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Characterization of the adaptive response of grapevine (cv. Tempranillo) to UV-B radiation under water deficit conditions

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ABSTRACT

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3 This work aims to characterize the physiological response of grapevine (*Vitis vinifera* L.) cv.
4 Tempranillo to UV-B radiation under water deficit conditions. Grapevine fruit-bearing cuttings
5 were exposed to three levels of supplemental biologically effective UV-B radiation (0, 5.98 and
6 9.66 kJ m⁻² day⁻¹) and two water regimes (well watered and water deficit), in a factorial design,
7 from fruit-set to maturity under glasshouse-controlled conditions. UV-B induced a transient
8 decrease in net photosynthesis (A_{net}), actual and maximum potential efficiency of photosystem
9 II, particularly on well watered plants. Methanol extractable UV-B absorbing compounds
10 (MEUVAC) concentration and superoxide dismutase activity increased with UV-B. Water deficit
11 effected decrease in A_{net} and stomatal conductance, and did not change non-photochemical
12 quenching and the de-epoxidation state of xanthophylls, dark respiration and photorespiration
13 being alternative ways to dissipate the excess of energy. Little interactive effects between UV-B
14 and drought were detected on photosynthesis performance, where the impact of UV-B was
15 overshadowed by the effects of water deficit. Grape berry ripening was strongly delayed when
16 UV-B and water deficit were applied in combination. In summary, deficit irrigation did not modify
17 the adaptive response of grapevine to UV-B, through the accumulation of MEUVAC. However,
18 combined treatments caused additive effects on berry ripening.

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36 Key words: photosynthesis, phenology, UV-B radiation, UV-B absorbing compounds, *Vitis*
37 *vinifera* L., water deficit
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1. INTRODUCTION

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3 Mediterranean vegetation is often exposed to high fluence rates of UV-B radiation (280-315
4 nm), because of cloudless summer sky. In addition, the absence of precipitation is considered
5 as a major limiting factor for plant growth and development during the summer months in this
6 area . In many regions, reduced water availability is frequently accompanied by increased UV-B
7 radiation levels [1]. Predicted scenarios of climate change over the next decades include a
8 pronounced decrease in precipitation, especially in the warm season, thus increasing the
9 probability of extreme drought events in the Mediterranean area [2]. Besides, changes in mean
10 cloudiness may affect the levels of solar radiation, including UV-B, reaching Mediterranean
11 ecosystems in the near future [3].
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22 The effects of UV-B radiation on leaf physiology have been extensively studied. They include
23 changes on leaf ultrastructure and anatomy, reduction in the concentration of photosynthetic
24 pigments and photosynthetic capacity, altered plant phenology and reduction of plant biomass
25 production [4]. In addition, when exposed to UV-B, plant cells usually produce reactive oxygen
26 species (ROS), regardless of the dose applied, as a result of the disruption of metabolic
27 activities or by the activation of membrane-localized NADPH-oxidase [5]. In order to cope with
28 oxidative damage, plants activate their antioxidant metabolism. Increases in superoxide
29 dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX)
30 activities have been found in many UV-B exposure studies [5]. Another of the direct UV-B
31 adaptive mechanisms best studied is the biosynthesis of UV-B absorbing compounds, mainly
32 flavonoids. UV-B responsive flavonoids located in the vacuoles of epidermal cells have the
33 potential to attenuate the penetration of UV-B radiation [6]. In addition, dihydroxy flavonoids
34 located in the chloroplasts of mesophyll cells may have a central role in the antioxidant defense
35 system, inhibiting the generation of ROS and reducing ROS once they have formed [7], thus
36 avoiding oxidative damage to DNA, structural proteins, lipids and other cellular compounds. The
37 chloroplast-located flavonols may have a very peculiar location which is of increasing
38 significance when excess of radiant energy-induced depletion of key components of the
39 antioxidant network system allows the diffusion of ROS out of the chloroplast [7].
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1 Evidence of interaction between UV-B exposure and drought stress in plants has emerged in
2 recent years. A series of similar defense mechanisms, as well as adverse changes, leading to
3 disruption of physiological processes, growth inhibition and yield reduction, take place in plants
4 exposed to single action of water deficit and UV-B radiation [1]. Therefore, combined action of
5 water deficit and UV-B radiation can modify the response patterns. Previous studies show that
6 UV-B radiation can interact with water deficit to induce protective mechanisms, i.e. increased
7 levels of UV-B absorbing compounds (flavonoids) and antioxidant enzymes (SOD, CAT and
8 APX), osmolyte accumulation (i.e. proline), increased leaf cuticle thickness or reduced stomatal
9 conductance. These responses may substantially decrease UV-B and/or drought sensitivity
10 when both factors are applied in combination [8, 9]. Furthermore, both UV-B and water deficit
11 cause the generation of ROS, nitric oxide, and induce the synthesis of abscisic acid, ethylene,
12 jasmonic and salicylic acid. These molecules, controlling plant reactions to both water deficit
13 and UV-B applied separately, may also be involved in cross-talk under their combined actions
14 [1, 10]. Nevertheless, responses are not always consistent between studies, thus leading in
15 some cases to contradictory results. This is the case of Sullivan and Teramura [11], Duan, et al.
16 [12] and Bernal, et al. [13], who found few significant interactions between UV-B and water
17 stress on plant growth and photosynthetic activity. On the other hand, when UV-B or water
18 deficit, applied alone, causes damages, their combined action usually became more
19 detrimental. For instance, combined stresses treatment led to the inhibition of soluble sugars
20 accumulation, restricting the capacity for osmotic adjustment, which caused negative additive
21 effect on rosette growth of *Arabidopsis thaliana* [14]. Additive negative effects on the growth of
22 grapevine and willows have also been reported [15, 16]. Thus, the coincidence of water deficit
23 and UV-B radiation, depending on their intensity, duration and sequence of occurrence, may
24 activate mechanisms allowing plants to cope under stress conditions or enhance harmful
25 effects. Such contrasting responses highlight the capacity for interaction between water deficit
26 and UV-B, but clearly demonstrate the need for further studies of these interactive responses.

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53 The aim of this work was to characterize the response of grapevine cv. Tempranillo to UV-B
54 radiation under water deficit conditions, focusing on plant development, photosynthetic
55 performance, antioxidant enzymes and UV-B absorbing compounds. Although the cultivar
56 Tempranillo, in particular, is probably well adapted to relatively high UV-B irradiance [17, 18],
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1 the study of the interactive effects of UV-B and water deficit may help to understand the
2 acclimation response of grapevine to UV-B within a climate change context, where the influence
3 of other co-occurring abiotic stress factors can modulate grapevine responses to UV-B
4 radiation. To this end, the fruit-bearing cutting model system for grapevine (fruiting plants
5 developed from rooted cuttings) was used, providing a feasible system to study the impact of
6 stress factors effects on grapevine physiology, without other confounding environmental
7 influences. This model allows the development of vegetative (roots, leaves and shoots) and
8 reproductive (inflorescences and clusters) organs as for the vineyard grapevines, under fully
9 controlled environmental conditions. Fruit-bearing cuttings respond like vineyard plants to
10 different cultural factors [19, 20]. Indeed, the fruit-bearing cuttings model has been previously
11 useful in the evaluation of the physiological response of grapevine to environmental or
12 developmental factors [17, 20-22].
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27 **2. MATERIAL AND METHODS**

28 *2.1. Plant material and growth conditions*

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Fruit-bearing cuttings of *Vitis vinifera* L. cv. Tempranillo were produced as described in
Martínez-Lüscher, et al. [17]. Briefly, three-node segments of grapevine cv. Tempranillo were
collected, from Station of Viticulture and Enology of Navarra (Olite, Spain). Rooting was induced
using indol butiric acid in a heat-bed (27°C) kept in a cool room (5°C) in darkness. When
cuttings developed enough roots, they were transplanted to 6.5 L pots containing 1:1 soil:peat
(v/v). Only one flowering stem was allowed to develop on each plant. Shoots were fixed to
horizontal rods compelling leaves to face upwards. Vegetative growth was controlled by
pruning, in order to not-exceed an optimal leaf area to grape mass ratio (ca. 12 leaves per
plant). The experiment was carried out in glass-houses [23] at the University of Navarra
(Pamplona, Spain, 42°48'14" N, 1°39'54" W) from April to September 2011. Throughout all the
experiment, growth conditions in the glass-houses were 25/15°C, 50/90% relative humidity (RH)
(day/night) and natural light. Glass-house walls and structure filtered up to 99.9% of UV-B, 35%
of UV-A and 15% of the photosynthetically active radiation (PAR) coming from the sun. A
supplemental system of high-pressure sodium lamps (SON-T Agro Philips, Eindhoven,

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Netherlands) was triggered when PAR dropped below a photon flux density of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ (14h photoperiod) [23].

2.2. Experimental design

When fruit-set was complete for all plants, three levels of biologically effective UV-B (0, 5.98 and $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$), calculated using the generalized plant action spectrum of Caldwell [24] as formulated by Green et al. [25], and two water availabilities (well watered and water deficit) were applied in a factorial design (12 plants per treatment) until maturity.

UV-B was applied by means of narrow band lamps TL100W/01 (311-313 spectrum peaking; Philips, Netherlands) hanging above the canopy. Spectral scans were performed with a double monochromator spectroradiometer (model SR9910, Macam Photometrics Ltd., Livingstone, Scotland) (Fig. S1). UV-A radiation emitted by the lamps accounted for less than 1% of the solar UV-A irradiance (W m^{-2}) reaching the plants during the irradiation period. In the treatment of $0 \text{ kJ m}^{-2} \text{ d}^{-1}$, lamps on top were unlit. The UV-B doses of 5.98 and $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ were applied using the same fluence rate (0.55 W m^{-2}), but during different exposure times: 3 h for the dose of $5.98 \text{ kJ m}^{-2} \text{ d}^{-1}$ and 3 h and 51 min for the dose of $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$. The applied biologically effective dose of $5.98 \text{ kJ m}^{-2} \text{ d}^{-1}$ corresponded to 86% of the maximum daily dose at the study site, whereas $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ is only reached in viticulture suitable sites of the southern hemisphere, such as Argentina, South Africa or Australia .

Soil water sensors (Watermark™ soil moisture sensors, Irrrometer, USA), placed in every pot, were used to control irrigation. Well watered plants were maintained at around 80% of substrate field capacity. Water deficit plants received only 50% of the water received by well-watered plants. Irrigation was performed with the nutritive solution described by Ollat et al. [19]. Nutrient solution concentrations were adjusted to provide the same amount of nutrients to each treatment batch.

Water status, gas exchange, chlorophyll fluorescence and MEUVAC were measured 7, 30 and 60 days after the beginning of the treatments. Maximum potential efficiency of photosystem II (Fv/Fm) was measured on days 1 to 7, 15, 30 and 60 after the beginning of the treatments. Plant growth, leaf photosynthetic pigments, antioxidant enzymes and lipid peroxidation were

1 measured after 60 days of treatment. Given the importance of comparing leaves of the same
2 physiological age in UV studies, young fully expanded leaves of the same age (around four
3 week old) were chosen. On day 7, these leaves had been fully developed before the beginning
4 of the treatments, under the glass-house conditions described previously. On day 30 and 60,
5 young fully expanded leaves developed during the treatments were chosen, as far as possible.
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10 2.3. *Plant growth parameters and phenological development*

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12 At maturity, leaf area was measured immediately after harvest with a leaf area meter (LI-
13 300 model; Li-Cor, Lincoln, USA). Leaf dry matter (DM) was determined after drying at 80°C for
14 2 days. Distance between shoot nodes was also determined. Three events (fruit-set, onset of
15 veraison and maturity) were selected for the study of phenological development, determined as
16 the elapsed time (days) between fruit-set to the onset of veraison, between the onset of
17 veraison to maturity and between fruit-set and maturity.
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26 2.4. *Leaf relative water content (RWC)*

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28 Relative water content (RWC) was estimated by a modification of the method of
29 Wheatherley [26], using leaf discs of 1 cm², and calculated as $RWC = 100 \times (FW - DW) / (TW - DW)$,
30 where FW, DW and TW denote fresh, dry and turgid weight, respectively. TW was
31 calculated after fully hydrating fresh leaf discs in darkness at 4°C for 24h. DW was determined
32 after drying at 80°C for 2 days in an oven.
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41 2.5. *Gas exchange and chlorophyll fluorescence*

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43 Gas exchange measurements were conducted using a portable photosynthesis system (GFS-
44 3000, Walz, Germany) with a 3 cm² cuvette. Dark respiration (R_D) measurements were
45 performed 2 h before the beginning of the light period. Environmental conditions in the
46 measurement chamber were: ambient CO₂ concentration=375 ppm, air temperature=25°C and
47 RH=50%. Gas exchange characteristics in illuminated leaves were measured at mid-morning
48 under the same environmental conditions in the cuvette, and with a photosynthetically active
49 photon flux density (PPFD) of 1200 μmol photons m⁻² s⁻¹. Net photosynthesis (A_{net}), stomatal
50 conductance (g_s), sub-stomatal CO₂ concentration (C_i) and R_D were calculated according to von
51 Caemmerer and Farquhar [27]. Chl fluorescence was measured immediately after the gas
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1 exchange measurements (in the dark and in the light), with a fluorescence module (PAM-
2 fluorometer 3055-FL, Walz, Germany) attached to the photosynthesis equipment. The
3 experimental protocol for analysis of Chl fluorescence quenching was performed according to
4 Martínez-Lüscher, et al. [17]. Parameters monitored were maximum potential PSII efficiency
5 ($F_v/F_m = (F_m - F_o)/F_m$), actual PSII efficiency ($\Phi_{PSII} = (F_m' - F_s)/F_m'$), photochemical quenching
6 ($qP = (F_m' - F_s)/(F_m' - F_o')$) and non-photochemical quenching ($NPQ = (F_m/F_m') - 1$). The electron
7 transport rate was calculated as: $ETR = \Phi_{PSII} \times PPFD \times 0.5 \times 0.84$, according to Krall and
8 Edwards [28], where 0.5 was used as the fraction of energy distributed between the two
9 photosystems [29] and 0.84 is the fractional leaf absorptance [30]. Multiplying 0.84 x 0.5 gives a
10 value of 0.42, a value very similar to the α term reported for grapevine cv. Tempranillo [31].
11 Photorespiration was estimated as: $R_L = 1/12(ETR - 4 \times (A_{net} + R_D))$, according to Valentini, et al.
12 [32].
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25 2.6. Sampling for biochemical assays

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27 The same leaves used for photosynthesis and Chl fluorescence measurements were used for
28 the biochemical analysis. Two leaf disks of 1 cm² were harvested immediately after gas
29 exchange and Chl fluorescence measurements, cut with a calibrated cork borer, wrapped in
30 aluminum foil, immediately plunged into liquid N₂ and stored at -80°C until photosynthetic
31 pigments and MEUVAC analyses. Then, the leaf was detached and frozen in liquid N₂ for lipid
32 peroxidation (thiobarbituric acid reacting substances, TBARS), antioxidant enzyme activities
33 and protein determinations.
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43 2.7. Methanol extractable UV-B absorbing compounds (MEUVAC) and photosynthetic 44 pigments

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46 Methanol extractable UV-B absorbing compounds were determined according to Dai et al. [33].
47 Frozen leaf disks were extracted with 10 mL of methanol:water:7M HCl (70:29:1 by volume) at
48 85°C in the dark for 15 min. The extracts were centrifuged at 2,000 g for 15 min, and the
49 absorbance spectrum was measured in a spectrophotometer UV/Vis (UVMini 1240, Shimadzu,
50 Kyoto, Japan). The amount of MEUVAC was expressed in arbitrary units as the area under the
51 absorbance curve in the UV-B interval ($AUC_{280-315}$), calculated per unit of leaf area.
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1 Leaf photosynthetic pigments were determined according to Larbi et al. [34]. Pigments were
2 extracted with acetone in the presence of sodium ascorbate, filtered (0.45- μ m) and quantified by
3 HPLC. Two eluents were used: mobile phase A (acetonitrile:methanol, 7:1, v/v) was pumped for
4 3.5 min, and then mobile phase B (acetonitrile:methanol:water:ethyl acetate, 7:0.96:0.04:8 by
5 volume) was pumped for 4.5 min. Modified triethylamine (0.7% by volume) was added to both
6 solvents to improve pigment stability. De-epoxidation state of the xanthophyll cycle was
7 calculated as: $DES=(A+Z)/(V+A+Z)$, where V, A and Z were antheraxanthin, violaxanthin and
8 zeaxanthin concentrations, respectively.
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10 *2.8. Determination of total soluble protein and antioxidant enzymes*

11 Frozen leaf tissue (1 g) was homogenized with 10 mL of 0.1 M potassium phosphate buffer (pH
12 7.0), containing 0.1 mM EDTA- Na_2 , 0.5 mM ascorbate and 1% PVPP (polyvinyl polypyrrolidone)
13 in an ice bath. The homogenate was filtered and centrifuged at 28,710 g and 4°C for 10 min.
14 The supernatant was used for determinations of protein content and antioxidant enzyme
15 activity. Total soluble protein concentration was determined as described by Bradford [35] using
16 bovine serum albumin as standard. Superoxide dismutase (SOD, EC 1.15.1.1) was determined
17 by the nitroblue tetrazolium (NBT) method [36] with some modifications. Guaiacol peroxidase
18 (GPX, EC 1.11.1.7) assay was performed using the method described by Pütter [37]. Catalase
19 (CAT, EC 1.11.1.6) activity was assayed by measuring the rate of H_2O_2 disappearance at 260
20 nm as described by Aebi [38] with some modifications. Ascorbate peroxidase (APX, CE
21 1.11.1.11) activity was determined as described by Nakano and Asada [39] with some
22 modifications. See Martínez-Lüscher et al. [17] for more details on enzyme activity
23 determination and methods modification.
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25 *2.9. Determination of lipid peroxidation*

26 Lipid peroxidation was estimated by measuring the concentration of thiobarbituric acid reacting
27 substances (TBARS), as described by Dhindsa et al. [36], in leaf samples taken 60 days after
28 the beginning of the experiments. Frozen leaf tissue (0.5 g) was homogenized with 10 mL
29 trichloroacetic acid (TCA) 0.1% (w/v) in an ice bath, filtered and centrifuged at 28,710 g and 4°C
30 for 10 min. Two mL of extract were mixed with an equal volume of either (i) 20% (w/v) TCA
31 solution or (ii) 20% TCA solution containing 0.5% (w/v) TBA. The mixtures were incubated into a
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1 hot bath (95°C) for 30 min and centrifuged at 10,000 g and 4°C for 10 min. The absorbance of
2 the supernatant was read at 532 nm and 600 nm. TBARS concentration was calculated as:
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4 $[(A_{532} \times 1000) - (A_{600} \times 1000)] / 155$ being 155 the extinction coefficient in $\text{mM}^{-1} \text{cm}^{-1}$ [40].
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6 7 2.10. *Statistical analyses*

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9 Statistical analysis was performed using XLstat-Pro (Addisoft). The data were subjected to a
10 three-factor analysis (ANOVA 3x3x2) to partition the variance into the main effects (stage, UV-B
11 and water deficit) and the interaction among them. In case of significant interaction among
12 factors, treatments were compared using the least significant difference (LSD) post-hoc test
13 (p<0.05).
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20 21 3. RESULTS

22 23 3.1. *Phenological development and plant growth parameters*

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25 UV-B radiation applied alone significantly delayed (up to 11 days in the case of the dose of 5.98
26 $\text{kJ m}^{-2} \text{d}^{-1}$) the phenological development of grapevine plants compared with those non-exposed,
27 particularly after veraison (Table 1). Water deficit significantly shortened (up to three days) the
28 period between fruit-set and the onset of veraison, but clearly delayed maturity, especially in the
29 plants exposed to 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ of UV-B (23 days). UV-B radiation significantly effected
30 reductions in shoot growth, measured as internodal length, irrespective of water regime applied,
31 whereas water deficit caused reductions in leaf area, leaf dry matter and shoot internodal length
32 (Table 1).
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43 3.2. *Leaf water status*

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45 Leaf stomatal conductance decreased as substrate water potential decreased, regardless of
46 the UV-B dose (Fig. 1A). Both UV-B and water deficit led to small reductions in the relative
47 water content of plants (between 2-5%, 1B). However, the effect of UV-B was more evident in
48 well irrigated plants compared with those subjected to water deficit on day 7 and 60 after the
49 beginning of the treatments (Fig. 1B).
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57 3.3. *Gas exchange and chlorophyll fluorescence*

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The treatment with $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ of UV-B transiently (day 7) caused reductions in A_{net} and g_s under well-watered and water deficit conditions (differences not significant in the last situation), and increased R_L and R_D only in the plants subjected to water deficit (Fig. 2). Φ_{PSII} and qP were lower after 7 and 30 days of exposure to $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ of UV-B, compared with non-exposed plants, only under well-watered conditions (Fig. 3). Water deficit, in general, significantly effected reductions in A_{net} , g_s , E , C_i , Φ_{PSII} and qP values throughout the experiment, with respect to well watered conditions (Fig. 2 and 3). By contrast, deficit irrigation caused increase in R_L and the ratio $\text{ETR}/A_{\text{net}}+R_D+R_L$, regardless of the dose of UV-B applied. NPQ was not significantly modified by UV-B or water deficit (Fig. 3). UV-B transiently effected decrease in F_v/F_m during the first eight days of exposure, recovering afterwards to control values (Fig. 4). There was no significant interaction between UV-B dose, water availability and developmental stage for most of the photosynthetic parameters measured, except for R_D (Fig. 2).

3.4. *Photosynthetic pigments and methanol extractable UV-B absorbing compounds (MEUVAC)*

Plants treated with UV-B radiation had higher concentrations of MEUVAC in leaves than those non-irradiated, after 30 and 60 days of exposure and under both water availabilities (Fig. 5). Water deficit did not affect the concentration of MEUVAC of plants irradiated with UV-B ($P_{(UV \times WA)} = 0.093$). Only in non-irradiated plants, the concentration of UV-B absorbing compounds under water deficit was significantly lower compared with well watered conditions on day 30 (Fig. 5).

UV-B radiation applied alone did not significantly modify the concentration of Chl and carotenoids after 60 days of treatment (Table 2). Water deficit significantly effected increase in Chl concentration per unit of leaf area, especially when combined with UV-B ($P_{(UV \times WA)} = 0.05$). When UV-B was applied in combination with water deficit, it caused increase in neoxanthin and decrease in zeaxanthin concentrations and the de-epoxidation stage of xanthophyll cycle, with respect to plants non-irradiated. Significant interactions between UV-B and water availability were observed for β -carotene, lutein, neoxanthin, zeaxanthin and DES.

3.5. *Total soluble proteins, antioxidant enzymes and lipid peroxidation*

1 UV-B radiation did not significantly modify the concentration of leaf soluble proteins,
2 whereas water deficit caused increase in TSP after 60 days of exposure (Table 3). Neither UV-
3 B nor water deficit significantly modified the levels of TBARS (Table 3). Plants irradiated with
4 UV-B had higher SOD activity, but similar CAT, GPX and APX activities, compared with those
5 non-irradiated, regardless of water availability (Table 3). Water deficit did not modify, in general,
6 the activity of antioxidant enzymes, and it only significantly effected decrease in CAT activity in
7 plants exposed to 5.98 kJ m⁻² d⁻¹. There were no significant interactions between UV-B and
8 water availability for any of these biochemical parameters (Table 3).
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16 **4. DISCUSSION**

17 *4.1. Grapevine acclimation to UV-B*

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19 The main effects of UV-B radiation reducing A_{net} were observed after 7 days of exposure on
20 well-watered plants (Fig. 2). Contrarily to previous studies on grapevine [6, 17], such decrease
21 was not accompanied by a reduction in the concentration of sub-stomatal CO₂ concentration.
22 Therefore, reduction in CO₂ availability did not seem to be the main factor limiting CO₂ fixation
23 in the plants exposed to UV-B. The slight down-regulation of PSII activity (Φ_{PSII}) observed on
24 day 7, through a decrease in the proportion of the reaction centers that remained open (qP),
25 may have contributed to the impaired A_{net} of UV-B exposed plants (Fig. 3). Along with the
26 decrease observed in A_{net} , UV-B transiently induced a significant decrease in the maximum
27 potential efficiency of PSII (Fv/Fm) during the first seven days of exposure (Fig. 4), thus
28 indicating that grapevine leaves experienced a certain degree of stress during this period,
29 recovering afterwards in absence of permanent PSII photoinhibition. The Fv/Fm is also a direct
30 measurement of the PSII efficiency that excludes the indirect effect of UV-B reducing stomatal
31 aperture and thereby limiting gas exchange [6].
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48 One of the most important molecular events underlying UV-B protection and acclimation in
49 plants, including grapevine, is the activation of the antioxidant system, which includes de
50 accumulation of non-enzymatic and enzymatic antioxidants [5, 17]. Among the non-enzymatic
51 antioxidants, the accumulation of phenolic compounds, mainly dihydroxy flavonoids [41], in the
52 vacuoles of epidermal cells reduces the penetration of UV wavelengths deeper into leaves [6].
53 These compounds are also accumulated in the chloroplasts and vacuoles of mesophyll cells,
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1 where they may have a functional role in photoprotection, as H₂O₂ and singlet oxygen
2 scavengers [7]. In the present study, the levels of MEUVAC in the plants exposed to UV-B for 7
3 days were not significantly higher than the levels of non-exposed plants (Fig. 5). These results
4 suggest that during the first days of UV-B exposure, grapevine leaves, which had been fully
5 developed without UV-B, were not able to accumulate enough amounts of phenolic compounds
6 to protect the photosynthetic apparatus from UV-B radiation, thus showing a transient
7 impairment of the photosynthetic performance (Fig. 2 to 4). Comont, et al. [14] reported that
8 whereas this protective response builds up gradually in the field, plants grown in zero UV-B
9 glasshouse conditions (as was this case) do not build such protection and/or the rapid capacity
10 to respond to UV-B. Nevertheless, after 30 and 60 days of exposure to UV-B, grapevine leaves
11 doubled their concentration of MEUVAC. The increased activity of the antioxidant system also
12 includes up-regulation of antioxidant enzymes such as SOD, CAT and peroxidases [5].
13 Elicitation of antioxidant enzymes response may be mediated by reactive oxygen species
14 and/or the UV-B specific photoreceptor signaling pathway [5]. In the present study, a significant
15 increase in SOD activity was observed after 60 days of UV-B exposure (Table 3), thus leading
16 to an increased capacity to scavenge oxygen free radical. Such increase in SOD, together with
17 the accumulation of MEUVAC, provided not only an effective protection of the photosynthetic
18 apparatus, but also protected the leaves against a potential oxidative damage induced by UV-B,
19 as suggests the low levels of TBARS (Table 3). All of these changes are indicative of UV-B
20 acclimation occurring following long-term exposures [17].
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41 Regarding the effect of UV-B radiation on plant water status, most of the studies on the
42 response of plants to UV-B show no remarkable changes in leaf RWC [8, 9]. In other cases,
43 UV-B has been reported to improve leaf water status, through production of osmolytes,
44 accumulation of dehydrins or increases in cuticle thickness [1]. By contrast, in the present study,
45 leaf RWC of UV-B treated plants was significantly lower (day 7 and 60) than that of plants non-
46 exposed to UV-B (Fig. 1B), which may suggest a decrease in cell turgidity. However, such
47 decrease in RWC cannot be explained by a higher loss of water through transpiration in UV-B
48 exposed plants (Fig. 2). The relationship between UV-B and changes in plant cell turgidity is not
49 clear and, our results do not allow us to give a conclusive explanation. The results agree with
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1 those of Yang and Yao [42] and Ziska, et al. [43], who observed decreases in leaf water status
2 induced by UV-B in different plant species.
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4 *Effects of water deficit and its interaction with UV-B on the photosynthetic performance of*
5 *grapevine*
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9 A significant decrease in A_{net} was observed after 7, 30 and 60 days of water deficit, regardless
10 of the UV-B dose applied (Fig. 2). Stomatal closure seemed to be the main limitation to
11 photosynthesis, as deduced from the impaired g_s , E and C_i values. Unlike that observed for UV-
12 B, the decrease of Φ_{PSII} induced by water deficit was smaller than that of A_{net} (Fig. 2 and 3), thus
13 reflecting an unbalance between photochemistry and CO_2 fixation, which may lead to an excess
14 of electrons generated in the photosynthetic electron transport chain. However, plants
15 compensated for the decrease in photosynthesis maintaining R_D and significantly increasing R_L
16 under water deficit conditions ($P_{(WA)} < 0.0001$) (Fig. 2). R_L and R_D are important mechanisms of
17 photoprotection on grapevine, particularly under conditions of low A_{net} , as it occurs under water
18 stress [44]. Therefore, the ratio of electrons generated to electrons consumed ($\text{ETR}/A_{\text{net}}+R_D+R_L$)
19 only increased from ca. 5 to 6-7 (Fig. 3). The results suggest that, although under water deficit
20 alternative sink for the excess of electrons exists (such as the Mehler reaction), the risk of water
21 stress-mediated oxidative damage was low. In fact, the concentration of proteins and Chl, main
22 targets of ROS, did not decreased after 60 days of exposure to water deficit, and the levels of
23 TBARS and the antioxidant enzyme activities (SOD, GPX, CAT and APX) were not increased
24 under water-stress. Similarly, Doupis, et al. [16] observed that the activity of antioxidant
25 enzymes in grapevine leaves did not significantly increase under water-stress. More recently,
26 Król, et al. [45] have reported that the antioxidant activity of grapevine leaves can even
27 decrease under long-term drought stress, as happened with CAT activity in the present study
28 (Table 3). Such decrease in CAT activity, may involve a decreased capacity to scavenge
29 oxygen peroxide under water deficit conditions. Nevertheless, the activity of other peroxidases
30 (APX and GPX) seemed to be high enough to compensate such decrease in CAT, as suggests
31 the lower values of lipid peroxidation.
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57 Regarding the combined effect of UV-B and water deficit, the results indicate that UV-B
58 radiation significantly affected leaf water status (RWC) and photosynthetic activity (A_{net} , Φ_{PSII}
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1 and Fv/Fm, during the first days of exposure), primarily when water availability was high, and
2 these effects were less evident under water deficit conditions, when plant water content and
3 photosynthetic performance was already reduced (Fig. 1 to 4). In addition, the combination of
4 UV-B and deficit irrigation did not cause pronounced additive effects, with respect to the
5 exposure to UV-B alone, on the activity of antioxidant enzymes and the accumulation of UV-B
6 protecting pigments, two major acclimation responses of plants to UV-B radiation (Table 3 and
7 Fig. 5). The results suggest that the adaptive response of grapevine to UV-B radiation was not
8 modified by water deficit, and agree with previous studies, in which few interactive effects of
9 UV-B radiation and water availability on water status, photosynthetic capacity and enzymatic
10 antioxidant system of different plant species were observed [10-12]. There was only one key
11 functional trait, R_D , which exhibited relatively strong synergistic effect from the combined
12 application of UV-B and water deficit on day 7. The increase in R_D , only observed in the plants
13 exposed to UV-B and deficit irrigation, coincided with the beginning of the period of greater
14 synthesis of UV-B protecting compounds (between day 7 and 30, Fig. 5). An increase in cell
15 respiration has been related to the greater need for energy to protect against UV-B and for the
16 repair of UV-B damage [46]. In addition, R_L increased especially in those plants grown under
17 UV-B and water deficit (not significant interaction in this case) (Fig. 2), in which the decrease in
18 the rates of carbon fixation with respect to Φ_{PSII} was more evident. The results suggest that
19 when UV-B and water deficit were applied simultaneously, R_L and R_D were the two major
20 photoprotective processes, whereas thermal energy dissipation was not involved in the
21 adaptation of grapevine leaves to the combination of these factors, as indicated by the absence
22 of changes in NPQ and DES (Fig. 3 and Table 2).
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4.2. Phenological response of grapevine to UV-B and water deficit

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47 One of the less studied aspects of the response of plants to the combination of UV-B and
48 drought is their role as regulators of plant phenology and reproductive success [14]. In the
49 present study, plants grown under a single environmental factor, UV-B radiation or water deficit,
50 took up to 11 and 13 days longer, respectively, to reach grape berry ripeness (21-23 °Brix)
51 (Table 1). A delay of plant developmental stages (flowering and ripening) induced by UV-B has
52 been reported for other species such as *A. thaliana* and wheat [14, 47]. Schultz [48] also
53 reported that grapevine phenology may be affected by UV-B radiation. With respect to the effect
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1 of water deficit, moderate drought, in general, promotes sugar accumulation, thus accelerating
2 grape ripening in the field [49, 50]. Nevertheless, the effect varies depending on the timing and
3 intensity of water stress imposition [49] and a delay in fruit maturity has been observed after
4 pre-veraison irrigation cutoff in Cabernet Sauvignon [51]. In the present work, the onset of
5 veraison was triggered earlier in berries under water deficit conditions, compared to well
6 watered plants. The results agree with Castellarin, et al. [50], who observed that water deficit
7 hastened the onset of ripening (veraison), which was associated to the accelerated
8 accumulation of anthocyanins and anthocyanin-related gene expression. Under water deficit, an
9 earlier increase in grape berry abscisic acid (ABA) concentration has been reported [22].
10 Besides its role as a stress-related signal, ABA has been pinpointed as the signal triggering
11 berry ripening, since a strong increase in berry ABA content is recorded at the end of the colour
12 turning period and during the initial stages of ripening [52]. ABA up-regulates genes associated
13 with the ripening process, such as those involved in anthocyanin biosynthesis [53] and acts
14 through the over- or under-expression of, mostly, the same proteins which are involved in the
15 ripening process, in the same direction as observed during ripening [54]. In the present study,
16 water deficit may have induced an earlier increase in the levels of ABA in the berries, thus
17 triggering the onset of ripening before than in well-irrigated plants. Even though they started the
18 ripening process sooner, sugar accumulation process was clearly slower in plants under water
19 deficit, thus delaying maturity. Since berry growth and ripening greatly depend on the import of
20 photoassimilates, the strong reduction in leaf area and photosynthetic activity observed in the
21 plants subjected to water deficit, probably may explain the slow-down in ripening.

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43 Interestingly, the water deficit induced delay in plant development was enhanced by UV-B
44 radiation, and ripeness was delayed up to 28 days, when UV-B and water deficit were
45 combined, with respect to the non-irradiated well-watered plants (Table 1). This delay was
46 mainly associated with a slow-down of the stage III of berry growth comprised between the
47 onset of veraison to maturity. Similarly, Comont et al. [14] reported that UV-B and drought
48 acting simultaneously caused a delay in phenology of *A. thaliana*, compared with drought alone.
49 In the present study, the treatment with $9.66 \text{ kJ m}^{-2} \text{ d}^{-1}$ of UV-B slightly caused reduction in A_{net}
50 in plants grown under water deficit conditions on day 7 and 30 (stages of fruit-set and veraison,
51 respectively). Such additive effect of UV-B, although not statistically significant, represented an
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1 additional decrease in A_{net} per unit of leaf area of between 10% and 23% (day 7 and 30,
2 respectively), with respect to the photosynthetic rates of non-irradiated water deficit plants.
3 Taken together with the decrease in total leaf area (19%, not significant differences), this may
4 have effected reductions in the total amount of carbon fixed per plant, and consequently
5 allocated to grape ripening. However, the decreases in A_{net} and leaf area may not be enough to
6 explain such phenological delay. Additionally, the effect of UV-B and water deficit increasing
7 respiratory and photorespiratory activities should be also considered. Plants use up to 50% of
8 recently formed C in respiration processes, which means that an important fraction of
9 photoassimilates could have been wasted through R_L and R_D , thus contributing to an impaired
10 carbon balance. Finally, the secondary metabolism activation cost, associated to the use of
11 photoassimilates to produce phenolics and other UV-B screening compounds, cannot be ruled
12 out in order to explain the delayed phenological development in the combined treatment.
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25 **5. CONCLUSIONS**

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27 The results suggest that under environmental controlled conditions, water deficit had larger
28 effects on grapevine growth and photosynthetic activity than UV-B, which only exhibited a
29 transient detrimental effect on the photosynthesis of plants. Little interaction effects between
30 UV-B and water deficit were detected on photosynthesis performance and UV-B radiation did
31 not strongly aggravate the impact of deficit irrigation on the photosynthetic activity of grapevine.
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33 In general, the acclimation process of grapevine leaves to a long-term exposure to UV-B,
34 through the accumulation of UV-B absorbing compounds, was not altered by water deficit.
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36 However, the combined exposure to UV-B and water deficit led to additive effects on plant
37 phenological development, slowing-down grape ripening, which was associated with an
38 impaired carbon balance under such conditions.
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Figure captions

Figure 1. Relationship between stomatal conductance (g_s , A) and substrate water potential and relative water content (RWC, B) of plants grown under 0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ of UV-B, well watered and water deficit. In Fig. B, columns are means ($n=9$) \pm SE. Different letters within the same parameter indicate significant differences ($p<0.05$).

Figure 2. Net photosynthesis (A_{net}), stomatal conductance (g_s), transpiration (E), sub-stomatal CO_2 concentration (C_i), photorespiration (R_L) and dark respiration (R_D) of plants grown under 0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ of UV-B, well watered (WW) and water deficit (WD), after 7, 30 and 60 days of treatment. Columns are means ($n=9$) \pm SE. Different letters within the same parameter indicate significant differences ($p<0.05$). Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B, water availability and developmental stage ($P_{(UV-B \times WA \times Stage)}$).

Figure 3. Actual PSII efficiency (Φ_{PSII}), non-photochemical quenching (NPQ) and photochemical quenching (qP), and ratio of electrons generated to electrons consumed in A_{net} , R_D and R_L ($\text{ETR}/A_{\text{net}}+R_D+R_L$) of plants grown under 0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ of UV-B, well watered (WW) and water deficit (WD), after 7, 30 and 60 days of treatment. Columns are means ($n=9$) \pm SE. Different letters within the same parameter indicate significant differences ($p<0.05$). Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B, water availability and developmental stage ($P_{(UV-B \times WA \times Stage)}$).

Figure 4. Maximum potential PSII efficiency (Fv/Fm) of plants grown under 0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$ of UV-B, well watered (WW) and water deficit (WD), throughout the experiment. Points are means ($n=9$) \pm SE. Significant differences ($p<0.05$) between UV-B doses and water regimes within the same date are indicated as * and \blacklozenge , respectively. Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B, water availability and developmental stage ($P_{(UV-B \times WA \times Stage)}$). Inset is a zoom of the seven first days of treatment.

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Figure 5. Leaf methanol extractable UV-B absorbing compounds (MEUVAC) of plants grown under 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B, well watered (WW) and water deficit (WD), after 7, 30 and 60 days of treatment. Columns are means (n=9) ± SE. Different letters within the same parameter indicate significant differences (p<0.05). Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B, water availability and developmental stage ($P_{(UV-B \times WA \times Stage)}$).

Figure S1. Spectral irradiance of the UV-B lamps.

Table(s)

Table 1. Phenological development and growth parameters of plants grown under 0, 5.98 and 9.66 kJ m⁻² d⁻¹, well watered and water deficit, at maturity. Values are means (n=12) and standard errors. Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B and water availability ($P_{(UV-B \times WA)}$).

	Well Watered			Water Deficit			$P_{(UV-B)}$	$P_{(WA)}$	$P_{(UV \times WA)}$
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹			
Elapsed time from fruit set to the onset of veraison (days)	41.2(1.3)	41.7 (0.7)	42.9(0.9)	40.0(0.8)	39.8(0.9)	40.1(1.0)	0.574	0.012	0.666
Elapsed time from the onset of veraison to maturity (days)	23.2(0.4)	34.6(5.4)	27.7(3.7)	37.7(2.9)	42.6(2.7)	51.3(3.0)	0.015	<0.001	0.074
Elapsed time from fruit set to maturity (days)	64.5(1.0)	76.0(5.7)	70.6(3.5)	77.7(3.4)	82.4(2.4)	91.6(2.8)	0.010	<0.001	0.113
Leaf area (dm ² plant ⁻¹)	13.5(0.7)	13.5(1.2)	13.5(0.5)	5.4(0.6)	4.6(0.5)	4.4(0.2)	0.748	<0.001	0.739
Leaf dry matter (g plant ⁻¹)	8.8(0.8)	10.1(1.1)	10.2(0.5)	3.7(0.4)	3.6(0.4)	3.5(0.2)	0.556	<0.001	0.348
Internodal length (cm)	8.0(0.3)	7.0(0.6)	6.6(0.3)	3.9(0.3)	2.8(0.2)	2.9(0.2)	0.001	<0.001	0.723

Table 2. Chlorophyll (Chl) and carotenoid concentrations and de-epoxidation state (DES) of xanthophyll cycle in leaves of plants grown under 0, 5.98 and 9.66 $\text{kJ m}^{-2} \text{d}^{-1}$, well watered and water deficit, at maturity. Values are means (n=6) and standard errors. Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B and water availability ($P_{(UV-B \times WA)}$). In case of significant interaction between factors, different letters within the same parameter indicate significant differences ($p < 0.05$) according to LSD test.

	Well Watered			Water Deficit			$P_{(UV-B)}$	$P_{(WA)}$	$P_{(UV \times WA)}$
	0 $\text{kJ m}^{-2} \text{d}^{-1}$	5.98 $\text{kJ m}^{-2} \text{d}^{-1}$	9.66 $\text{kJ m}^{-2} \text{d}^{-1}$	0 $\text{kJ m}^{-2} \text{d}^{-1}$	5.98 $\text{kJ m}^{-2} \text{d}^{-1}$	9.66 $\text{kJ m}^{-2} \text{d}^{-1}$			
Chl a+b ($\mu\text{mol m}^{-2}$)	602.7(25.8)	581.3(24.1)	501.8(34.1)	557.3(45.5)	720.8(34.8)	638.0(64.6)	0.114	0.029	0.050
Chl a+b ($\mu\text{mol g}^{-1} \text{DW}$)	8.21(0.54)	8.54(0.60)	7.22(0.39)	9.00(0.45)	8.31(0.49)	8.94(0.40)	0.543	0.064	0.147
Chla/Chlb	2.96(0.04)	3.04(0.03)	3.12(0.06)	3.09(0.03)	3.02(0.05)	3.01(0.07)	0.686	0.986	0.056
β -Carotene ($\text{mmol mol}^{-1} \text{Chl}$)	88.9(1.2) ab	86.5(1.0) b	91.5(1.3) a	85.8(1.3) b	89.9(1.1) a	88.6(1.2) ab	0.092	0.539	0.012
Neoxanthin ($\text{mmol mol}^{-1} \text{Chl}$)	43.1(0.03) a	41.2(0.9) ab	41.8(0.6) ab	40.1(0.8) b	42.6(1.0) a	42.7(0.7) a	0.742	0.838	0.026
Lutein ($\text{mmol mol}^{-1} \text{Chl}$)	138.9(1.0) ab	130.7(3.3) b	135.5(1.9) ab	132.9(4.6) ab	139.5(1.6) a	134.6(3.3) ab	0.953	0.791	0.042
Violaxanthin (V) ($\text{mmol mol}^{-1} \text{Chl}$)	57.1(3.5)	53.1(1.2)	55.2(3.2)	52.3(5.7)	59.9(2.1)	56.9(2.4)	0.845	0.544	0.208
Antheraxanthin (A) ($\text{mmol mol}^{-1} \text{Chl}$)	7.0(0.4)	6.4(1.3)	8.5(1.2)	10.1(2.5)	6.8(0.6)	6.0(0.5)	0.293	0.751	0.121
Zeaxanthin (Z) ($\text{mmol mol}^{-1} \text{Chl}$)	5.6(0.5) ab	6.0(1.4) ab	9.7(2.2) ab	10.1(2.7) a	6.8(0.8) ab	5.2(1.1) b	0.628	0.831	0.032
V+A+Z ($\text{mmol mol}^{-1} \text{Chl}$)	69.6(3.6)	67.3(1.6)	73.4(2.2)	72.5(7.0)	73.0(1.9)	68.0(2.9)	0.970	0.721	0.311
DES (A+Z)/(V+A+Z)	0.18(0.01) ab	0.18(0.03) ab	0.25(0.04) ab	0.28(0.06) a	0.18(0.02) ab	0.16(0.02) b	0.405	0.901	0.048

Table 3. Total soluble proteins (TSP), lipid peroxidation, determined as thiobarbituric acid reacting substances (TBARS) concentration, superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT) and ascorbate peroxidase (APX) activities in leaves of plants grown under 0, 5.98 and 9.66 kJ m⁻² d⁻¹ of UV-B, well watered and water deficit, at maturity. Values are means (n=5) and standard errors. Main effects of UV-B ($P_{(UV-B)}$), water availability ($P_{(WA)}$) and interaction between UV-B and water availability ($P_{(UV-B \times WA)}$).

	Well Watered			Water Deficit			$P_{(UV-B)}$	$P_{(WA)}$	$P_{(UV \times WA)}$
	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹	0 kJ m ⁻² d ⁻¹	5.98 kJ m ⁻² d ⁻¹	9.66 kJ m ⁻² d ⁻¹			
TSP (mg m ⁻²)	8.6(0.1)	9.8(0.6)	10.9 (0.2)	12.1(0.01)	12.8 (1.0)	14.3(0.2)	0.145	0.001	0.969
TSP (mg g ⁻¹ DW)	12.0(0.18)	13.4(0.85)	15.1 (1.60)	18.9(2.24)	16.2 (1.22)	19.8(2.18)	0.232	0.001	0.445
TBARS (nmol MDA g ⁻¹ DW)	130.1(36.9)	98.3(11.2)	87.3(5.1)	94.7 (8.8)	92.6(14.6)	106.4(34.1)	0.412	0.692	0.485
SOD (USOD mg ⁻¹ g ⁻¹ DW min ⁻¹)	380.9(43.9)	840.6(52.2)	850.1(42.6)	526.8(64.3)	842.9(70.6)	943.1(39.2)	< 0.001	0.080	0.413
GPX (nmol guaiacol g ⁻¹ DW min ⁻¹)	31.3(8.3)	28.6(6.8)	30.1(9.4)	23.7(6.1)	32.3(6.4)	41.3(11.2)	0.577	0.683	0.538
CAT (mmol H ₂ O ₂ g ⁻¹ DW min ⁻¹)	2.48(0.52)	2.59(0.16)	2.37(0.37)	2.33(1.08)	2.07(0.46)	2.28(1.02)	0.583	0.002	0.051
APX (μmol asc g ⁻¹ DW min ⁻¹)	15.0(1.62)	16.9(3.4)	14.8(1.96)	13.8(1.1)	12.7(1.2)	12.8(1.6)	0.889	0.131	0.742

Figure(s)

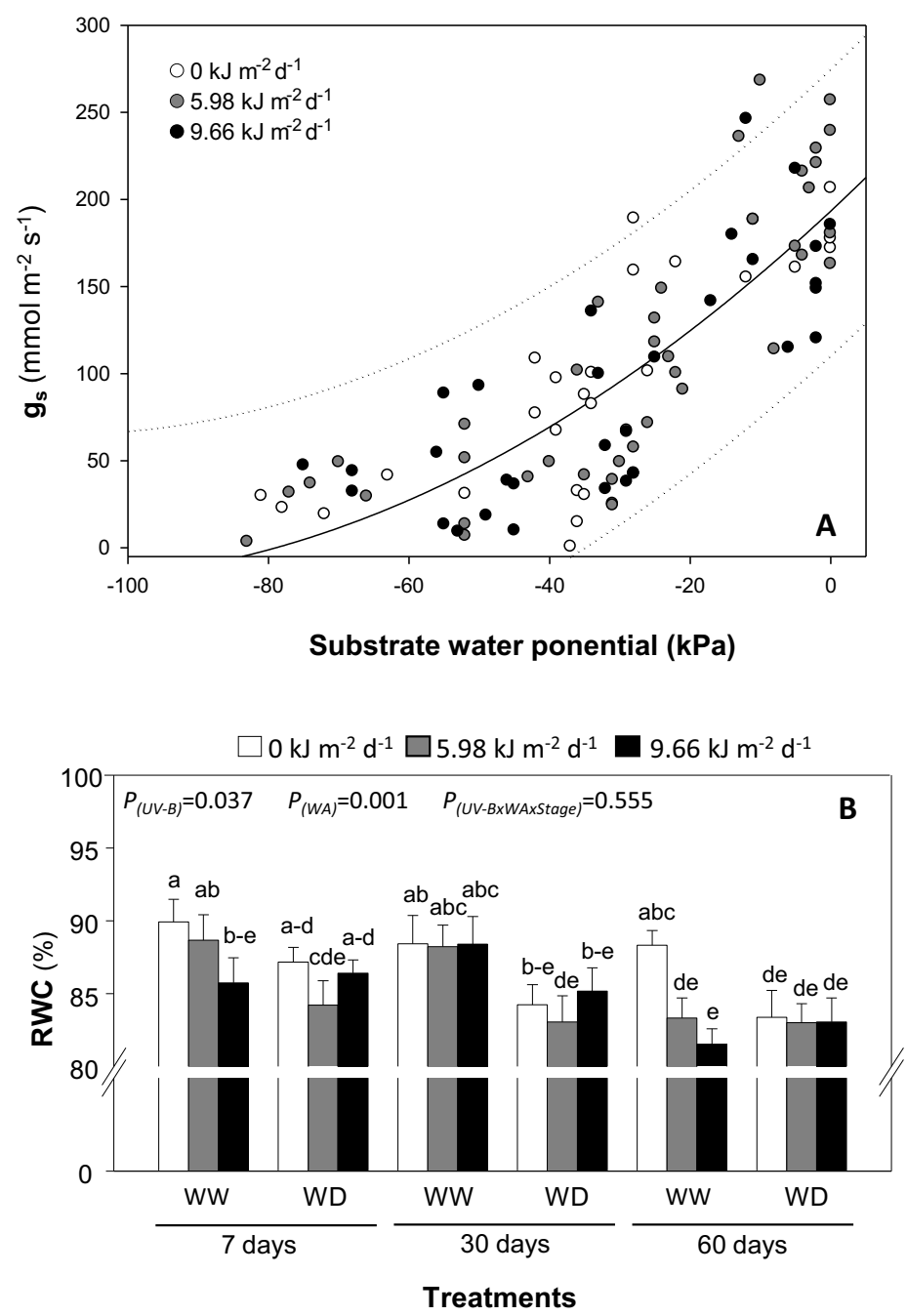


Figure 1

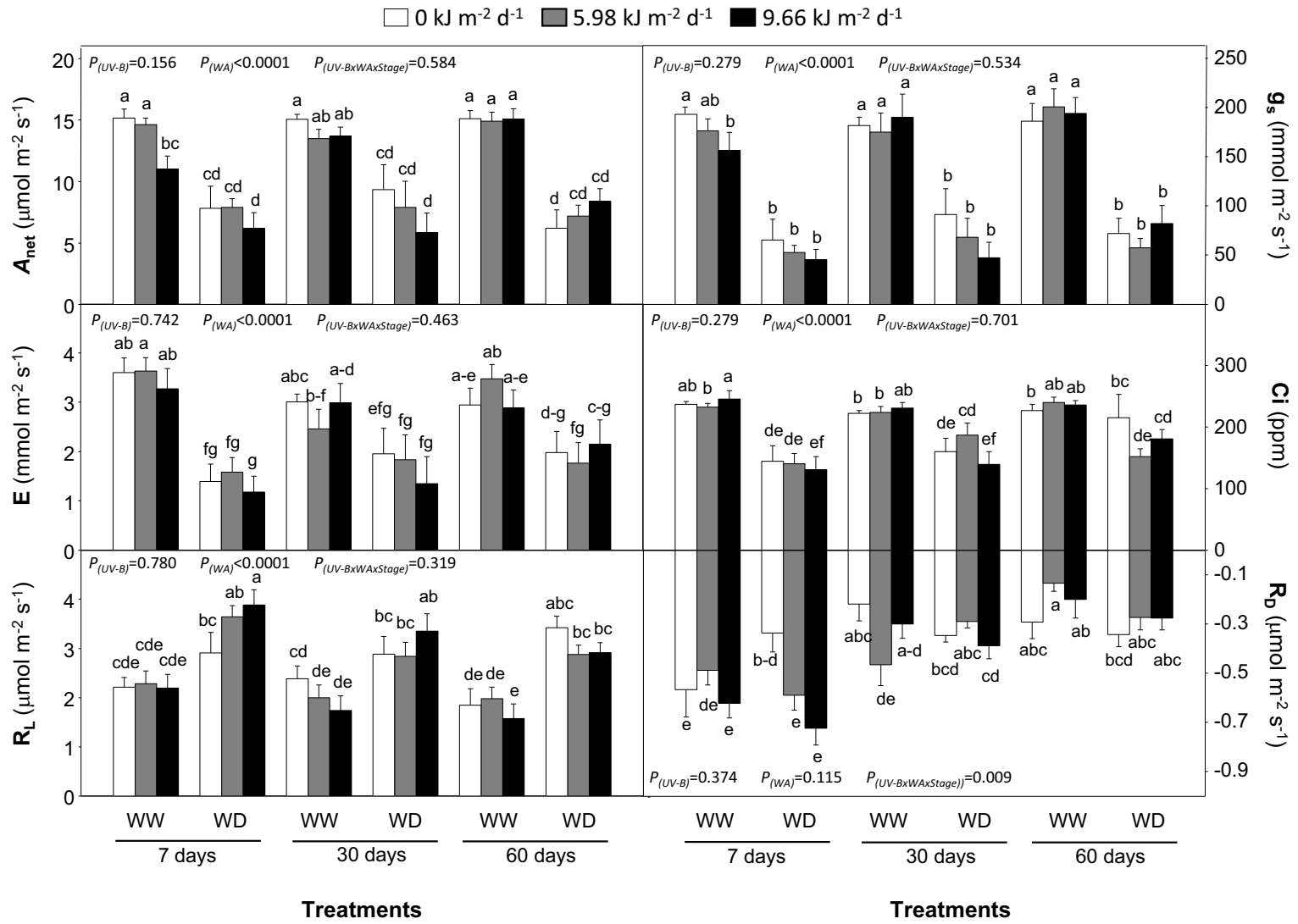


Figure 2

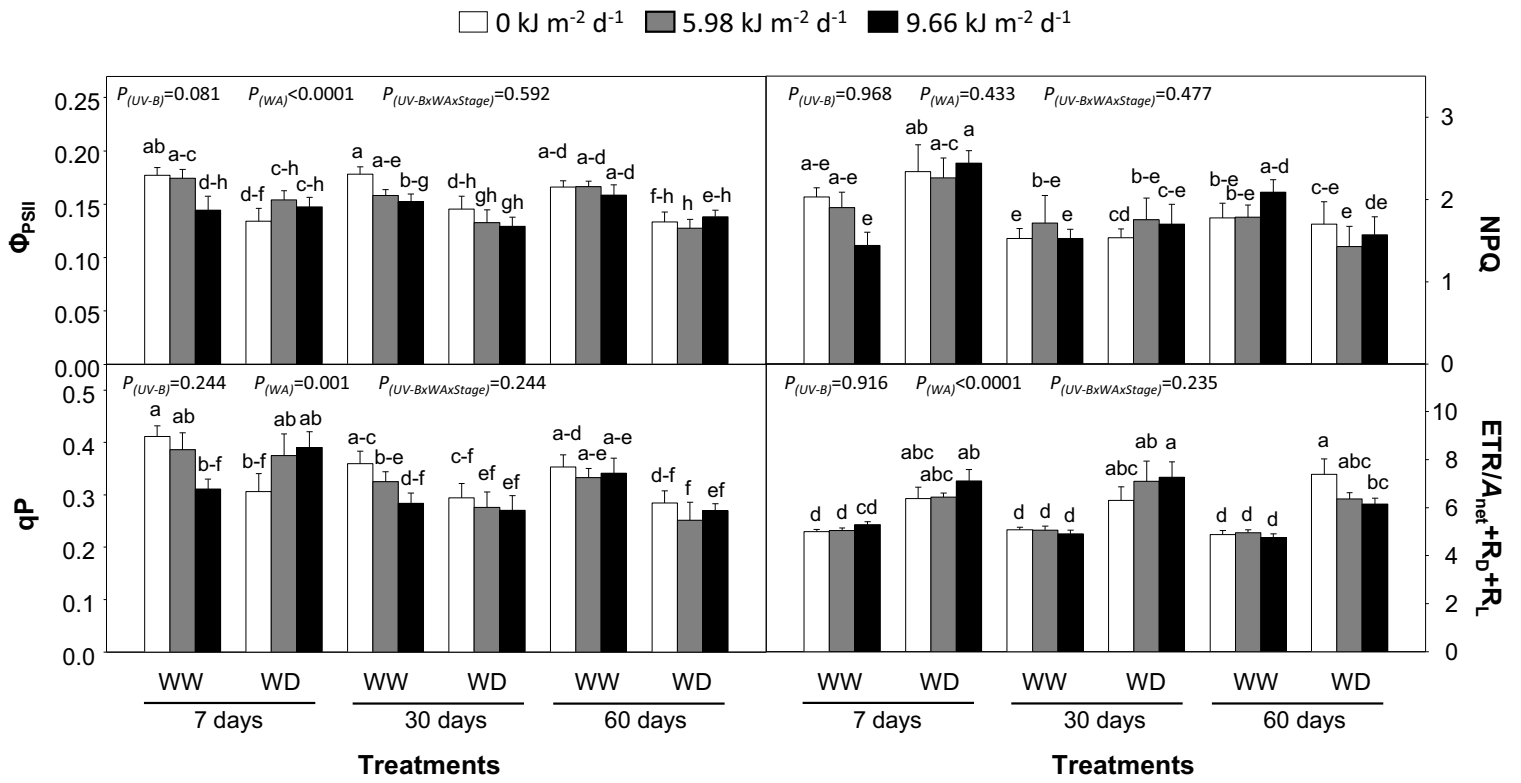


Figure 3

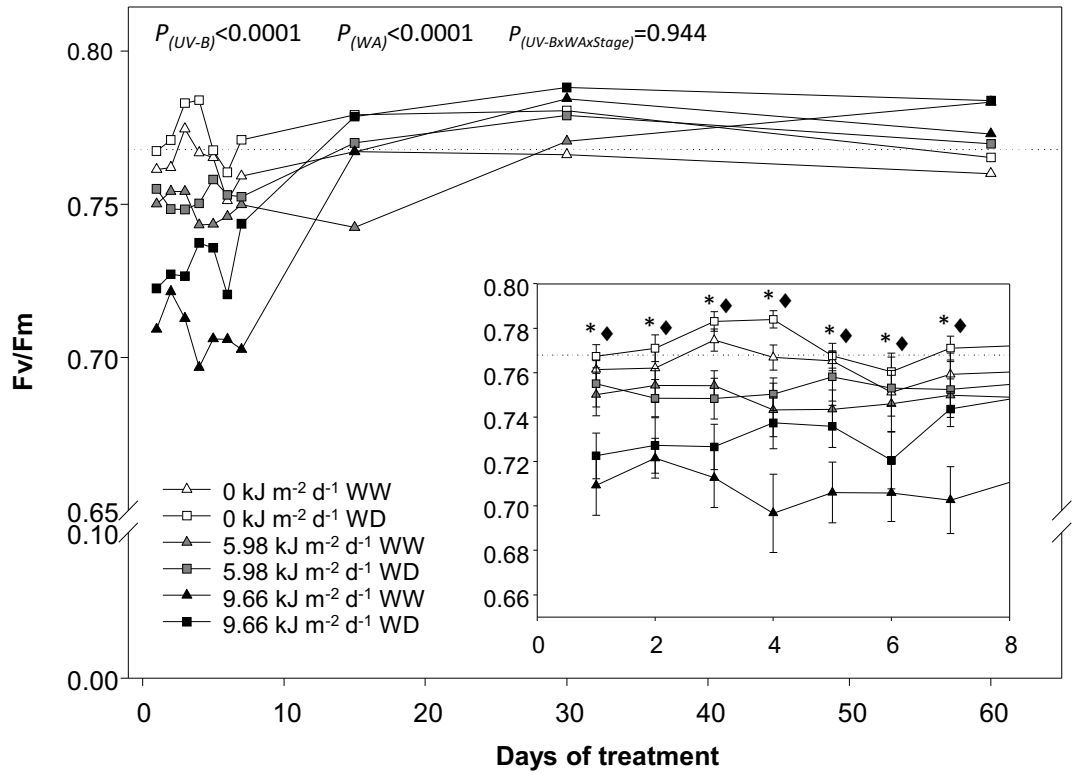


Figure 4

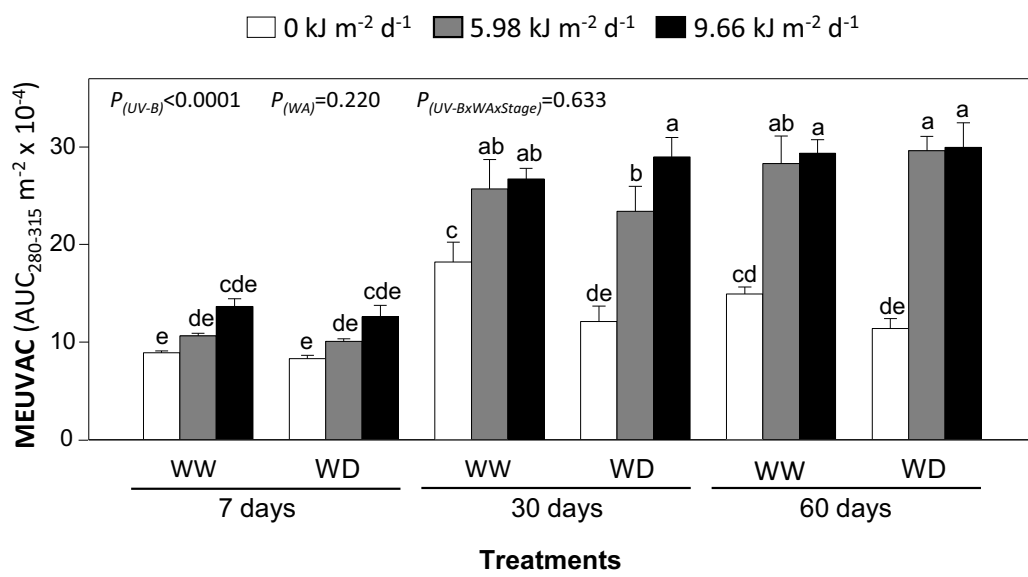


Figure 5

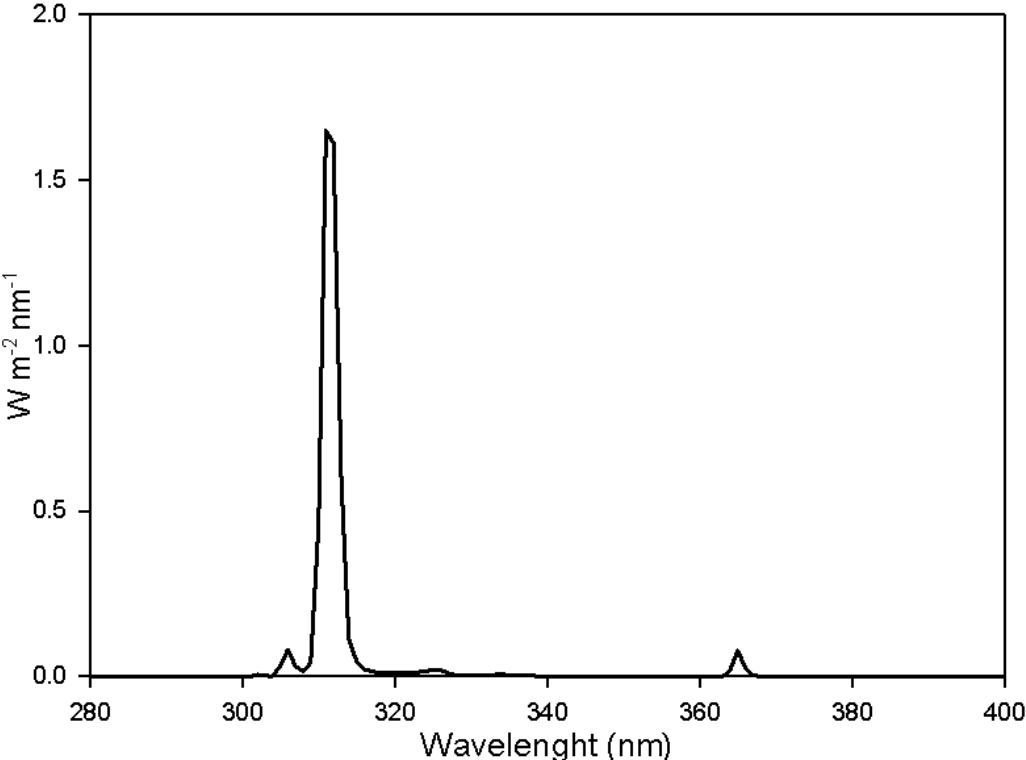


Figure S1. Spectral irradiance of the lamps used as UV-B source

Highlights

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UV-B and water deficit had little interactive effects on grapevine photosynthesis performance

The impact of UV-B on leaf photosynthesis was overshadowed by the effects of water deficit

Deficit irrigation did not modify the adaptive response of grapevine to UV-B radiation

UV-B and water deficit caused additive effects delaying the phenological development of grape berries