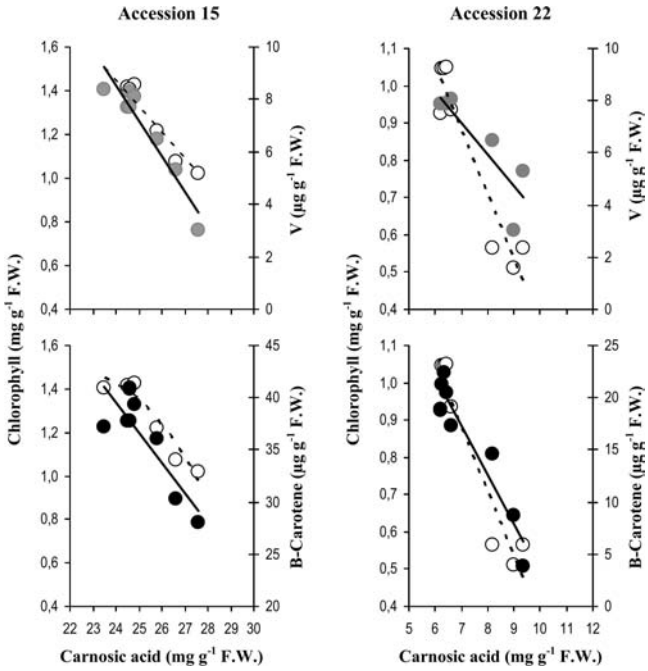

Figure 2. Relationship between carnosic acid concentration levels and total chlorophyll (open circles), violaxanthin (grey circles), and β-carotene (filled circles) levels in rosemary plants subjected to cold stress conditions. For accession 15 regression analysis showed regression coefficients of 0.865, 0.901, and 0.701 ($P = 0.001$; 0.001; 0.006) for chlorophyll, violaxanthin, and β-carotene. Accession 22 plants showed regression coefficients of 0.887, 0.751, and 0.897 ($P = 0.001$; 0.003; 0.001) for chlorophyll, violaxanthin, and β-carotene.

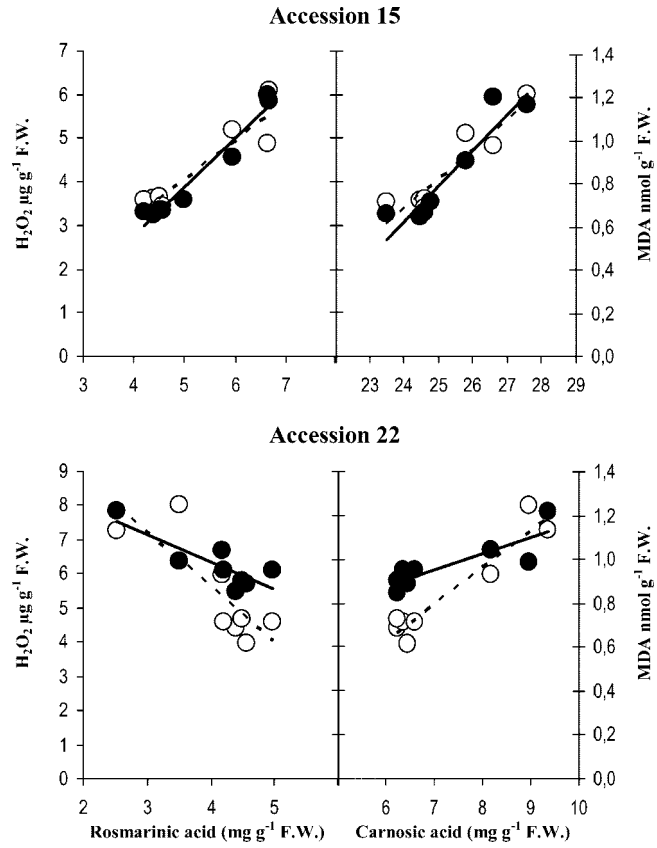

Figure 3. Relationship between rosmarinic and carnosic acids and hydrogen peroxide H_2O_2 (filled circles) and MDA (open circles) levels in rosemary plants subjected cold stress conditions. For accession 15 regression analysis showed regression coefficients for rosmarinic acid of 0.940 and 0.914 ($P = 0.001$) and for carnosic acid 0.901 and 0.838 ($P = 0.002$). Accession 22 plants showed regression coefficients for rosmarinic acid of 0.652, 0.613 ($P = 0.009$; 0.013) and for carnosic acid 0.679 and 0.895 ($P = 0.007$; 0.002).

peroxide and MDA levels. However, an opposite result was found in accession 22 stressed plants, negatively correlated with hydrogen peroxide and MDA levels during the whole experiment. If the observed variations of rosmarinic acid represent an antioxidant response against increased levels of ROS, then the source of these ROS and the contribution/interaction of other secondary metabolites or low molecular weight antioxidants becomes an important question, especially to fully understand the results showed by accession 22 plants.

Cold stressed plants exhibited higher concentration levels of carnosic acid, which were positively correlated with hydrogen peroxide and MDA levels in both accessions. In previous reports, but under drought conditions, both carnosic acid and carnosol showed a decrease in their concentrations due to their consumption to oxidize compounds in rosemary chloroplasts (*9*). However, in this report carnosic acid and carnosol concentration levels increased and decreased, respectively, in cold treated plants and in both accessions. These results suggest a consumption of carnosic acid and carnosol to oxidized compounds and also an increase in the biosynthesis of carnosic acid, thus raising the antioxidant protection within rosemary chloroplasts, despite total chlorophyll, β-carotene, and violaxanthin losses.

In summary, rosemary accessions growing in environmental control cabins, simulating typical chilling periods of southern U.K., exhibited increases in their carnosic acid levels. Our results are in agreement with previous studies under field conditions (*3*, *9*), showing similar variations in carnosic acid concentration levels during the winter months. It is therefore suggested that carnosic acid biosynthesis could be induced during the autumn and winter

months in rosemary plants. Moreover, their capacity to withstand these chilling temperatures may be related to the accessions ability to dissipate the excess of energy absorbed by chlorophylls using the xanthophylls cycle, together with the contribution of other low molecular weight antioxidants such as carnosic acid (*9*). On the other hand, in this report we demonstrate that not all rosemary accessions are equally well adapted to chilling temperatures. In fact, accession 22 cold treated plants showed severe losses in chlorophyll, β-carotene, and even xanthophylls (including zeaxanthin and antheraxanthin), despite no visual symptoms of leave injury being observed. Finally, more research is needed to understand rosmarinic acid variations in rosemary plants under stress conditions.

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