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REGULAR ARTICLE

Differences in antioxidant mechanisms in grapevines subjected to drought and enhanced UV-B radiation

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Abstract

The differences in antioxidant properties in grapevines (*Vitis vinifera* L. cv Romeiko) exposed to either drought, enhanced levels of UV-B radiation or to the combined application of the two abiotic stressors were studied. Two-year-old grapevines grown outdoors in 25 L pots containing peat:perlite:sand (3:1:1) were used. The following treatments were applied: i) well-watered, under ambient UV-B level treatment; (ii) water-stressed, under ambient UV-B level treatment; (iii) well-watered treatment under enhanced UV-B and (iv) water-stressed treatment under enhanced UV-B. Results indicated that predawn leaf water potential (Ψ_{PD}) decreased progressively in water-stressed treatments, irrespective of the level of the UV-B radiation. All treatments exhibited a close relation between photosynthetic rate (Pn) and stomatal conductance (gs), suggesting that stomatal closure is the dominant limitation to photosynthesis. Both drought and enhanced UV-B radiation caused a significant increase in hydrogen peroxide content (H_2O_2) and lipid peroxidation (TBARS). However, the accumulation of H_2O_2 was more pronounced in plants exposed to enhanced UV-B radiation. Independent of water supply, enhanced UV-B radiation significantly increased the activities of superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), quaiacol peroxidase (GPX, EC 1.11.1.7) and catalase (CAT, EC 1.11.1.6) as well as carotenoids content while the expression of antioxidant enzymes was lower in plants exposed only to drought conditions.

Key words: UV-B radiation, drought, antioxidants, gas exchange, *Vitis vinifera*

Introduction

In the Mediterranean zone, high levels of ultraviolet-B (UV-B: 280-320 nm) radiation and drought are a typical combination of abiotic stresses that plants often have to cope with during the growing season. In particular, the depletion of the earth's stratospheric ozone layer that occurred in the last decades has led to an increase in levels of UV-B radiation that reach the earth's surface. Many studies have already indicated significant effects of high UV-B doses in plants at physiological, morphological, anatomical and biochemical level (Rozema et al., 1997; Jansen et al., 1998). On the other hand, drought is considered to be an important environmental constraint limiting plant growth and yield worldwide (Flexas and Medrano, 2002; Chaves et al., 2003).

Studies on interaction between high levels of UV-B and drought indicate that the two abiotic factors can induce various responses in plants that could be either additive, synergistic or antagonistic (Gwynn-Jones et al., 1999; Alexieva et al., 2001). It is suggested that the type of responses exhibited by a plant organism to the combination of these two abiotic stresses is unique, depending on the genotype, nature, intensity and duration of the stresses applied (Alexieva et al., 2003).

A common consequence of plants exposure to environmental stresses is the over-production of highly reactive oxygen species (ROS), mostly in chloroplasts, mitochondria and peroxisomes (Beis and Patakas, 2012). The accumulation of high ROS concentrations could result in RNA and DNA damage, enzyme inhibition, protein oxidation and membrane lipid peroxidation (Mittler, 2002; Scandalios, 2002). Plants have developed an efficient antioxidant system for protection against these toxic effects of ROS. In particular, the activation of certain antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and quaiacol

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peroxidase (GPX) consists a significant component of plants ROS defence network. Moreover, chlorophyll loss (due to chlorophyll degradation or chlorophyll synthesis deficiency) reduces the amount of photons absorbed by leaves under unfavourable environmental conditions, thus enhancing the photoprotective and antioxidant leaf capacity (Smirnoff, 1993). Additionally, several non-enzymatic antioxidant molecules such as carotenoids are reported to contribute in effectively dissipating the excess of excitation energy under stress conditions by quenching triplet state chlorophyll molecules and scavenging singlet oxygen and other toxic ROS that are formed within the chloroplasts (Berli et al., 2010). However, the relative contribution of these antioxidant mechanisms in the protective responses of grapevine plants exposed to drought, enhanced UV-B and/or the combination of these two stressors remained obscure.

Thus the aim of this study is to elucidate possible differences in grapevines enzymatic and non-enzymatic defense mechanisms in response to drought and enhanced UV-B radiation.

Materials and Methods

Plant material and treatments

This study was carried out in the Institute for Olive Tree and Subtropical Plants in the city of Chania, Crete, Greece (35° 32' 00" N, 24° 04' 09" E). The climate is of Mediterranean type with hot and dry summers and mild and rainy winters. A total of twenty four, two-year-old, grapevine plants of the local red variety Romeiko, (*Vitis vinifera* L.) grafted onto 110-R rootstocks were used. All plants were grown in 25 L pots containing a peat:perlite:sand potting mix (3:1:1, v/v). Canopy management practices included: winter pruning to 3 nodes per vine, removal of the extra shoots immediately after budbreak (only the most robust shoot was allowed to grow on each plant) and vertical shoot positioning. The plants were divided into four groups, each one consisting of six plants. In each group of plants, one of the following treatments was applied over a 15-day period: (i) well-watered (WW) treatment, in which the plants were irrigated daily to soil capacity while being exposed to ambient UV-B radiation (WW-ambient UV-B treatment); (ii) water-stressed (WS) treatment, in which the plants were receiving every day, 50% of the amount of irrigation water provided to well-watered plants, under ambient UV-B levels (WS-ambient UV-B treatment), (iii) well-watered treatment under supplemental UV-B, in which the well-watered plants were exposed to ambient plus 30% UV-B radiation (WW+30% UV-B treatment); and (iv) water-stressed treatment under supplemental UV-B, in which the water-stressed plants were exposed to ambient plus 30% UV-B radiation (WS+30% UV-B treatment). Irrigation water was applied with drip emitters while enhanced UV-B radiation was supplied by UV-emitting fluorescent tubes (Philips, Ultraviolet-B, TL40W/12RS, Holland), covered with preheated, for 8 hours, 0.115 mm thick cellulose acetate (CA) film (Clarifoil, Binley, Coventry, UK) which transmits essentially no UV shorter than 290 nm. The CA sheets were changed every 20 hrs of operation, to avoid ageing effects of the filter. The irradiance in the UV-B band was checked daily with a broadband radiometer (SKU 430, Skye Instruments Ltd., Powys, UK). The supplemental biological effective UV-B dose ($UV\text{-}B_{BE}$) - estimated according to the generalised plant action spectrum (Caldwell, 1971) - corresponded to 19% ozone depletion (Green et al., 1974; 1980; Green, 1983). The supplemental UV-B level, as well as Caldwell's generalised plant action spectrum were selected to correspond with other studies in areas with similar latitude (35^o) (Levizou and Manetas, 2001).

Leaf water potential determination and gas exchange measurements

Predawn leaf water potential (Ψ_{PD}) was used as a sensitive indicator of grapevine water status (Williams and Araujo, 2002). Ψ_{PD} values were determined with a pressure chamber (PMS Instrument Company, Corvallis, Oregon) according to the method of Scholander et al. (1965). The readings were taken, beginning at 4:30 a.m. and ending before sunrise, using fully expanded leaves. In particular, the fourth to sixth mature leaf from the shoot apex was used. Leaf photosynthetic rate (Pn) and stomatal conductance (Gs) were recorded with a Li-6400, portable photosynthesis system (Li-Cor Bioscience Inc, Lincoln, Nebraska, US), on fully expanded, healthy leaves in the morning (between 09:30 and 11:00) at saturated light intensities (PPFD greater than 1000 $\mu mol\ m^{-2}\ sec^{-1}$).

Enzymatic antioxidant activity, lipid peroxidation and hydrogen peroxide measurements

SOD, GPX, APX and CAT activities were determined on six leaves per treatment which were collected three times during the experimental period. The extraction medium consisted of 0.1 M K-P (potassium phosphate) buffer (pH 7.6), containing 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2), 0.5 mM ascorbate and

1% PVPP (polyvinylpolypyrrolidone). 0.3 g of leaf tissue was homogenised in 1.5 mL of the extraction buffer and the homogenate was centrifuged at 13 000 x g for 30 min. The supernatant was used for assaying the activities of the enzymes. The absorbance of the crude enzymes extract was measured with a Hitachi U-1100 spectrophotometer (Hitachi Ltd., Tokyo, Japan). SOD isoforms (EC 1.15.1.1) activity was determined using the methodology described by Becana et al. (1986) based on the capacity of the enzyme extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) to blue formazan. GPX (EC 1.11.1.7) assay was performed using the method described by Costa et al. (2002). GPX activity was calculated following the oxidation of guaiacol at 470 nm using an extinction coefficient of 26.6 $mMcm^{-1}$. APX (EC 1.11.1.11) activity was determined according to Nakano and Asada (1981), by measuring the change in absorbance at 290 nm ($E = 2.8\ mMcm^{-1}$). CAT (EC 1.11.1.6) activity was assayed according to Cakmak and Marschner (1992). Soluble protein content was measured according to the method of Bradford (1976), using Sigma-Aldrich, Total Protein Kit, Micro (product code TP0100, Sigma-Aldrich, Saint Louis, Michigan, USA). For the determination of hydrogen peroxide (H_2O_2) and lipid peroxidation (TBARS), leaf tissue (0.5 g) obtained from the same leaves that were used for the antioxidant enzyme assays, 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. H_2O_2 content was measured spectrophotometrically after reaction with potassium iodide (KI) (Alexieva et al., 2001). The absorbance was measured at 390 nm using a solution consisting of TCA 0.1% and pure catalase reagent as a blank, to ensure zero interference. In order to calculate the amount of hydrogen peroxide, a standard curve was prepared with known H_2O_2 concentrations. TBARS (Thiobarbituric Acid Reactive Substances) index is taken as the measure of in vivo lipid peroxidation of plant tissue. TBARS were estimated by the method of Hodges et al. (1999). The absorbance of the supernatant was read at 532 nm with the values for non-specific absorption at 600 nm and 440 nm subtracted.

Photosynthetic pigments analysis

Chlorophyll (Chl-a and Chl-b) and carotenoids (Car) were extracted from leaves with 80% acetone. Chl and Car content was determined spectrophotometrically according to the method of Lichtenthaler and Welburn (1983).

Statistical analysis

All data were subjected to a two-way ANOVA using the SPSS 15.0 (SPSS Inc., Chicago, Illinois, USA). The LSD test was used for the comparison of treatments average values. Statistical comparisons were considered significant at $P < 0.05$.

Results and Discussion

The more rapid decrease in photosynthetic rate exhibited by the plants exposed to higher UV-B intensities (Figure 1A) could be attributed to changes in stomatal conductance (Figure 1B). Indeed, stomatal conductance was significant lower in plants exposed to elevated UV-B intensities irrespective the water regime applied. Since stomatal conductance strongly depends on leaf water relations (Beis and Patakas, 2010), it was expected Ψ_{PD} values to be lower in plants under enhanced UV-B radiation (compared with WS-ambient UV-B treatment), as a consequence of the rapid stomatal closure (Figure 2). However, this was not evident in our results where WW+30% treatment exhibited higher values in predawn leaf water potential (Figure 2). The latter indicates that high levels of UV-B could probably affect stomatal function through an alternative mechanism, which is not directly related to leaf water status. This is consistent with the results obtained by Negash and Bjorn (1986) and Nogués et al. (1999).

On the other hand, the reduction of stomatal conductance is known to induce a serious decline in CO_2 concentration in chloroplasts, which in turn increases plant's susceptibility to photoinhibition (Beis and Patakas, 2012). In fact, under limiting conditions of CO_2 fixation, the rate of reducing power production could overcome the rate of its use in photosynthetic electron transport chain, thus damaging the photosynthetic apparatus. In order to prevent the production of excess reducing power plants have developed different protection mechanisms including reduction of light absorbance by adjusting chlorophyll content and/or antenna size as well as by increasing thermal dissipation (NPQ) of excess absorbed light in the light-harvesting complexes, through the xanthophyll and the lutein cycle (Müller et al., 2001; Li et al., 2009). In our results a reduction in chlorophyll photosynthetic pigments was evident in all stress treatments, being more pronounced in plants exposed to enhanced UV-B intensities irrespective the water regime applied (Fig. 3A and 3B). This is consistent with the results reported for several other species indicating a significant decrease in total chlorophyll content in plants

grown under high UV-B intensities (Liu et al., 2011). In contrast, carotenoids concentration was increased under enhanced UV-B radiation (Fig. 3C). These results are consistent with those of Majer and Hideg (2012) and Berli et al. (2010) who also indicated an increase in carotenoids in grapevine plants exposed to enhanced UV-B levels. This increase in carotenoids content could play an important photoprotective role either by dissipating excess excitation energy as heat or by singlet oxygen scavenging (Majer and Hideg, 2012). However, recent studies suggested that no singlet oxygen was produced in response to oxidative stress caused by high UV-B doses and consequently no correlation exist between total carotenoids content and singlet oxygen scavenging capacities (Majer and Hideg, 2012). Thus, the exact role of carotenoids under enhanced UV-B conditions remains to be elucidated.

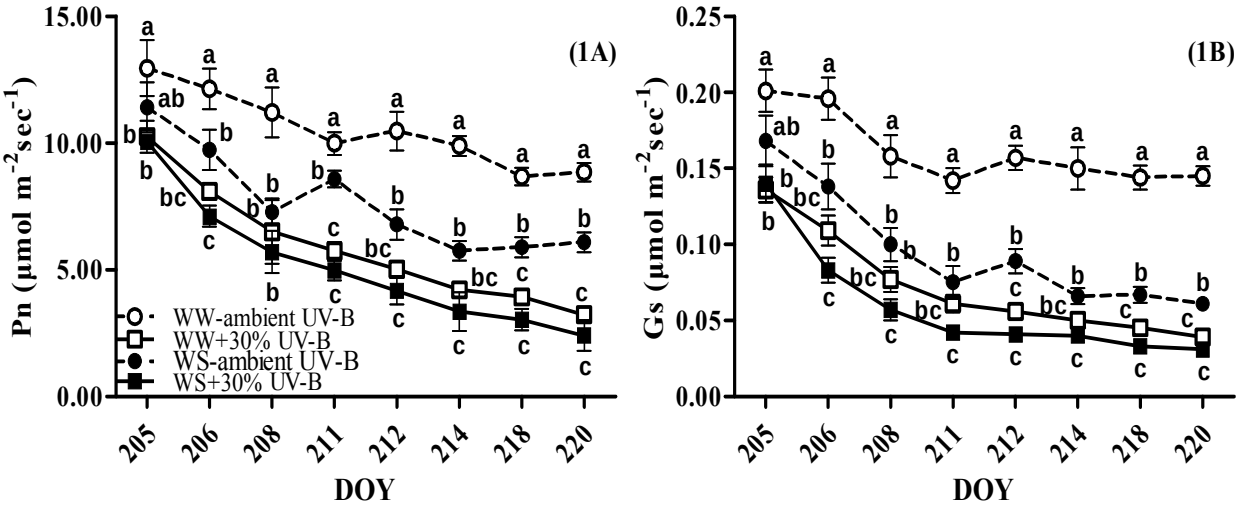

Figure 1. Changes in leaf photosynthetic rate-Pn (A) and stomatal conductance-Gs (B) throughout the experimental period (DOY-Day Of Year) in all treatments.
Each symbol represents the average ± standard error of 4 values. Different letters indicate significant difference at $P < 0.05$.

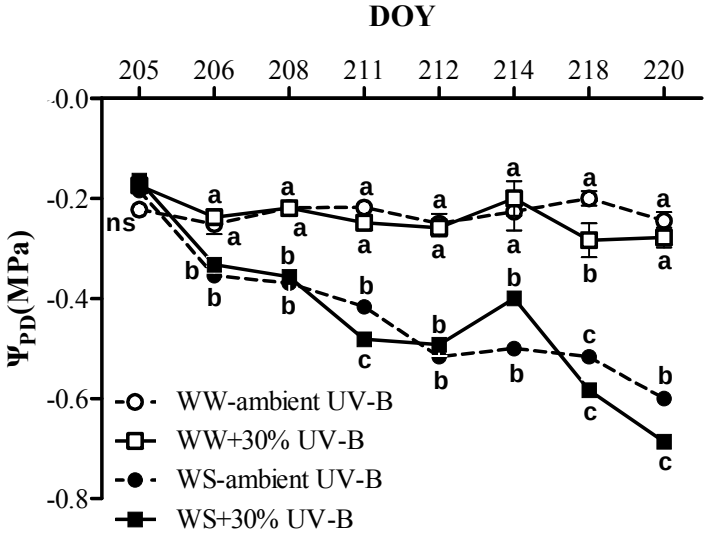

Figure 2. Changes in leaf predawn water potential (Ψ_{PD}) during the experimental period (DOY-Day Of Year) in all treatments.
Each point represents the mean value ± standard error of 4 measurements. Different letters indicate significant difference at $P < 0.05$.

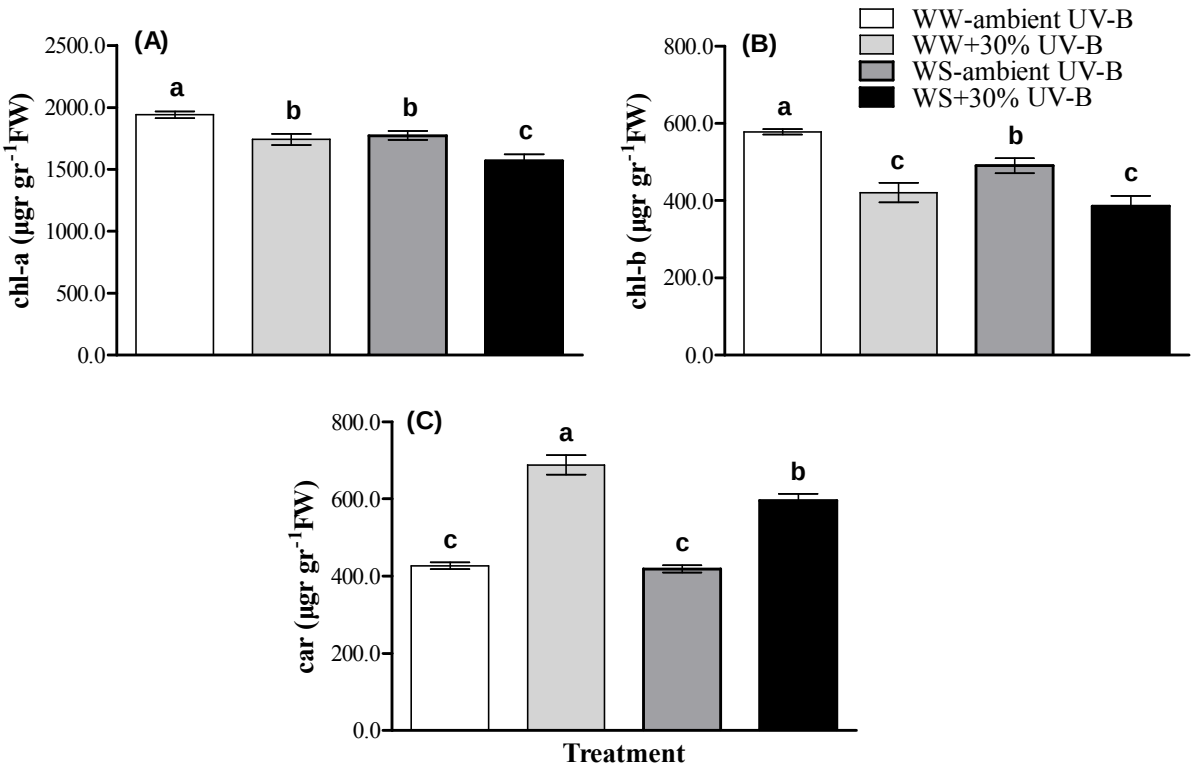

Figure 3. Average values of chlorophyll-a (chl-a), chlorophyll-b (chl-b) and total carotenoids (car) throughout the experimental period in all treatments (n=18).
Means with different letters are significantly different ($P < 0.05$).

Changes in environmental conditions result in metabolic imbalances that can induce an oxidative stress in cells by promoting the generation and accumulation of Reactive Oxygen Species (ROS). In order to cope with oxidative damage, plants have developed an antioxidant system that includes the up-regulation of different enzymes (CAT, GPX, APX, SOD) (Foyer and Noctor, 2005). SOD isoforms constitute the first line of antioxidant defence and have been identified as a family of metalloenzymes, catalyzing the scavenging of O_2^- to H_2O_2 (Noctor and Foyer, 1998). CAT isoforms are localized predominantly in peroxisomes and glycoxysomes and are heme proteins that convert H_2O_2 to water and oxygen (Dat et al., 2000), and various peroxidases (POX) such as APX and GPX are localized throughout the cell (mainly in cell walls and vacuoles) and decompose H_2O_2 by oxidation of substrates such as phenolic compounds and/or antioxidant's metabolites (Gaspar et al., 1991). In our results a significant increase in SOD activities was evident in plants exposed to enhanced UV-B radiation irrespective the water regime applied (Table 1). SOD rapidly converts O_2^- to H_2O_2 which can then be converted to water by CAT, APX and GPX. GPX and APX activities followed similar patterns to those of SOD, exhibiting higher values in plants exposed to elevated UV-B radiation (Table 1). On the other hand, CAT activity significantly increased only in plants exposed to the combination of elevated UV-B and drought (Table 1). However, the observed significant increase of all antioxidant enzymes in this treatment was proved to be ineffective to scavenge and reduce H_2O_2 concentration and consequently to protect cells from lipid peroxidation (Table 1). Considering that lipid peroxidation could be used as an abiotic stress intensity index, it can be concluded that the plants exposed to the elevated UV-B radiation and particularly the plants exposed to the combination of elevated UV-B and drought experienced more severe stress conditions compared to those exposed to drought.

Table 1. Effects of drought and enhanced UV-B radiation on the antioxidant enzymes activities as well as on H_2O_2 (μmol $gr^{-1}FW$) concentration and lipid peroxidation.
Data represent average values ± standard error (n=18). Different letters in the same column indicate significant difference at P < 0.05.

Treatments	Biochemical Parameters					
	SOD	GPX	APX	CAT	H_2O_2	TBARS
WW-ambient UV-B	49.21 ± 1.05b	0.11 ± 0.01b	0.23 ± 0.01b	8.56 ± 0.13b	1.11 ± 0.06c	12.38 ± 1.58c
WW+30% UV-B	95.25 ± 5.76a	0.30 ± 0.04a	0.38 ± 0.04a	9.34 ± 0.23b	3.74 ± 0.49ab	43.61 ± 4.82a
WS-ambient UV-B	69.38 ± 2.22b	0.15 ± 0.02b	0.27 ± 0.03b	9.34 ± 0.19b	2.66 ± 0.30b	26.63 ± 2.80b
WS+30% UV-B	110.76 ± 8.24a	0.44 ± 0.07a	0.44 ± 0.06a	10.65 ± 0.40a	4.35 ± 0.41a	49.11 ± 5.76a

SOD (U mgr-1protein); GPX (μmol quaiacol mgr-1protein min-1); APX (μmol ascorbate mgr-1protein min-1); CAT (μmol H_2O_2 mgr-1protein min-1); H_2O_2 (μmol gr-1FW); TBARS (μmol gr-1FW)

Conclusions

Exposure of grapevines to enhanced levels of UV-B radiation resulted in a more pronounced oxidative stress compared to drought. The grapevines antioxidant defence mechanism includes the activation of several antioxidant enzymes as well as changes in photosynthetic pigments and carotenoids.

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