

The effects of drought and supplemental UV-B radiation on physiological and biochemical traits of the grapevine cultivar “Soultanina”

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ABSTRACT

Aim: In the Mediterranean region, grapevines usually undergo drought and high UV-B intensities during their summer growth season. The present study was conducted in order to evaluate the effects of these two abiotic stressors on the physiological and biochemical characteristics of a major Greek raisin variety (*Vitis vinifera* L. cv Soultanina).

Methods and results: The experimental plants were three-years-old, grafted onto 110R rootstock and grown outdoors in 25 L pots containing a peat:perlite:sand (3:1:1, v/v/v) potting mixture. Grapevines were subjected to two irrigation treatments: (1) Well-Watered (plants were uniformly irrigated on a daily basis to soil substrate capacity), and (2) Water-Stressed (plants were equally irrigated with 50 % of the amount of water provided to Well-Watered plants), and to two levels of UV-B radiation: (1) ambient UV-B radiation, and (2) ambient plus 15 % UV-B radiation. Although the combination of drought and supplemental UV-B radiation appeared to have synergistic effects on gas exchange characteristics and H₂O₂ production, the development of biochemical limitations to photosynthesis was not detectable. Compared to the other stress treatments, WW±15 % UV-B plants exhibited higher stomatal conductance (g_s) and photosynthetic rate (P_N).

Conclusion: Under elevated UV-B radiation, superoxide dismutase (SOD) activation, chlorophyll degradation and enhanced synthesis of carotenoids all helped the plant to maintain its physiological functions, while in Water-Stressed plants irrespective of the level of UV-B, a more pronounced role of abscisic acid (ABA) and trans-zeatin-riboside (t-ZR) in mediating stomatal responses was revealed.

Significance of the study: Our results imply that the environmental conditions were not stressful enough to report the occurrence of non-diffusional limitations to photosynthesis. In addition, two different adaptive responses in relation to the applied abiotic stressor were shown.

KEYWORDS

water limitation, UV-B radiation, ABA, cytokinin, gas exchange, photosynthetic pigments, antioxidants

INTRODUCTION

Viticulture is an activity of primary socio-economic importance worldwide; it is present in more than 40 countries, the global vineyard covers an area of 7.4 million hectares, and total production amounts to 77.8 million tonnes of fresh grapes and 292.3 million hectolitres (mhl) of wine (OIV, 2019). Terroir - a concept that considers the influence of soil, landscape, climate and genotype-related cultural and oenological practices on yield, quality and typicality of the products - recognizes that environmental factors have a major effect on the ability of a region to produce quality grapes. Despite the fact that grapevines are widely grown in the dry and semi-dry climates of the Mediterranean region and are traditionally non-irrigated, vine-growers must adjust their viticultural practices, as well as the appropriate locations for grape growing (i.e., examine the feasibility of moving the vineyards to cooler areas), according to model predictions for future climate in Mediterranean Europe (Carvalho and Amâncio, 2019). Climatic scenarios suggest that a lengthened summer drought, along with a gradual increase in temperature and occurrences of heat waves, will strike the Mediterranean region in the upcoming decades (Stocker, 2013). They also predict that Mediterranean plants will be subject to increased solar radiation and, in particular, ultraviolet-B radiation (280-315 nm) levels, due to a reduction in average cloud cover (Bais *et al.*, 2015). Consequently, plant water demand will increase, the genotoxic effects of high UV-B doses will be intensified, and thus grapevines will probably need to activate a variety of defense mechanisms to tolerate the limited water availability and the enhanced levels of ultraviolet radiation.

Drought is a multidimensional stress factor that adversely affects the development and yield of higher plants through morphological (e.g., reduction in growth rate and changes in leaf anatomy), physiological (e.g., extremely negative plant water potential and reduced carbon assimilation), and biochemical and molecular (e.g., accumulation of compatible organic solutes, production of a series of antioxidant enzymes, and changes in endogenous phytohormone contents) alterations (Wang *et al.*, 2003). However, subjecting grapevines to mild water stress is a common practice in modern viticulture aiming to control vegetative growth and to improve grape quality. Despite evidence of hydraulic vulnerability segmentation in the annual and the perennial

parts of *Vitis vinifera* genotypes, observations using visualisation methods have revealed that grapevines are hydraulically vulnerable, losing 50 % of stem hydraulic conductivity when stem water potential values are close to -1.7 MPa (Charrier *et al.*, 2016).

High acute doses of UV-B radiation can act as genetic stressors, with effects on plants including reduction in growth and photosynthetic capacity, direct DNA damage, release of Reactive Oxygen Species (ROS), membrane degradation and alterations in the synthesis of pigments, hormones and proteins (Frohnmeyer and Staiger, 2003). Nevertheless, “realistic”, non-damaging levels of UV-B have been shown to be acclimatory, since they regulate certain gene expressions associated with the morphogenetic and metabolic functions of plants and act as important environmental signals (Jenkins, 2014). This view is supported by the recent characterisation of a specific ultraviolet-B radiation receptor (UV RESISTANCE LOCUS 8 (UVR8)), which acts as an intrinsic chromophore and regulates the expression of key genes in UV-B responses (Loyola *et al.*, 2016). Previous studies have reported that, when exposed to high fluence rates of UV-B, grapevines suffered a down-regulation in photosynthetic performance (Martínez-Lüscher *et al.*, 2015), a pronounced decrease in growth rate and biomass production (Doupis *et al.*, 2011), a buildup of reactive oxygen species, and modifications in chlorophylls and carotenoids levels (Doupis *et al.*, 2012). Although most of the aforementioned studies are not characterised by a realistic simulation of UV-B irradiance, but instead try to highlight the contrasting effects between (unrealistic) high and ambient levels of UV-B radiation, (a) the long-term exposure of grapevines to the maximum (current) UV-B levels (as the full recovery of the ozone layer is not expected until after 2050, even in the absence of further CFCs emissions (Solomon, 2019)), (b) the previously mentioned predicted decrease in mean cloudiness (and the consequent increase in absorbed UV-B radiation), and (c) since moving vineyards to higher altitudes (with an increase in altitude every 1,000 meters, UV levels increase by 10 to 12 %) can be an effective adaptation to a warmer climate, the evaluation of grapevine response to stressful levels of UV-B should be seriously considered and coordinated with detailed experimental data.

Plants have evolved various adaptive responses to minimise the effects of unfavourable environmental conditions. Among chemical

signals, the phytohormone abscisic acid (ABA) - a sesquiterpene that is synthesised in plastids by a C40 carotenoid precursor - is a predominant stress response molecule which plays a key role in the regulation of multiple physiological and biochemical adaptation processes that are common in plants under certain abiotic or biotic stress conditions. The concentration of ABA increases rapidly in roots, shoots and leaves when the soil dries out (Beis and Patakas, 2015). ABA synthesis is associated (directly or interacting with hydraulic signals and/or other chemical protectants) with decreased stomatal conductance, buffering the drop in xylem water potential and improving water-use efficiency. In addition, ABA regulates the expression of various sets of drought-responsive genes, such as those involved in the synthesis of aquaporins (Lovisolò *et al.*, 2010) and dehydrins (Yang *et al.*, 2012), and in the accumulation of osmolytes (Ju *et al.*, 2020). ABA biosynthesis in grapevines is also induced by elevated levels of UV-B, and it has been reported that ABA participates in the activation of defense mechanisms triggering the accumulation of flavonoids and carotenoids, compounds that filter part of the UV-B energy excess and scavenge free oxygen radicals (Berli *et al.*, 2010). Furthermore, ABA is known to stimulate major antioxidant enzymes that catalyse the reactions associated with the protection of grapevine tissues from high UV-B induced-ROS production (Alonso *et al.*, 2016).

Cytokinins (such as zeatin) are adenine derivatives root-born hormones that influence fundamental aspects of plant growth and development (cell division and enlargement, leaf senescence, root elongation, and branching and chloroplast biogenesis (Ryu and Cho, 2015)), as well as plant responses to environmental stresses (Cortleven *et al.*, 2019). In general, cytokinins are considered to be negative regulators in plant drought responses, and previous publications have reported the occurrence of a dynamic cross-talk between cytokinins and ABA (Beis and Patakas, 2015) in drought stress signaling networks, showing that cytokinins can act as antagonists of ABA on stomatal behaviour, modifying plant acclimation processes under water shortage. Accordingly, UV-B radiation has been suggested to indirectly control the expression of cytokinin oxidase (CKX), a key enzyme in cytokinin degradation, and high levels of UV-B have been shown to reduce the endogenous cytokinin status in cucumber (Kataria *et al.*, 2005) and pea (Vaseva-Gemisheva *et al.*, 2004). In rice, however,

the effect of UV-B on cytokinins has been found to be dependent on the duration of the exposure (Lin *et al.*, 2002).

The objective of the present study was to evaluate whether chemical signals, such as abscisic acid (ABA) and trans-zeatin riboside (t-ZR), affect the gas exchange characteristics and photosynthetic pigments composition of the table grapevine cultivar, Soultanina (syn. Thompson seedless) under the influence of drought and high levels of UV-B radiation. The study also aimed to elucidate the relative contribution of phytohormones and antioxidant enzymes to plant adaptation mechanisms in response to the above stressors, either applied alone or in combination.

MATERIALS AND METHODS

1. Plant material and experimental design

The experiment was conducted during the growing season of 2008 at the facilities of the Institute of Olive Tree, Subtropical Plants and Viticulture in Chania, Crete, Greece (35° 32' 00" N, 24° 04' 09" E). Twenty four two-year-old grapevines (*Vitis vinifera* L. cv Soultanina) grafted onto 110R rootstock (*V. rupestris* x *V. berlandieri*) were grown outdoors in 25 L pots containing a mixture of peat, perlite and sand (3:1:1v/v). All plants were pruned to a single bud spur during winter and trained to a vertical shoot positioning trellis system, while laterals and clusters were removed immediately after formation. For two months, the pots were uniformly irrigated to soil capacity once a day using a drip irrigation system. Thereafter, the plants were divided into four groups of six plants selected on the basis of their growth similarities as follows: (a) well-watered (control) treatment, in which the plants were watered daily to soil capacity and subjected to ambient UV-B levels (WW-ambient UV-B treatment), (b) water-stressed treatment, in which the plants received, on a daily basis, 50 % of the amount of irrigation water provided to well-watered plants and were subjected to ambient UV-B levels (WS-ambient UV-B treatment), (c) well-watered treatment under supplementary UV-B, in which the plants were grown under ambient plus 15 % UV-B radiation (WW ± 15 % UV-B treatment), and (d) water-stressed treatment under enhanced UV-B, in which the water-stressed plants were exposed to ambient plus 15 % UV-B radiation (WS ± 15 % UV-B treatment). The above treatments were applied over a 15-day period.

UV-B irradiation was obtained by using UV-emitting fluorescent lamps (Philips, Ultraviolet-B, TL40W/12RS, Eindhoven, The Netherlands), each mounted on a stand at a distance of 30 cm above the plant canopy and wrapped with preheated 0.115 mm cellulose acetate sheets (Clarifoil, Coventry, UK) to exclude any UV-C transmission. The cellulose acetate film was replaced every 20-25 hours of operation. The UV-B irradiance was continuously recorded using a broadband radiometer (SKU430, Skye Instruments Ltd., Powys, UK). The average unweighted UV-B radiation close to the experimental site was 0.26 W m⁻². Calculated according to the generalised plant action spectrum (Caldwell, 1971), the applied $\pm 15\%$ UV-B biological effective dose (UV-B_{BE}) was 10.22 kJ m⁻² day⁻¹, thus simulating an approximate 10% reduction in the ozone layer above the city of Chania, according to the empirical model described by Green (1983). After the 15-day period, the plants were fully rehydrated. This experiment was repeated three times on the same group of 24 plants during the summer of 2008. All data presented in this paper refer to the last experimental cycle (from 23 July to 7 August).

2. Determination of leaf water potential

Using a Scholander-type pressure chamber (PMS Instrument Company, Corvallis, Oregon, USA), predawn (05:00 to 06:30 local standard time) leaf water potential (Ψ_{PD}) was determined every two days on three fully developed leaves (from different plants) per treatment.

3. Gas exchange measurements

A Li-6400, portable photosynthesis system (LiCor Bioscience Inc., Lincoln, Nebraska, USA) was used to measure net CO₂ assimilation rate (P_N) and maximum stomatal conductance (g_s), every second day, in the morning (between 08:30 and 10:30) at a saturating light intensity (photosynthetic photon flux density (PPFD) greater than 1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$), and with a reference for CO₂ concentration of 380 $\mu\text{mol mol}^{-1}$. Photosynthetic characteristics were measured on four intact leaves per treatment collected from the same plants prior to use for the determination of leaf water potential.

4. Determination of abscisic acid (ABA) and trans-zeatin riboside (t-ZR) concentrations

For ABA and t-ZR extraction, three randomly sampled leaves from each treatment (one leaf per plant) were collected four times during the experimental period (DOY 205, 210, 215 and 220). The leaves were frozen in liquid nitrogen, and

stored at $-80\text{ }^\circ\text{C}$ until further laboratory analysis. Leaf ABA concentration was determined according to the methodology of Lovisolo *et al.* (2002) with minor modifications: 1 g of the frozen leaf was ground into powder, homogenised in 10 mL of ice-cold 80% (v/v) aqueous methanol solution and then kept in the dark at 4 $^\circ\text{C}$ for 48 hours to prevent ABA photo-oxidation. The suspension was centrifuged twice at 8,000 x g for 20 mins, and the supernatant was filtered through a reversed phase C₁₈Sep-Pak cartridge (Waters, Milford, USA) and prewashed with 5 mL of 80% methanol to remove phenolics and pigments. Subsequently, methanol was removed by distillation under vacuum, with warming in a water bath, and the aqueous residue was extracted three times against an equal volume of ethyl acetate at pH 3.0. Ethyl acetate of the combined solution was removed under vacuum and the remaining aqueous phase was collected for t-ZR quantification. The dry residue was resuspended in TRIS-buffered saline solution (150 mM NaCl, 1 mM MgCl₂, 50 mM TRIS, pH 7.8) and ABA was detected immunologically using a Phytodetek Kit (Agdia, Elkhart, USA) following the procedure provided by the manufacturer.

The Extraction protocol for t-ZR was conducted on the same homogenised samples used for the ABA determinations according to the method of Turnbull *et al.* (1997) with minor modifications. After adjustment to pH 9.0 with 1 M NaOH, the remaining aqueous phase from the ABA purifications was partitioned four times with water-saturated n-butanol. The n-butanol fraction was reduced to dryness under vacuum and the residue was resuspended in TRIS-buffered-saline solution (150 mM NaCl, 1 mM MgCl₂, 50 mM TRIS, pH 7.8) and stored at $-20\text{ }^\circ\text{C}$ for further analysis. The quantification of trans-zeatin riboside was accomplished using a Phytodetek-t-ZR immunoassay kit (Agdia, Elkhart, USA). All assays were performed in triplicate and according to the manufacturer's guidelines.

5. Measurement of chlorophyll and carotenoids

Chlorophyll (Chl-a and Chl-b) and carotenoids (Car) were extracted from the same leaves used for the ABA and t-ZR determinations using 80% acetone. Chl and Car content was determined spectrophotometrically (Hitachi U-1100 spectrophotometer, Hitachi Ltd., Tokyo, Japan) at 470, 644.8 and 661.6 nm according to the method of Lichtenthaler and Wellburn (1983).

6. Determination of SOD activity and H₂O₂ content

Total SOD (EC 1.15.1.1) activity was determined on six leaves per treatment, which were collected three times during the experimental cycle (days 1, 7 and 16) and immediately frozen at -80 °C. SOD isoforms assay was performed following the methodology described by Becana *et al.* (1986). For the determination of hydrogen peroxide, leaf tissue (0.5 g) obtained from the same leaves used for SOD assay was homogenised with 5 mL trichloroacetic acid (TCA) 0.1 % (w/v) in an ice bath. The homogenate was centrifuged at 10,000 x g for 30 min and H₂O₂ content was measured spectrophotometrically after a reaction with potassium iodide (KI) (Alexieva *et al.*, 2001). More details about the methods used to determine SOD activity and H₂O₂ content are provided in Doupis *et al.* (2011).

7. Data analysis

A multifactorial analysis of variance (ANOVA) was performed to assess the main effects and the interactions between drought and enhanced UV-B

radiation, using the SPSS 19.0 statistical package for Windows “repeated measures” feature (SPSS Inc., Chicago, IL, USA). In the case of significant differences, means were compared using Tukey’s test (P = 0.05). The figures were produced using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Predawn leaf water potential (Ψ_{PD}) decreased progressively under drought stress, reaching -0.6 MPa after 15 days, irrespective of the UV-B radiation regime (Figure 1B). Throughout the experimental period, Ψ_{PD} values in well-watered plants ranged from -0.17 to -0.36 MPa. The onset of the difference in Ψ_{PD} values between WW and WS plants became significant 10 days after the imposition of the different irrigation treatments.

Significant effects of both water application and UV-B radiation on net photosynthetic rate (P_N) and stomatal conductance (g_s) were revealed (Table 1). In the WW-ambient UV-B and WW \pm 15 % UV-B treatments, photosynthetic rates followed very similar patterns as the experiment progressed,

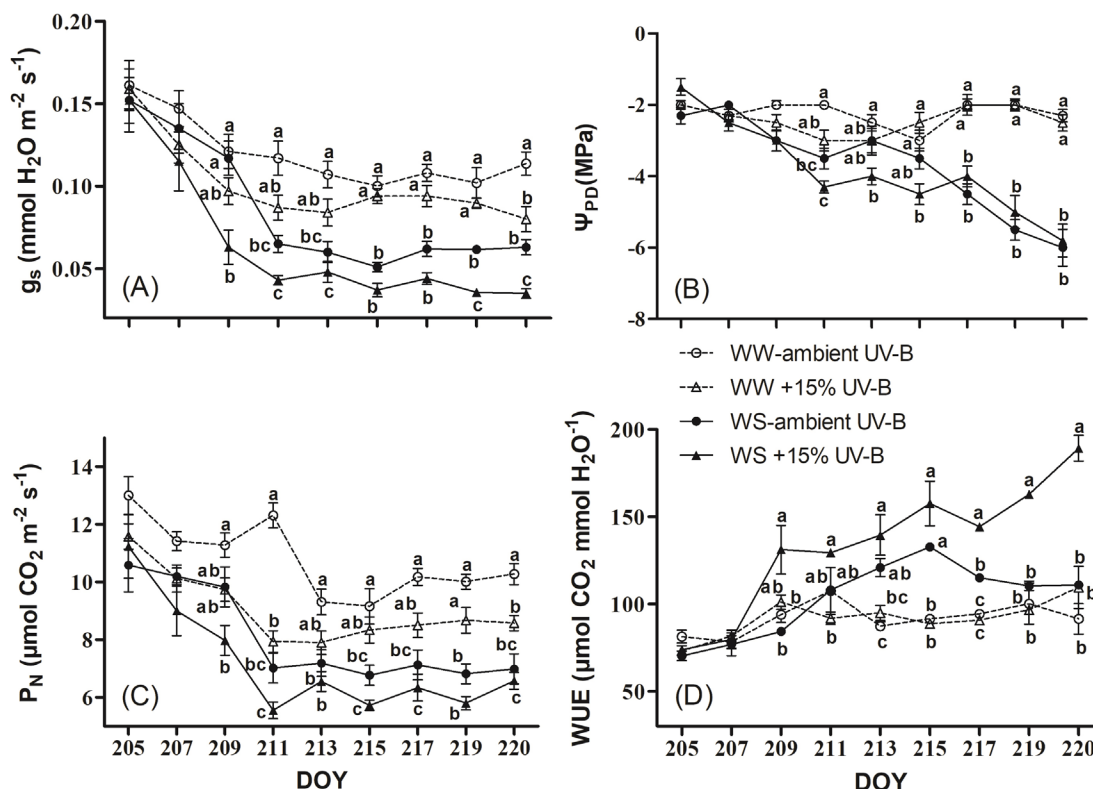


FIGURE 1. Changes in predawn leaf water potential (Ψ_{PD}) (B), stomatal conductance (g_s) (A), net photosynthetic rate (P_N) (C) and Water Use Efficiency (D) in Sultania plants during the experimental period (DOY 205-220).

Each point is the mean \pm SE of 3 or 4 replicates. Different letters indicate statistically significant differences at $p \leq 0.05$. WW, well-watered; WS, water-stressed.

TABLE 1. Effects of water limitation, UV-B radiation and their combination on predawn leaf water potential (Ψ_{PD}), net photosynthetic rate (P_N), stomatal conductance (g_s) and water use efficiency (WUE).

Source of variance	Treatment	Parameters			
		Ψ_{PD} (MPa)	P_N ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	g_s ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$)	WUE
Irrigation	WW	$-2.33 \pm 0.06a$	$9.91 \pm 0.22a$	$0.110 \pm 0.004a$	$91.92 \pm 1.74b$
	WS	$-3.77 \pm 0.18b$	$7.63 \pm 0.26b$	$0.074 \pm 0.006b$	$118.75 \pm 4.62a$
Radiation	Ambient UV-B	-2.97 ± 0.17	$9.42 \pm 0.28a$	$0.102 \pm 0.005a$	$97.58 \pm 2.49b$
	$\pm 15\%$ UV-B	-3.13 ± 0.17	$8.12 \pm 0.26b$	$0.082 \pm 0.005b$	$113.09 \pm 4.76a$
Irr. x Rad.	WW-ambient UV-B	$-2.23 \pm 0.08a$	$10.77 \pm 0.27a$	$0.102 \pm 0.005a$	$91.82 \pm 2.57b$
	WW $\pm 15\%$ UV-B	$-2.42 \pm 0.10a$	$9.04 \pm 0.25b$	$0.101 \pm 0.005ab$	$92.02 \pm 2.39b$
	WS-ambient UV-B	$-3.70 \pm 0.27b$	$8.06 \pm 0.34bc$	$0.085 \pm 0.008bc$	$103.33 \pm 4.01b$
	WS $\pm 15\%$ UV-B	$-3.84 \pm 0.26b$	$7.20 \pm 0.38c$	$0.064 \pm 0.008c$	$134.17 \pm 7.26a$
$P_{(\text{irrigation})}$		0.000	0.000	0.002	0.000
$P_{(\text{radiation})}$		0.473	0.012	0.035	0.008
$P_{(\text{Irr. x Rad.})}$		0.923	0.313	0.854	0.059

Different letters in the same column indicate significant differences at $P \leq 0.05$. Data represent average values \pm SE ($n = 27$ for Ψ_{PD} and WUE and 36 for P_N and g_s). $P_{(\text{irrigation})}$, significance of the irrigation effect; $P_{(\text{radiation})}$, significance of the UV-B radiation effect; $P_{(\text{irr. x rad.})}$, significance of the irrigation x UV-B radiation interaction effect; WW, well-watered; WS, water-stressed.

while WS-ambient UV-B plants exhibited a steeper, but non-significant, decline in P_N values compared to WW $\pm 15\%$ UV-B plants (Figure 1C, Table 1). Significant photosynthetic limitations were reported for the combined application of drought and enhanced UV-B radiation, with P_N values being recorded at the end of the period of the stressors imposition, being 59 % of the control treatment. Moreover, water deficit and supplemental UV-B affected both gas exchange characteristics in a similar way, as shown by the parallel decline in stomatal conductance and photosynthetic rate throughout the experimental period (Figure 1A, Figure 1C). WS $\pm 15\%$ UV-B treatment exhibited a large reduction in g_s values during the experiment, leading to higher Water Use Efficiency (WUE) (Figure 1D).

At the end of the experimental period, leaf ABA accumulation in the WS treatment (irrespective of the UV-B level) was three to four times higher than in the WW treatment (Figure 2A), whereas UV-B radiation (applied alone or in combination) did not induce any significant increase in the foliar ABA concentration (Table 2). Accordingly, water availability seemed to have a more important role in reducing t-ZR concentration in leaves

than UV-B radiation, since WS plants exhibited a rapid reduction in t-ZR levels, while enhanced UV-B did not produce any significant effect on t-ZR content (Figure 2B). Both ABA production and t-ZR decrease resulted in the higher ABA/t-ZR ratio exhibited by the WS-ambient UV-B plants (Figure 2C, Figure 2D).

Both drought and enhanced UV-B radiation caused a significant increase in SOD activity (Table 2). However, the activation of the antioxidant enzyme was more pronounced in plants exposed to the high level of UV-B radiation. Additionally, hydrogen peroxide production significantly increased when plants were either exposed to water limitation or to the combination of both stresses (Table 2).

A reduction in chlorophyll photosynthetic pigments was evident in all stress treatments, being more prominent in plants exposed to the combined application of the stressors treatment (Figure 3A, Figure 3B and Table 3).

In addition to the stronger effect on chlorophyll degradation and irrespective of the water regime applied, UV-B radiation stimulated the accumulation of carotenoids in leaves of the plus 15 % UV-B plants (Figure 3C, Figure 3D).

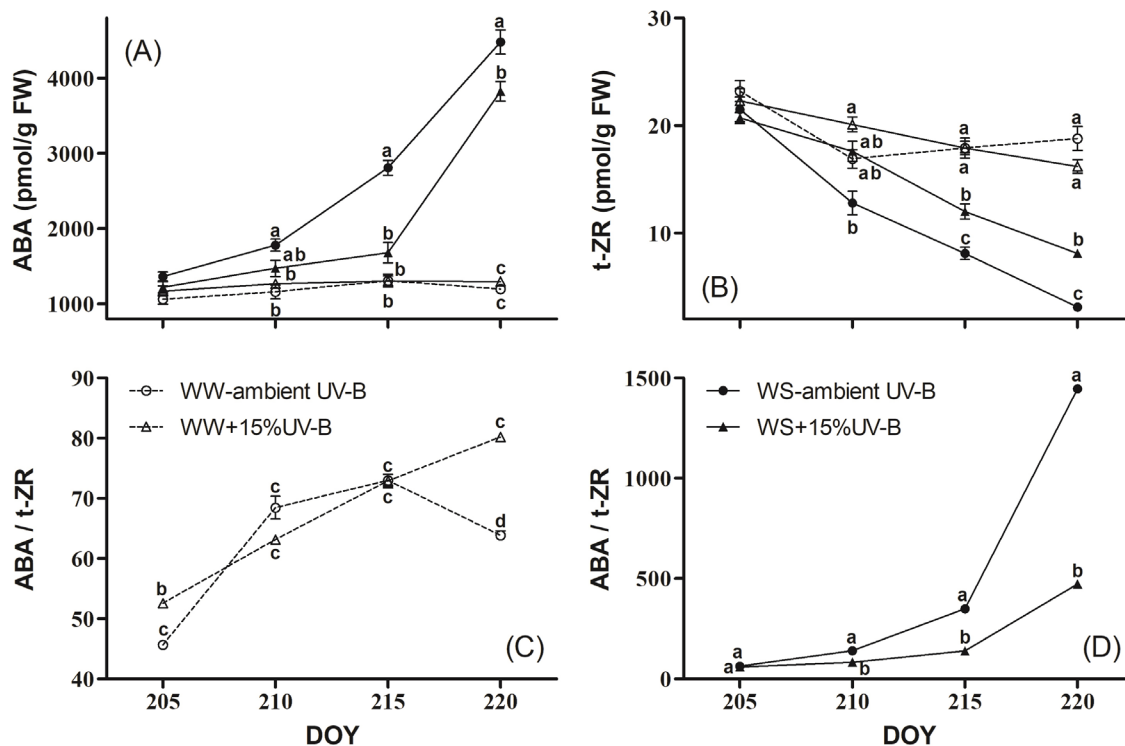


FIGURE 2. Changes in leaf ABA concentration (A), trans-zeatin riboside (t-ZR) (B), and in the ratio ABA/t-ZR (C and D) in Soultanina plants during the experimental period (DOY 205-220).

Each point is the mean \pm SE of 3 replicates. Different letters indicate statistically significant differences at $p < 0.05$. WW, well-watered; WS, water-stressed.

TABLE 2. Effects of water limitation, UV-B radiation and their combination on leaf ABA concentration, trans-zeatin riboside (t-ZR), ABA/t-ZR ratio, SOD and H_2O_2 content.

Source of variance	Treatment	Parameters				
		ABA (pmol/g FW)	t-ZR (pmol/g FW)	ABA/t-ZR	SOD (U/mg protein)	H_2O_2 (μ mol/g FW)
Irrigation	WW	1221.75 \pm 26.42a	19.16 \pm 0.56a	64.96 \pm 2.23a	71.67 \pm 4.10b	2.04 \pm 0.18b
	WS	2329.75 \pm 243.41b	12.99 \pm 1.30b	344.10 \pm 91.52b	82.78 \pm 3.32a	3.32 \pm 0.32a
Radiation	Ambient UV-B	1895.50 \pm 233.36a	15.29 \pm 1.36	281.13 \pm 93.80a	64.50 \pm 2.37b	2.12 \pm 0.25b
	\pm 15% UV-B	1656.00 \pm 175.84b	16.86 \pm 0.95	127.93 \pm 27.67b	89.96 \pm 2.61a	3.24 \pm 0.29a
Irr. x Rad.	WW-ambient UV-B	1181.75 \pm 41.77b	19.20 \pm 0.84a	62.73 \pm 3.17b	56.95 \pm 1.93c	1.48 \pm 0.12c
	WW \pm 15% UV-B	1261.75 \pm 29.83b	19.12 \pm 0.77a	67.18 \pm 3.15b	86.40 \pm 3.67a	2.61 \pm 0.22bc
	WS-ambient UV-B	2609.25 \pm 365.74a	11.37 \pm 2.08bb	499.53 \pm 167.90a	72.05 \pm 2.4b	2.76 \pm 0.38ab
	WS \pm 15% UV-B	2050.25 \pm 316.93ab	14.60 \pm 1.50ab	188.68 \pm 50.28ab	93.51 \pm 3.51a	3.88 \pm 0.47a
$P_{(irrigation)}$		0.000	0.000	0.000	0.001	0.000
$P_{(radiation)}$		0.026	0.079	0.000	0.000	0.002
$P_{(Irr. \times Rad.)}$		0.047	0.068	0.000	0.188	0.988

Different letters in the same column indicate significant differences at $P \leq 0.05$. Data represent average value \pm SE ($n = 12$ for ABA and t-ZR and $n = 18$ for SOD and H_2O_2). $P_{(irrigation)}$, significance of the irrigation effect; $P_{(radiation)}$, significance of the UV-B radiation effect; $P_{(irr. \times rad.)}$, significance of the irrigation x UV-B radiation interaction effect; WW, well-watered; WS, water-stressed.

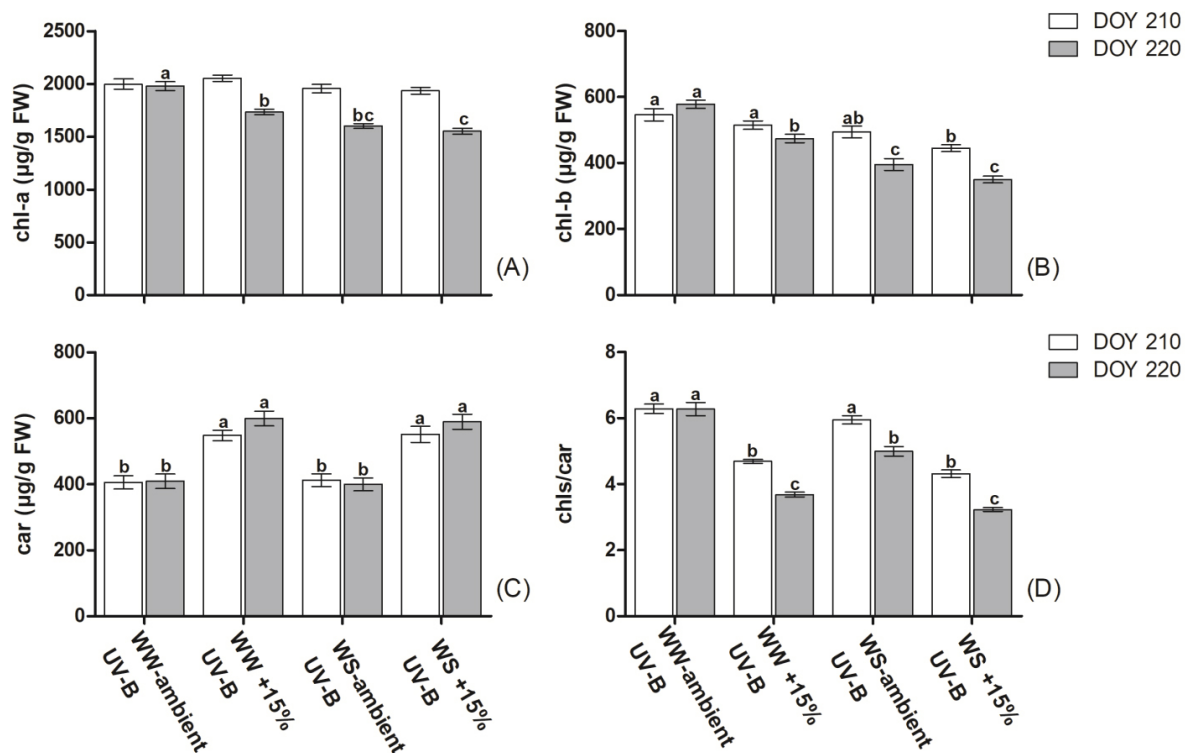


FIGURE 3. Average values of chlorophyll-a (chl-a) (A), chlorophyll-b (chl-b) (B), total carotenoids (car) (C) and of the chl-b/car ratio throughout the experimental period (DOY 210 and DOY 220) in all treatments ($n = 3$). Different letters indicate statistically significant differences at $p < 0.05$ for each DOY. WW, well-watered; WS, water-stressed.

DISCUSSION

Throughout the experimental period, Ψ_{PD} values were significantly reduced in accordance with the water supply, while UV-B radiation had no additional effect on leaf water relations (Figure 1B). Several authors have reported that both of these abiotic stressors can impose significant limitations on photosynthetic productivity (for a review, see Alonso *et al.*, 2016) by restricting the diffusion of CO_2 into the leaf as a result of stomatal closure (diffusional limitations), and/or by inhibiting PSII photochemical apparatus and CO_2 metabolism (photochemical limitations). In our study, the combination of drought and enhanced UV-B radiation (WS \pm 15 % UV-B treatment) appeared to have a synergistic effect on gas exchange parameters (Table 1), which is consistent with previous reports (Doupis *et al.*, 2011; Doupis *et al.*, 2016); however, the simultaneous and parallel decline in P_N and g_s values (Figure 1A, 1C), as well as the high WUE values (Figure 1D) exhibited by the combined stressors treatment, suggest that photochemical inhibition did not occur and that stomatal closure is the main factor responsible for the reductions in CO_2 assimilation.

The WW \pm 15 % UV-B treatment showed better performance compared to the other stress treatments, since WW \pm 15 % UV-B plants maintained higher values of photosynthetic rate and stomatal conductance. On the other hand, the gas exchange characteristics appeared to be more sensitive to water limitation than supplemental UV-B, suggesting that (a) when applied alone, the plus 15 % UV-B level, compared to water potential progressive drop to -0.6 MPa, was not stressful enough to report the occurrence of significant limitations to photosynthesis, and/or (b) the underlying defense mechanisms under drought stress are less effective in maintaining grapevine photosynthesis compared to UV-B adaptation strategies. Such contradictory results in the literature, concerning the impact of drought and high UV-B on photosynthetic performance, are usually attributed either to the fact that the response of the photosynthetic apparatus depends on plant species and developmental stage, or to differences in the experimental set-up (time, duration and severity of stress exposure) (Martínez-Lüscher *et al.*, 2015). With the results from a transcriptomic and metabolomic analysis on grapes irradiated with UV-B, Loyola *et al.* (2016)

recently showed that, upon low or high UV-B exposure, plants could regulate the expression of two photomorphogenic genes that play a part in the UV signaling pathway, a mechanism that allows the grapevine to acclimatise rapidly to UV-B. In any case, under the present experimental conditions, water limitation seemed to have a more adverse effect on the photosynthetic characteristics of grapevine than supplemental UV-B radiation.

Water deficit and elevated doses of UV-B radiation can have negative impacts on plant growth and performance. Numerous studies have shown that plants have evolved various adaptive mechanisms, including hormonal signaling cascades, that enable them to acclimatise to drought and/or high UV-B (Schachtman and Goodger, 2008; Lu *et al.*, 2009). On day 15 of our experiment, foliar ABA rose above 4 nmol g⁻¹ FW in the WS-ambient UV-B treatment, in comparison with nearly 1 nmol g⁻¹ FW ABA that was recorded for WW ± 15 % UV-B plants (Figure 2A). This indicates that drought stress may be the predominant factor in triggering foliar ABA accumulation. The combined action of the water deficit and UV-B radiation modified the ABA-induction response pattern throughout the experimental period, thus providing evidence that ABA is not an important mediator in grapevine defense responses under high levels of UV-B radiation. The lower leaf ABA concentration obtained in the supplemental UV-B treatments comes in disagreement: (a) with the study by Berli *et al.* (2010), who reported that ABA biosynthesis in grapevine is induced by high UV-B irradiance, and (b) the enhanced stomatal closure exhibited by the plus 15 %UV-B treatments (Figure 1A), since lower stomatal conductance values have been reported to be closely associated with an increase in leaf ABA concentration (Beis and Patakas, 2015). Although ABA is well-known for playing a crucial role in regulating stomatal movements under unfavourable conditions, previous studies have revealed a discontinuity in the crosstalk between UV-B and ABA-induced stomatal closure (He *et al.*, 2011). In addition, Nogués *et al.* (1999) concluded that UV-B radiation can have a direct effect on stomatal behaviour, through interfering in the osmotic solute flux from guard cells, and in the resultant changes in guard cell turgor and stomatal aperture, or by directly repressing the plasmalemma ATPase proton pump. It is also important to mention that numerous and diverse publications on the effects of UV-B on stomatal aperture and conductance, report that UV-B can not only stimulate stomatal closure, but also stomatal opening; this is a contradiction that

likely reflects the heterogeneity of experimental conditions and plant material (Jansen and van en Noort, 2000). Our results suggest that an ABA-independent signaling pathway is involved in the UV-B-induced stomatal closure in grapevine leaves. Accordingly, in our experiment, the similar leaf t-ZR levels in WW-ambient UV-B and WW ± 15 % UV-B plants showed that t-ZR, the most important of the isoprenoid cytokinins, had a non-significant contribution in alleviating UV-B radiation stress, through the control of stomatal conductance and the optimization of Water Use Efficiency (Figure 1A, 1D), since during the experiment leaf t-ZR levels in WW-ambient UV-B and WW ± 15% UV-B plants were very similar (Figure 2, Table 2).

The slight activation of the first-line of enzymatic antioxidants defense (SOD), together with the less pronounced increase in H₂O₂ in WS-ambient UV-B plants (Table 2), indicate that the up-regulation of the enzymatic antioxidant system is not the preponderant mechanism of adaptation under drought conditions. Conversely, under supplemental UV-B radiation (when applied alone), superoxide dismutase seemed to offer significant protection against ROS production, while the combination of the two stresses was synergistic and led to a marked increase in H₂O₂ accumulation (Table 2). This is in line with previous studies reporting that grapevines exposed to a combination of elevated UV-B and drought experienced more severe stress conditions than those exposed to single applications of the abiotic stressors (Doupis *et al.*, 2011; Doupis *et al.*, 2012).

The photoprotection of photosystem II (PSII) is necessary to avoid stress-induced damage to the photosynthetic machinery due to the imbalance between light capture and its utilisation, and the concomitant formation of Reactive Oxygen Species (ROS). Plants have developed a wide array of defense responses in order to restrict the formation of ROS and to scavenge any excessively produced ROS, thus avoiding damage related to active oxygen. As in our study (Figure 3; Table 3), a decrease in chlorophyll content in the presence of enhanced UV-B radiation (Kakani *et al.*, 2003) and under water limitation (Ashraf and Harris, 2013) was evident in most of the plant species reviewed. Although chlorophyll bleaching has been considered a typical symptom under the influence of abiotic stresses, our results suggest that chlorophyll reduction, and the resulting lowered capacity for the light harvesting of PSII (and the subsequent limited generation

TABLE 3. Effects of water limitation, UV-B radiation and their combination on chlorophyll-a (chl-a), chlorophyll-b (chl-b), total carotenoids (car) and the pigment ratio chlorophylls to carotenoids.

Source of variance	Treatment	Parameters			
		chl-a ($\mu\text{g/g FW}$)	chl-b ($\mu\text{g/g FW}$)	car ($\mu\text{g/g FW}$)	chls/car
Irrigation	WW	1907.16 \pm 24.25	541.92 \pm 9.26a	482.94 \pm 15.79	5.22 \pm 0.20b
	WS	1800.22 \pm 65.89	456.4 \pm 15.56b	484.19 \pm 15.12	4.76 \pm 0.20a
Radiation	Ambient UV-B	1910.87 \pm 62.95	524.34 \pm 14.78a	424.51 \pm 7.86b	5.74 \pm 0.13b
	\pm 15 % UV-B	1796.51 \pm 30.50	473.99 \pm 14.60b	542.63 \pm 10.62a	4.24 \pm 0.15a
Irr. x Rad.	WW-ambient UV-B	1965.57 \pm 18.41	570.86 \pm 8.84a	421.46 \pm 10.92b	6.05 \pm 0.10a
	WW \pm 15 % UV-B	1848.75 \pm 38.74	512.98 \pm 11.30ab	544.42 \pm 15.35a	4.40 \pm 0.20b
	WS-ambient UV-B	1856.17 \pm 125.27	477.82 \pm 21.01bc	427.55 \pm 11.73b	5.44 \pm 0.21a
	WS \pm 15 % UV-B	1744.27 \pm 43.53	434.9 \pm 22.08 ^c	540.83 \pm 15.33a	4.09 \pm 0.22b
$P_{(\text{irrigation})}$		0.219	0.001	0.955	0.011
$P_{(\text{radiation})}$		0.192	0.013	0.001	0.000
$P_{(\text{Irr. x Rad.})}$		0.976	0.647	0.829	0.309

Different letters in the same column indicate significant differences at $P \leq 0.05$. Data represent average values \pm SE ($n = 12$). $P_{(\text{irrigation})}$, significance of the irrigation effect; $P_{(\text{radiation})}$, significance of the UV-B radiation effect; $P_{(\text{irr. x rad.})}$, significance of the irrigation x UV-B radiation interaction effect; WW, well-watered; WS, water-stressed.

of chloroplast-derived ROS), may be a defense mechanism against irreversible photodamage (Herbinger *et al.*, 2002). In this respect, it should be noted that at the end of the experiment the maximal potential quantum yield of the PSII (Fv/Fm) values was around 0.83 and very similar in all stress treatments (data not shown), thereby supporting the absence of biochemical impairments of the photosynthetic apparatus.

It is also worth mentioning that a significant increase in carotenoid accumulation in both of the elevated UV-B treatments was observed (Fig 3C; Figure 3D), hence suggesting that isoprenoid molecules, such as carotenes and xanthophylls, participate in the ability for grapevine to adapt to increased UV-B radiation. Similar reports on the effects of UV-B on carotenoids (Berli *et al.*, 2010; Doupis *et al.*, 2012) underline the important role of specific carotenoids in maintaining the integrity of the photosynthetic membranes under oxidative stress by quenching the excited singlet and triplet state of chlorophyll, extending the range of the light absorbed by the photosynthetic machinery, and deactivating toxic ROS. On the other hand, since drought stress did not stimulate carotenoids synthesis (Figure 3C), we can assume that pigment formation may not be an important facet of grapevine drought tolerance.

In conclusion, when subjected to a gradual water deficit, grapevine showed a more pronounced

impairment of gas exchange characteristics than when exposed to supplemental UV-B radiation. The combination of drought and enhanced UV-B radiation appeared to have synergistic effects on net photosynthetic rate and stomatal conductance. No biochemical limitations to photosynthesis exist in stressed plants, and stomatal closure seems to be the main factor limiting photosynthetic activity. Moreover, our results revealed different adaptive responses to the abiotic stressor applied: Under water limitation, abscisic acid and trans-zeatin-riboside regulation appeared to have an important role in grapevine defense responses; whereas under high UV-B radiation, the modifications in the synthesis of the photosynthetic pigment pool and the activation of antioxidant enzymes may have been protective mechanisms against irreversible photodamage to PSII.

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