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UV-B radiation effects on foliar concentrations of rosmarinic and carnosic acids in rosemary plants

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Abstract

The influence of two different dosages of UV-B radiation on foliar concentrations of rosmarinic and carnosic acids was studied. The results showed that UV-B radiation significantly increased the concentrations of both rosmarinic and carnosic acids, as well as other rosemary compounds, such as naringin and carnosol. However, the increase of rosmarinic and carnosic acid levels were not concomitant with an increase in the DPPH radical-scavenging activity of rosemary extracts from UV-B-treated plants when compared with control plants. The significance of these results is considered.

Keywords: *Rosmarinus officinalis*; DPPH; Antioxidant; Diterpene; Phenolic acids

1. Introduction

Plants that grow in Mediterranean and tropical environments, as well as those adapted to high altitudes, have developed a number of mechanisms to protect themselves from UV-B radiation. There is now a substantial body of evidence showing the significant effects of UV-B radiation on flavonoids and hydroxycinnamic acids (Bieza & Lois, 2001; Burchard, Bilger, & Weissenbock, 2000; Liakoura, Manetas, & Karabourniotis, 2001; Olsson, Veit, & Bornman, 1999; Tattini, Gravano, Pinelli, Mulinacci, & Romani, 2000), two group of compounds which can act as sun-screens, thus providing protection for the plants against UV-B radiation (Kolb et al., 2001; Mazza et al., 2000). In addition, these natural products also have antioxidant properties (Larson, 1995). For example, certain flavonoids, as well as hydroxycinnamic acids, can act as powerful one electron scavengers of free radicals (Grace, Logan, & Adams, 1998; Rice-Evans, Miller, & Paganga, 1997) and also as two electron donors to the H_2O_2-scavenging peroxides of plant cells (Takahama, 1986; Yamasaki, 1997).

These antioxidant properties have raised food industry interest in plant phenolics. It is widely demonstrated that herbs extracts have high antioxidant activity, and among them, *Rosmarinus officinalis* is always one of the best performers. Several studies of its antioxidative components have indicated that the most active compounds are the diterpene, carnosic acid, and the phenylpropanoid, rosmarinic acid; in fact, there are many reports analysing the antioxidant activity of rosemary extracts and their compounds (Aruoma, Halliwell, Aeschbach, & Löliger, 1992; Cuvelier, Bondety, & Berset, 2000; Cuvelier, Richard, & Berset, 1996; Frankel, Huang, Aeschbach, & Prior, 1996; Hopia, Huang, Schwartz, German, & Frankel, 1996; Luis & Johnson, 2005). In addition, several reports have been published, analysing not only the distribution of rosmarinic and/or carnosic acids during growth and vegetative development of rosemary leaves, but also the effects of drought on carnosic acid levels and the influence of

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drought, in combination with UV-B radiation, on the rosemary photosynthetic capacity (Del Baño et al., 2003; Hidalgo, Ubera, Tena, & Valcárcel, 1998; Ibañez et al., 2003; Luis & Johnson, 2005; Munné-Bosch & Alegre, 2000, 2003; Munné-Bosch, Schwarz, & Alegre, 1999; Nogués & Baker, 2000). However, to our knowledge, this is the first report in which the effects of UV-B radiation on both rosmarinic and carnosic acid concentrations were studied in rosemary plants.

Therefore, the aims of this study were as follows: (a) to identify and characterise the most abundant compounds (rosmarinic and carnosic acids) in rosemary extracts (cultivar Sissinghurst English). All samples were analysed by HPLC (equipped with diode array detection); and (b) to study the effect of UV-B on the DPPH antiradical activity and the concentration of rosmarinic and carnosic acids in rosemary extracts.

2. Materials and methods

2.1. Materials

All solvents used in the experiments were of HPLC grade and were purchased from Fisher Scientific (UK). The standards caffeic and vanillic acids were purchased from Sigma–Aldrich Company Ltd. (UK). Rosmarinic acid was purchased from ICN Pharmaceuticals, Ltd. (UK). Carnosic acid was obtained from the National Herb Centre (Banbury, UK). Naringin, apigenin, hispidulin and cirsimaritin were obtained from the Phytochemistry Laboratory, Department of Botany, The University of Reading (UK).

Rosmarinus officinalis L. plants (cultivar Sissinghurst English) were selected at the National Herb Centre (Banbury, UK). Rosemary 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. 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 watered daily with tap water and twice a week with Hoagland solution.

UV-B fluorescent tubes, Philips TL12 (Starna Industries, UK), were used as a source of UV-B radiation. Cellulose diacetate, 125 μm, was purchased from A. Warne and Co. Ltd. (UK), while Polyester Mylard-D was purchased from Secol Ltd. (UK). UV-B radiation was measured with a scanning spectroradiometer (Bemtham TM 300 Monochromator).

2.2. Methods

2.2.1. UV-B treatments

For the UV-B radiation treatments, all plants were placed in a transparent exposure cabinet in the glasshouse at The University of Reading for two weeks. Glasshouse and cabinet transmission of UV-A radiation, supplemented by UV-B lamps, ensured that the UV-A exposure was maintained for photorepair and for flavonoid biosynthesis (Teramura & Ziska, 1986). The exposure cabinet was divided into two independent sections, one with UV-B radiation (tubes covered with cellulose diacetate) and one without (tubes covered with polyester). Both sections were regularly exchanged to minimise any differences other than the UV-B treatment. The biological UV-B dosages, according to the generalised plant action spectrum (normalised to 300 nm; Nogués & Baker, 2000) for the UV-B treated plants, were 5.4 and 31 kJ m^{-2} d^{-1}, while the control plants received 0.001 kJ m^{-2} d^{-1}.

2.2.2. Extraction and HPLC analysis of extracts

After two weeks of the experiment, fresh plant material (1 g) was ground 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 more 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, or a Waters 490 E programmable multiwavelength detector, were 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.

After 68 min, the gradient was recycled to initial conditions and held for 10 min before a new injection. The flow rate was 1 ml min^{-1} and the detection was set at 280 nm, a wavelength at which all compounds could be detected and quantified. Identification of individual compounds was based on comparison of the actual retention time to those of reference authentic standards. Carnosol was quantified as carnosic acid and all other compounds as themselves. The values obtained for carnosol, using carnosic acid, were recalculated using a relative response factor of 1.36 at 280 nm to obtain an accurate estimate of carnosol content (Thorsen & Hildebrandt, 2003).

2.2.3. DPPH antiradical assay

The free radical-scavenging activity was estimated using the stable DPPH radical (Lu & Foo, 2001). Freshly made DPPH radical (200 μM) (Sigma–Aldrich) was mixed with methanolic extracts of rosemary main secondary metabolites to start the reaction. Rosemary extracts were also tested using fresh plant material ground in liquid nitrogen and extracted with methanol at room temperature in a sonic bath as described previously. A control, containing no tested compounds or extracts, was included. The absorbance at 517 nm of DPPH was measured in a spectropho-

tometer (Ciba-Corning UK, 2800 Spectroscan) against a blank of pure methanol after 30 min at room temperature. The DPPH radical-scavenging capacity was estimated from the difference in absorbance, with or without tested compounds or extracts, and expressed as a percentage of DPPH scavenged in solution. The IC_{50} value represents the concentration of an individual compound required to quench 50% of DPPH under experimental conditions. All the tests were done in triplicate.

2.2.4. Statistical analysis

Experiments were repeated at least three times and the data were analysed statistically. All results are given as means ± standard deviation (SD). Differences between variables were tested for significance by the Student's *t*-test ($p < 0.05$).

3. Results and discussion

Although numerous phenolics, flavonoids, and diterpenes have been reported for rosemary, only eight different compounds were present in the cultivar Sissinghurst English extracts in sufficient amount to be identified and quantified (Table 1). Results from the HPLC analysis showed rosmarinic acid (2.08 mg g^{-1} fresh weight biomass) and carnosic acid (12.12 mg g^{-1} fresh weight biomass) to be the predominant compounds, which agreed with previous studies (Cuvelier et al., 1996; Zheng & Wang, 2001), followed by naringin and carnosol, both with similar values (0.57 and 0.58 mg g^{-1} fresh weight biomass, respectively).

Despite that both UV-B radiation treatments increased the concentrations of caffeic acid, rosmarinic acid, naringin, cirsimaritin, carnosol and carnosic acid in rosemary irradiated plants, two compounds, vanillic acid and hispidulin, exhibited an opposite response, decreasing their concentrations when they were exposed to both UV-B radiation treatments (Table 2). However, significant differences were found when rosemary plants were irradiated with a UV-B dose of 31 kJ m^{-2} d^{-1} (T2, in Table 2). These plants showed higher concentrations of caffeic acid, rosmarinic acid, carnosol, and carnosic acid (2.87 ± 0.016, 4.87 ± 0.104, 3.11 ± 0.02, and 32.9 ± 0.76 mg g^{-1} fresh weight, respectively), than control plants and than UV-B-irradiated plants with a dose of 5.4 kJ m^{-2} d^{-1} (T1, in Table 2). These results corroborate previous studies where UV-B radiation produced substantial differences in flavonoid and phenolic acids and esters between the control and UV-B-irradiated plants. In addition, there have been several reports on UV-B promotion of terpenoid production, particularly members of the Lamiaceae family (Johnson, Kirby, Naxakis, & Pearson, 1999; Karousou, Grammatikopoulos, Lanaras, Manetas, & Kokkini, 1998; Maffei & Scannerini, 2000). However, to our knowledge, this is the first study reporting UV-B radiation effects on diterpenes such as carnosic acid and carnosol.

Table 3 shows the DPPH antiradical capacites of caffeic acid, vanillic acid, rosmarinic acid, naringin and carnosic acid. Rosmarinic acid exhibited an excellent DPPH radical-scavenging activity with an IC_{50} value of 27 μM under experimental conditions. Carnosic and caffeic acids showed similar DPPH scavenging capacities with IC_{50} values of 32 and 38 μM, respectively, both significantly higher than vanillic acid and naringin (both >200 μM). In this study, UV-B radiation induced higher foliar concentrations of caffeic acid, rosmarinic acid, naringin, cirsimaritin, carnosol and carnosic acid, concentrations that should have a positive effect in the total antiradical capacity of rosemary

Table 1
Identified compounds and concentration levels in rosemary control plants

Compound	Retention time	UV λ_{max}	Concentration
Vanillic acid	8	260.292	0.004 ± 0.0002
Caffeic acid	9	296.324	0.012 ± 0.0006
Naringin	20	284.334	0.570 ± 0.028
Rosmarinic acid	23	290.330	2.080 ± 0.104
Apigenin	37	267.340	ND
Hispidulin	38	270.336	0.020 ± 0.0010
Cirsimaritin	43	274.334	0.080 ± 0.0040
Carnosol	52	284	0.580 ± 0.0219
Carnosic acid	57	284	12.180 ± 0.609
Total phenolics			15.020 ± 0.769

Retention times are expressed in minutes, UV λ_{max} in nm and concentrations in mg g^{-1} fresh weight biomass. ND, not detected.

Table 2
UV-B radiation effects on rosemary identified compounds

Compound	Control plants	T1	T2
Vanillic acid	0.004 ± 0.0002	0.009 ± 0.0006*	0.005 ± 0.0009*
Caffeic acid	0.01 ± 0.0006	0.04 ± 0.002*	2.87 ± 0.016*
Naringin	0.57 ± 0.028	0.93 ± 0.03*	1.05 ± 0.02*
Rosmarinic acid	2.08 ± 0.094	2.33 ± 0.106*	4.87 ± 0.104*
Hispidulin	0.02 ± 0.0005	0.009 ± 0.0007*	0.006 ± 0.0004*
Cirsimaritin	0.08 ± 0.0004	0.15 ± 0.009*	0.16 ± 0.0001*
Carnosol	0.58 ± 0.021	0.98 ± 0.02*	3.11 ± 0.02*
Carnosic acid	12.1 ± 0.30	18.0 ± 0.60*	21.5 ± 0.50*
Total phenolics	15.2 ± 0.66	23.2 ± 0.36*	32.9 ± 0.76*

T1 and T2 represent 5.4 and 31 kJ m^{-2} d^{-1} of UV-B radiation, respectively. An (*) indicates significant difference from control plants ($p < 0.05$).

Table 3
DPPH radical-scavenging activity of rosmarinic and carnosic acids at three selected concentrations compared with caffeic acid, vanillic acid and naringin

Compound	Concentrations			IC_{50} (μM)
	20 μM	50 μM	100 μM	
Rosmarinic acid	37.3 ± 1.4	82.5 ± 1.3	93.3 ± 1.4	27
Caffeic acid	27.5 ± 0.5	71.7 ± 1.2	94.9 ± 1.9	38
Vanillic acid	0.0	1.8 ± 0.2	3.80 ± 0.6	>200
Naringin	0.3 ± 0.01	0.7 ± 0.01	1.70 ± 0.2	>200
Carnosic acid	30.5 ± 1.2	82.7 ± 1.9	96.3 ± 1.8	32
Ascorbic acid	21.2 ± 0.9	75.0 ± 1.9	96.5 ± 1.4	47

The data were expressed as percentage of DPPH· scavenged and were the means ± SD ($n = 3$).

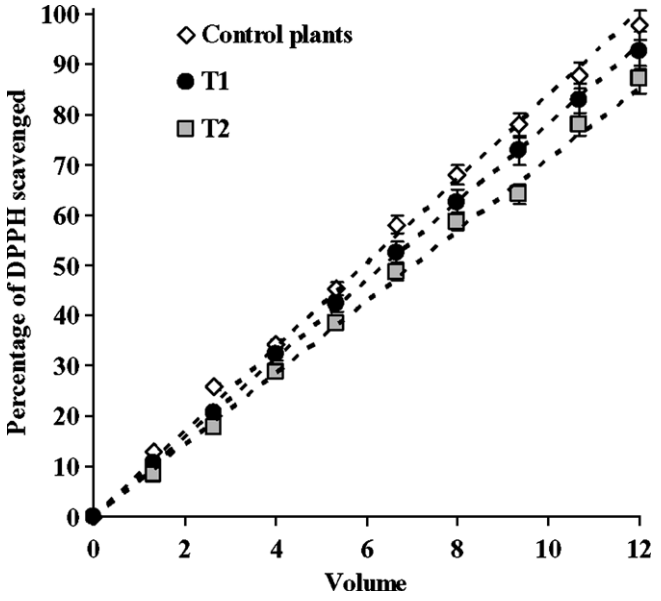

Fig. 1. DPPH·-scavenging activity of control and UV-B treated plants (T1 = 5.4 kJ m^{-2} d^{-1} and T2 = 31 kJ m^{-2} d^{-1} of UV-B radiation). Volume represents the μl ml^{-1} of the extract used. The data are the mean ± standard deviation for $n = 3$ different determinations. Significant differences were found between control and UV-B (T2 = 31 kJ m^{-2} d^{-1}) irradiated plants.

extracts. However, Fig. 1 shows a slight decrease in the DPPH antiradical capacity of rosemary plants treated with 31 kJ m^{-2} d^{-1} of UV-B radiation.

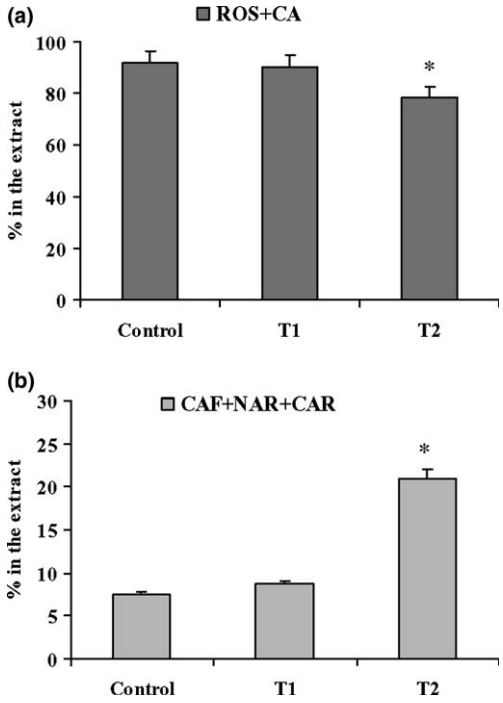

Fig. 2. Proportions (%) of (a) rosmarinic and carnosic acids and (b) caffeic acid, naringin, and carnosol in rosemary control and UV-B-treated plants extracts. T1 = 5.4 kJ m^{-2} d^{-1} and T2 = 31 kJ m^{-2} d^{-1} of UV-B radiation. An (*) indicates significant difference from control plants ($p < 0.05$). ROS, rosmarinic acid; CA, carnosic acid; CAF, caffeic acid; NAR, naringin; CAR, carnosol.

Altogether, these results indicated that UV-B radiation treatments, which considerably increased the amounts of rosmarinic and carnosic acids, did not necessarily improve the DPPH antiradical capacity of UV-B treated plants. A possible explanation of this result could be the decrease in rosmarinic and carnosic acids proportions in rosemary extracts, and an increase of weaker DPPH radical-scavengers, such as naringin and cirsimaritin (Fig. 2). In addition, the diterpene carnosol whose proportion increased considerably in UV-B-irradiated plants (31 kJ m^{-2} d^{-1}, T2) compared with the control, may contribute to minimise the $DPPH^{\cdot -}$-scavenging differences between control and UV-B irradiated plants due to its high DPPH·-scavenging activity (Miura, Kikzaki, & Nakatani, 2002). However, further studies are necessary to corroborate this hypothesis and to identify any possible synergistic effect between the rosemary compounds.

4. Conclusion

The present study provides a workable system to obtain consistent and high levels of polyphenolics from leaves of rosemary plants. Results from the experiments have shown that rosemary plants subjected to enhanced levels of UV-B radiation gave higher yields of rosmarinic and carnosic acids, than did the control and non-treated plants. The primary advantage of this system is that we can obtain a good yield of antioxidant products, such as rosmarinic and carnosic acids, from plants growing under glasshouse conditions minimising the environmental influence on their concentrations. However, further studies are needed to corroborate the UV-B radiation beneficial effects on rosmarinic and carnosic acid concentrations, particularly in different cultivars and accessions of rosemary plants.

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