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Abstract: The impact of UV irradiation was studied on photosynthesis, photosystem II photochemical yields and antioxidant responses using green-house grown grapevine (Vitis vinifera L. cv. Chardonnay) leaves. Supplemental UV irradiation (290-400 nm) was centred in the UV-B region, and corresponded to 8.95 kJ m-2 d-1 global (280-400 nm) or 8.04 kJ m-2 d-1 UV-B (280-315 nm) biologically effective dose. UV irradiation was applied daily and its effects were evaluated after 4-days. Younger (1-3 weeks-old) leaves (YL) and older (4-6 weeks-old) leaves (OL) were affected differently, UV irradiation decreased their photochemical yields to 78% and 56%, respectively. Unlike OL, YL responded by an increase in UV-B absorbing pigment, anthocyanin and total phenolics contents. UV irradiation increased total antioxidant capacities in YL but not in OL. YL were also different in their ability to increase speci□c hydroxyl radical and singlet oxygen neutralizing capacities in response to the supplemental UV irradiation, which is reported here for the □rst time. Our results suggest that the ability of maintaining photosynthesis under supplemental UV is not necessarily determined by base levels of antioxidants but rather by their inducibilities in response to the irradiation and emphasise the importance of comparing leaves of the same age in UV studies. Correlations between various antioxidant capacities, pigment contents and photosynthesis parameters were also examined. However, no single element of the defence system can be picked up as decisive factor of sensitivity to UV.

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Developmental stage is an important factor that determines the antioxidant responses of young and old grapevine leaves under UV irradiation in a green-house

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Abstract

The impact of UV irradiation was studied on photosynthesis, photosystem II photochemical yields and antioxidant responses using green-house grown grapevine (*Vitis vinifera* L. cv. Chardonnay) leaves. Supplemental UV irradiation (290-400 nm) was centred in the UV-B region, and corresponded to 8.95 kJ m^{-2} d^{-1} global (280-400 nm) or 8.04 kJ m^{-2} d^{-1} UV-B (280-315 nm) biologically effective dose. UV irradiation was applied daily and its effects were evaluated after 4-days. Younger (1-3 weeks-old) leaves (YL) and older (4-6 weeks-old) leaves (OL) were affected differently, UV irradiation decreased their photochemical yields to 78% and 56%, respectively. Unlike OL, YL responded by an increase in UV-B absorbing pigment, anthocyanin and total phenolics contents. UV irradiation increased total antioxidant capacities in YL but not in OL. YL were also different in their ability to increase specific hydroxyl radical and singlet oxygen neutralizing capacities in response to the supplemental UV irradiation, which is reported here for the first time. Our results suggest that the ability of maintaining photosynthesis under supplemental UV is not necessarily determined by base levels of antioxidants but rather by their inducibilities in response to the irradiation and emphasise the importance of comparing leaves of the same age in UV studies. Correlations between various antioxidant capacities, pigment contents and photosynthesis parameters were also examined. However, no single element of the defence system can be picked up as decisive factor of sensitivity to UV.

Key words: antioxidant, grapevine leaf, photosynthesis, reactive oxygen species, UV-B irradiation

Abbreviations

AA, ascorbic acid; ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); Fv/Fm, maximum photochemical yield of PS II in the dark adapted state; GA, gallic acid; FRAP, ferric reducing antioxidant power; HTPA, 2- hydroxyterephthalate; OL, old leaves; PAR, photosynthetically active radiation; PPFD, photosynthetic photon flux density; PS, photosystem; ROS, reactive oxygen species; TEAC, Trolox equivalent antioxidant capacity; TPA, terephthalate; 1,4-benzenedicarboxylic acid; Trolox, 6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid; Y(II)-55, effective photochemical yield of PS II at 55 µmol photons m^{-2} s^{-1} PPFD; YL, young leaves

1. Introduction

Sunlight contains high energy ultraviolet (UV, 290-400 nm) radiation which affects photosynthesis in various ways. Many studies have shown that high doses of UV, specially that of UV-B (280-315 nm) are damaging to plants. Cellular components such as proteins and nucleic acids absorb this radiation, resulting in biomass reduction, impaired photosynthesis and other chloroplast functions, decreased protein synthesis, damage to DNA, reviewed [1-2]. Effects of UV-B radiation include oxidative stress [3-4], and reactive oxygen species (ROS) have been shown to participate directly in the damage induced by high UV-B doses [5-7]. On the other hand, many effects of UV-B radiation concern morphogenetic changes in plants rather than damage, especially in response to lower UV doses [8-9]. In addition, UV-B affects the secondary metabolism of plants indicating that solar UV-B is to be regarded as an environmental challenge rather than a damage-inducing source of stress [10-11]. Plants protect themselves from this potentially harmful radiation by altering metabolic functions and a number of studies confirmed the role of UV-B in regulation of gene expression [9, 12-14]. Due to variations in experimental conditions, data available on the effect of UV-B radiation and the antioxidant response indicate considerable differences between plant tissues and/or plant species [15-19], but all studies agree on the critical role of effective ROS neutralizing in responses to UV-B. High, damaging ROS concentrations under high UV doses can be detected by spin trapping EPR [5, 7, 20] or fluorescent probes [6, 21] allowing comparative studies of ROS and ROS scavenging. Currently available ROS detecting methods are, however, not sensitive enough to identify ROS directly under milder stress conditions, leaving only the possibility of assuming that increased antioxidant activities reflect a situation requiring stronger control over the increased level of ROS. In addition to measuring ROS specific antioxidant enzymes, plant studies have also borrowed methods from medicine and food chemistry to assess total antioxidant potentials of a plant extracts [22-25]. Comparisons of these general and ROS specific antioxidant capacities are of interest. Traditionally, conclusions on the chemical nature of ROS are only drawn when specific enzymes are measured (for example superoxide dismutase or peroxidases) but ROS which are not targeted by specialised enzymes can not be studied this way. To overcome this issue, two newly developed methods measuring specific singlet oxygen [26] and hydroxyl radical [27] scavenging capacities of plant extracts were used in the present study.

The overall aim of this work was to study the role of antioxidant responses of grapevine leaves in acclimation to supplemental, UV-B centred broad band UV irradiation. In order to compare samples with different antioxidant potentials, we chose young and old (but not senescent) leaves of the same plant, and analysed photosynthesis, photochemical efficiency, pigment and antioxidant responses to UV. Using water-based leaf extracts, changes in general (total) antioxidant capacities and in specific ROS neutralizing abilities were compared and possible correlations of these were also sought. Grapevine (*Vitis vinifera* L. cv. Chardonnay) was chosen as plant material, due to its economic importance and the sensitivity of the cultivar to UV radiation [28]. Vines are advantageous for irradiation studies, as these can be trained horizontally allowing the exposure of leaves of different ages to the same dose.

2. Materials and methods

2.1. Plant material

Grapevine (*Vitis vinifera* L. cv. Chardonnay) plants were grown in 20 cm diameter pots, in a mixture of garden soil and vermiculate. Plants were grown in the absence of UV, in a glass-roofed green-house under natural daylight, which provided ca 200 µmol photons m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at noon. During growth, individual shoots were climbed on horizontal rods compelling most of the leaves facing upwards, with adaxial surfaces at the same distance from irradiation sources, regardless of their ages. In this study, fully developed 1-3 weeks-old leaves (the first three fully developed leaves from the tip of the shoot) represented young leaves (YL) while 4-6 weeks-old non-senescent leaves (chosen from the fourth to the ninth leaves from the tip of the shoot) were regarded as old leaves (OL). For antioxidant measurements water extracts were made by first grinding 4 leaf disks (approx. 40 mg) to powder in liquid nitrogen, then in 2 mL ice cold phosphate buffer (50 mM, pH 7.0). After centrifugation (500 x g for 5 min at 4 ^{o}C), supernatants were collected and stored at -70 ^{o}C until use. Singlet oxygen scavenging capacity measurements required more concentrated leaf extracts, and the above procedure was modified by extracting 9 leaf disks (approx. 90 mg) into 2 mL of the same buffer and centrifuging for 10 min at 3000 x g at 4 ^{o}C. The Folin-Ciocalteu reagent was purchased from Ferak Berlin GmbH (Berlin, Germany). All other chemicals were from Sigma-Aldrich (Sigma-Aldrich Kft Budapest, Hungary).

2.2. Supplemental UV radiation

For a 4-day supplemental UV treatment plants were moved to an other location in the green-house, where visible light was lower, due to the shading of UV tubes. Here both UV irradiated plants and controls were kept under 50 µmol photons m^{-2} s^{-1} PPFD between 7 AM and 7 PM. In addition to PPFD, UV treated plants were exposed to 0.84 W m^{-2} irradiance (integrated UV-B dose, measured with a Cole-Palmer radiometer, model 97503-00, and a broad range 312 nm centred sensor) from Q-Panel UVB-313EL tubes daily, between 9 AM and 3 PM. A cellulose diacetate filter (Courtaulds Chemicals, Derby, UK) was used to exclude any effect of shorter wavelength (< 280 nm) UV radiation. Spectral distribution of UV irradiance from the tube panel (Fig.1) was measured with a Newport OSM-400UV/VIS spectrometer (Newport Corporation, Irvine CA, USA), courtesy of Dr. Andreas Albert (Helmholtz Zentrum München, Germany). The applied UV irradiance corresponded to 8.95 kJ m^{-2} d^{-1} global (280-400 nm) or 8.04 kJ m^{-2} d^{-1} UV-B (280-315 nm) biologically effective dose, calculated using the Biological Spectral Weighting Function developed by Flint and Caldwell [29]. The applied biologically effective UV-B dose corresponded to approximately 107% of ambient daily UV-B in the northern hemisphere (latitude 46◦), in summer [30]. However, PPFD in our experiments was lower than in the field therefore results of this study cannot be directly extrapolated to outdoor conditions. PPFD was measured with a LI-250 radiometer (LI-COR Environmental, Lincoln, Nebraska USA).

2.3. Photosynthesis and photochemical yield measurements

Photosynthesis (gas exchange) measurements were performed on leaves attached to the vines. Photochemical yields were measured on detached leaves and were completed within 30 min after removal from the plants. Following these, 0.6 cm diameter disks were cut from the leaves which were used immediately for pigment analysis and for making leaf extracts. Photosynthesis was characterized by CO_2 uptake (CO_2 µmol m^{-2} s^{-1}) and stomatal conductance (mmol H_2O m^{-2} s^{-1}) measured on intact leaves at 300 µmol photons m^{-2} s^{-1} PPFD using a LI-6400 Portable Photosynthesis System (LI-COR Environmental, Lincoln, Nebraska USA). Photochemical yields were calculated from chlorophyll fluorescence yields measured on excised leaves using the MAXI-version of the Imaging-PAM (Heinz Walz GmbH, Effeltrich, Germany). Leaves were first kept in the dark for 20 min, which was followed by measurements of F_o, the minimal fluorescence yield of dark adapted samples and F_m, the maximal fluorescence yield obtained with the help of a saturation pulse. Maximum (potential) photochemical quantum yield of photosystem (PS) II was calculated as $F_v/F_m = (F_m-F_o)/F_m$.

Following this, leaves were illuminated with 55 µmol photons m^{-2} s^{-1} PPFD blue light for 5 min, then fluorescence yields F and F_m' were measured again before and after a saturating pulse. The effective PS II quantum yields of illuminated samples were calculated as Y(II)-55 = $(F_m' - F)/F_m'$ [31].

2.4. Pigment analysis

Two discs (d=0.6 cm) from each leaf were extracted into 80% acetone overnight at 4°C in darkness, ground and centrifuged (3000 x g, 5 min, 4°C) then chlorophyll (chlorophyll *a*+*b*) and carotenoid contents were calculated based on absorbances at 664.6, 646.6 and 440.5 nm according to [32]. Two other leaf disks were extracted into acidified methanol under the same conditions and used for spectrophotometric determination of total UV-B absorption ($\sum A_{280-315}$) [33] and anthocyanin content based on peak absorption at A_{530} [34]. All absorption measurements were carried out using a Shimadzu UV-1601 spectrophotometer.

2.5. Trolox equivalent antioxidant capacity (TEAC)

The TEAC assay is based on the ability of hydrogen donating antioxidants to convert 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid, the $ABTS^{+\bullet}$ radical) into colourless ABTS [35-36]. To form a cation radical, 0.1 mM ABTS, 0.0125 µM horse radish peroxidase and 1 mM H_2O_2 were mixed in a 50 mM phosphate buffer (pH 6.0). After 15 min, 100 µL leaf extract was added to 1 mL ABTS radical solution and the decrease in absorbance was measured at 730 nm after 2 min. A calibration curve was measured with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and TEAC values were given in µmol Trolox equivalents cm^{-2} leaf units.

2.6. Total phenolics content

Total phenolics content was determined with the Folin-Ciocalteu method as described by Veliglu et al. [37]. For each sample, 40 µL plant extract was mixed with 500 µL Folin-Ciocalteu reagent (previously diluted 10-times with distilled water) and allowed to stand at room temperature for 5 min, then 500 µL Na_2CO_3 (60 g L^{-1}) was added to the mixture. After 90 min incubation at room temperature, 725 nm absorbance was measured. A calibration curve was made with gallic acid (GA) and results were expressed in µmol GA equivalents cm^{-2} leaf units.

2.7. Ferric reducing antioxidant power (FRAP)

The FRAP assay was carried out according to a modification of the original medicinal biochemical assay [38] by Szőllősi and Szőllősi Varga [39]. The FRAP reagent was prepared by mixing 25 mL of acetate buffer (300 mM, pH 3.6), 2.5 ml of tripyridyltriazine (TPTZ) solution (10 mM TPTZ in 40 mM HCl) and 2.5 mL of $FeCl_3$ (20 mM in water solution). For each sample, 40 µL plant extract was added to 1 mL freshly mixed FRAP reagent. After 30 min incubation the increase in 593 nm absorbance due to the formation of the blue-coloured ferrous form (Fe^{2+}-TPTZ complex) was measured. A calibration curve was made with ascorbic acid (AA) and results were expressed as µmol AA equivalents cm^{-2} leaf units.

2.8. Singlet oxygen scavenging capacity

Specific singlet oxygen ($^1O_2$) scavenging capacity measurements were based on the method of Kraljic and El Moshni [40]. According to this, p-nitrosodimethylaniline (RNO) is bleached by a product of the reaction between $^1O_2$ and histidine which can be followed by monitoring the decrease in RNO absorption at 440 nm. Reaction mixtures contained 10 µM methylene blue, 15 µM RNO and 10 mM histidine in 50 mM phosphate buffer (pH 7.0). $^1O_2$ scavenging capacities of plant extracts were measured based on their abilities to inhibit the above reaction [26] and were quantified as µM Trolox equivalents cm^{-2} leaf units.

2.9. Hydroxyl radical scavenging capacity

Specific hydroxyl radical ($^{\bullet}OH$) scavenging capacity was determined by measuring the ability of leaf extracts to inhibit the formation of the strongly fluorescent 2-hydroxyterephthalate (HTPA) in a reaction between terephthalate (1,4-benzenedicarboxylic acid, TPA) and $^{\bullet}OH$ [41-42]. HTPA fluorescence was measured at room temperature with a Quanta Master QM-1 spectrofluorometer (Photon Technology Inc., Birmingham, New Jersey, USA), using 315 nm excitation and 420 nm emission. The 2.5 mL reaction mixture contained 500 µM TPA, 10 µM EDTA, 10 µM $FeSO_4$, 100 µM AA and 100 µM H_2O_2 in a 50 mM Na-phosphate buffer (pH 7.2). Hydroxyl radical scavenging of each plant extract was characterized by its half-inhibitory concentration on HTPA formation as described earlier [27]. The method was calibrated with ethanol, which is a strong $^{\bullet}OH$ scavenger and specific $^{\bullet}OH$ neutralising capacities of leaf extracts were given as mM ethanol equivalents cm^{-2} leaf units.

2.10. Statistics

Photosynthesis, leaf photochemistry, pigment contents and antioxidant capacities were measured in 3-9 leaves of similar age (YL or OL) and condition (PAR, PAR+UV). In figures data points/bars and error bars indicate average values and standard deviations, respectively. The significance of differences between data sets was analyzed using unpaired t-tests. Significantly different data sets ($p < 0.05$) are labelled with different letters. Correlations between two different biochemical or biophysical parameters were characterized by the R^2 parameter of a weighted linear least squares fit using one parameter as x and the other as y variable and reciprocals of x and y standard deviations as weights. Linear fits as well as other calculations were done with the software SigmaPlot (Systat Software Inc., San Jose, CA, USA) which was also used for drawing the figures.

3. Results

Effective photochemical yields of photosynthesis were slightly lower in young leaves (YL) than in old leaves (OL) when these were kept under photosynthetically active radiation (PAR) only, without UV-B (Fig.2A). Supplemental UV-B irradiation affected these differently, reducing them by 22% and 44% in YL and OL, respectively. Y(II)-55 values represent effective, light acclimated quantum yields, measured at relatively low, 55 µmol photons m^{-2} s^{-1} PPFD, which is very close to the PPFD applied during the four days of UV-B irradiation (50 µmol photons m^{-2} s^{-1}). In the absence of UV-B, potential (maximum) quantum yields (F_v/F_m) were the same in YL and OL and were affected by UV-B in the same way as Y(II)-55 (Fig.2B). Under this low PPFD, CO_2 gas exchange was very low, therefore photosynthesis measurements were performed under higher, 300 µmol photons m^{-2} s^{-1} PPFD. Chlorophyll fluorescence based yield measurements indicated that photochemical electron transport was still operational at this PPFD, even in UV-B irradiated leaves (data not shown). Figure 3 shows that YL had higher stomatal conductance and CO_2 uptake than OL. Stomatal conductance was low in all studied leaves, which is characteristic to green-house grown grapevine [43]. UV-B irradiation caused a strong decrease in both CO_2 uptake (Fig.3A) and stomatal conductance (Fig.3B) in all leaves.

Supplemental UV-B irradiation did not affect total chlorophyll contents either in YL or in OL (Fig.4A). While the carotenoid content increased slightly in OL in response to UV-B, in YL there was no change in the amount of this pigment (Fig. 4B). More UV-B absorbing compounds were present in OL than in YL even in the absence of UV-B radiation. While their amount did not change in OL upon UV-B irradiation, it increased significantly, by ca. 75% in

YL (Fig.4C). Anthocyanin levels were similar in control YL and OL, and supplemental UV-B increased this parameter to a small extent in YL only (Fig.4D).

Changes in three parameters generally used to describe total antioxidant capacities were also followed in the leaves. These are total phenolics [37], Trolox equivalent antioxidant capacity (TEAC) [35] and ferric reducing antioxidant power (FRAP) [38]. In control samples, both total phenolics and TEAC were much (ca 57 and 51%, respectively) lower in YL than in OL, but there were no significant differences in FRAP values (Fig.5). In response to the supplemental UV-B irradiation, values of all three total antioxidant capacity indicators increased in YL as compared to control YL, but no such increase was observed in OL. ROS specific antioxidant capacity measurements showed that control OL had approximately 28% higher hydroxyl radical scavenging potential than YL, while control YL and OL were not different in singlet oxygen scavenging capacities (Fig.6). Similarly to general antioxidant capacities, supplemental UV-B irradiation brought about marked changes in specific ROS scavenging in YL but not in OL. UV-B caused a smaller (1.3-fold) increase in hydroxyl radical (Fig.6A) and a larger (almost 7-fold) increase in singlet oxygen (Fig.6B) neutralizing capacities of YL.

4. Discussion

Depending on the applied doses and the presence of other environmental factors, UV-B radiation documentedly affects grapevine leaves in many ways. A recent study on an Argentinean cultivar, *Vitis vinifera* cv. Malbec demonstrated a variety of general protective responses, for example the induction of pathways regulating synthesis of UV-B absorbing compounds such as the phenylpropanoid pathway, the induction of different antioxidant defence systems and the activation of pathways commonly associated with pathogen defence and abiotic stress responses [44]. In our study, a relatively weak UV-B irradiation was applied to supplement low PPFD. These conditions resulted in intense stomata closure and strong limitation of photosynthesis (Fig.3) and decreased the photochemical yield (Fig.2). The effect of the applied 4-days UV-B irradiation was strong, but did not result in any loss of pigments (Fig.4) and was reversible (data not shown). We found that leaf photochemistry was more decreased in OL than in YL. Leaf age and developmental stage have already been shown to affect responses to UV-B in Brassicaceae [45] and a citrus species [46], but not in grapevine so far.

In a recently reported experiment using an other grapevine cultivar (cv. Malbec), Berli et al. [47] showed a similar effect of natural solar and UV-B supplemented solar irradiation on pigments that what we observed in YL, namely no changes in chlorophyll content and the accumulation of UV-B absorbing compounds and anthocyanins. They also showed evidence for lipid peroxidation and the activation of peroxidases by UV-B. Although the two experiments can not be compared directly, due to differences in plant material (cultivar, age) and PAR conditions accompanying UV-B (high solar PPFD used by Berli et al. and much lower PPFD in our study), both show the activation of the antioxidant system. Moreover, they demonstrate that supplemental UV-B is capable of this activation under diverse conditions. The increased hydroxyl radical neutralizing capacities of YL in our experiment (Fig.6A) may contribute to preventing the lipid peroxidation observed by Berli et al. [47]. We did not identify specific chemicals contributing to this capacity so far, but some of the UV-B absorbing compounds or phenolics which were present in UV-B exposed YL at higher concentrations than in controls (Figs. 4C and 5A) are likely candidates. To support the dual, UV screening and antioxidant role of UV-B absorbing compounds [48], Pollastrini et al. [49] recently reported an increase in epidermal polyphenols acting both as UV-B absorbents and antioxidants in grapevine cv. Sangiovese in a field experiment. Kolb et al. [50] showed that during the exposure of green-house grown grapevine (cv. Silvaner) to solar UV-B, epidermal screening was capable of protecting PS II photochemistry but not net photosynthesis, a scenario very similar to what we observed in young Chardonnay leaves (Figs. 2 and 3).

We found that activities of water-soluble leaf antioxidant parameters were stimulated by the supplemental UV-B radiation in YL but not in OL (Figs. 4-6). It is important to note that both fresh weight per leaf area and total extractable protein per leaf area were the same for all studied samples (data not shown), therefore differences in parameters expressed as per leaf area reflect true differences and not morphological responses. Interestingly, although total phenolics, FRAP, hydroxyl radical scavenging and UV-B absorbing pigment contents were initially higher in OL than in YL, OL were not capable of increasing these in response to supplemental UV-B (Figs. 4-6). Furthermore, there were no large differences between concentrations/ activities which appear protective in YL but insufficient in OL, demonstrating that acclimation to UV-B is not determined by quantities but rather by the ability to adjust these. Our results also point to hazards of leaf pooling (mixing leaves of different developmental stages) in studies of plant UV responses. Connections between UV-B screening and antioxidant compounds can be studied by either a comparative chemical

analysis of the extracts or studying correlations between their responses. In the present study, we chose the latter approach.

Although uncertainties (standard deviations) of some values were high, we were looking for linear correlations between various sets of antioxidant, pigment and photosynthesis parameters. For most parameter pairings this was not found and in some cases connections appeared non-linear. Figure 7 shows correlations which appeared close to linear, with $R^2 > 0.75$. A good ($R^2 = 0.847$) positive linear correlation was found between maximum (F_v/F_m) and effective (Y(II)-55) photochemical yields (Fig. 7A), indicating that the UV-B induced limitation of photochemistry was not solely due to stomata closure which does not affect F_v/F_m, but internal PS II factors could also be involved. With the exception of anthocyanins, pigment concentrations did not appear to be linked to changes in leaf photochemistry. Anthocyanin content had a weak negative linear ($R^2 = 0.762$) correlation with both Y(II)-55 (Fig. 7B) and F_v/F_m (data not shown), suggesting that although their concentrations increased in YL to a small extent, anthocyanins were not among the key components of PS II protection in the present experiment. Anthocyanin related protection of photosynthesis was demonstrated under stress by high PAR [51]. Because PPFD was relatively low in our experiment, the observed increase in anthocyanins is unlikely to be aimed at protection against PAR and it is more likely to be the consequence of a general increase in the biosynthesis of UV absorbing phenolics in response to the supplemental UV-B radiation.

From among the three studied total antioxidant capacity parameters, FRAP was not linearly correlated with either TEAC or total phenolics. TEAC, however, had a very good ($R^2 = 0.934$) positive linear correlation with total phenolics (Fig. 7C). This is different from what we found earlier in senescing tobacco leaves, where FRAP and total phenolics were strongly correlated and TEAC appeared less related to the two other parameters [24]. This difference between results of the two experiments which are only common in changes of antioxidant levels is not surprising. Nevertheless, it exemplifies that similarities or differences between results of the three methods measuring total antioxidant capacities are not due to those in the chemistry involved but rather to ratios of various antioxidants in the studied samples. Moreover, it should be emphasised that results of various total antioxidant capacity methods are not interchangeable. A positive linear correlation could also be found between TEAC (or total phenolic content) and total UV-B absorption as well (the former is shown in Fig. 7D, $R^2 = 0.973$), which is probably due to the fact that most phenolics absorb in the UV-B region [50], although our data do not report on non-extractable antioxidants, such as cell wall bound

phenolics. Both hydroxyl radical and singlet oxygen scavenging capacities were positively and linearly (R^2 = 0.822 and 0.953, respectively) correlated with TEAC (Figs. 7E and 7F). As TEAC is based on reactivity to a synthetic free radical [35], this result shows that synthesis of compounds reactive to both ROS as well as to the ABTS radical were stimulated in young Chardonnay leaves by the supplemental UV-B radiation. On the other hand, the lack of a linear correlation between FRAP and either hydroxyl radical or singlet oxygen scavenging, which was also found earlier [24] confirms that these specific antioxidant capacities are not decisive main constituents of FRAP.

The pivotal role of antioxidant responses in stress adaptation has already been well documented [53-55], although we are unaware of any similar study on UV-B acclimated young and old vine leaves. In addition to the search for correlations between responses of various antioxidant parameters, the novel aspect of our study is the possibility to dissect the role of specific ROS scavenging. Our results show that in YL both hydroxyl radical and singlet oxygen scavenging capacities increased markedly in response to supplemental UV-B irradiation. As discussed above, hydroxyl radical neutralizing was lower in untreated YL than in OL, but in response to UV-B it increased to the level found in untreated OL. Under strong oxidative stress conditions achieved by irradiating isolated thylakoid membranes with high doses of UV-B, hydroxyl radicals were directly detectable [5] and later we have shown that these ROS are by-products rather than specific initiators of UV-B-induced membrane damage [56]. Results presented here suggest that in leaves exposed to lower UV-B doses in combination with low PAR effective hydroxyl radical scavenging is important. YL which increased this specific antioxidant capacity were able to maintain more efficient photochemistry under supplemental UV-B than OL which did not adjust this.

In experiments presented here, singlet oxygen scavenging capacity showed the largest UV-B induced increase among the studied antioxidant parameters. Although most carotenoids are known to be capable of singlet oxygen scavenging [57] and specific carotenoids were shown to protect cyanobacteria against UV-B photodamage [58] we found no correlation between total carotenoid contents and singlet oxygen scavenging capacities of grapevine leaves. Our earlier studies showed that singlet oxygen was not produced in response to strong oxidative stress achieved by high UV-B doses [59] and in a spectral comparison of the ROS generating efficacies of UV we showed that longer wavelengths (UV-A) irradiation was more efficient in triggering detectable amounts of singlet oxygen [21]. These studies were, however, performed not only at high UV doses (ca 10-times higher than in the present study), but without photosynthetically active light. Although we lack direct evidence, the data shown

here suggest that under low, supplemental UV-B irradiance singlet oxygen may be present, because its efficient neutralizing lessens the loss of photosynthetic function under these conditions. A marked increase in response to supplemental UV-B irradiation in hydroxyl radical or singlet oxygen scavenging capacities implies that these specific ROS neutralizing capacities are important factors and should be included in studies on leaf UV responses.

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Figure captions

Figure 1.
Spectral distribution of irradiance from the Q-Panel UVB-313EL tube panel (covered with cellulose diacetate filter) applied in the experiments presented here. Inset: UV region of the same spectrum.

Figure 2.
(A) Effective photochemical yield of PS II at 55 µmol photons m^{-2} s^{-1} PPFD (Y(II)-55) and (B) maximum photochemical yield of PS II in the dark adapted state (F_v/F_m) of young (YL) and old (OL) grapevine leaves without (PAR, open bars) and with (PAR+UV, grey bars) supplemental UV irradiation (n= 4-8).

Figure 3.
(A) Photosynthesis and (B) stomatal conductance of young (YL) and old (OL) grapevine leaves without (PAR, open bars) and with (PAR+UV, grey bars) supplemental UV irradiation (n= 4-6).

Figure 4.
(A) Chlorophyll *a*+*b*, (B) carotenoid, (C) UV-B absorbing pigment and (D) anthocyanin content of young (YL) and old (OL) grapevine leaves without (PAR, open bars) and with (PAR+UV, grey bars) supplemental UV irradiation (n= 4-9).

Figure 5.
(A) Total phenolic content, (B) Trolox equivalent antioxidant capacity and (C) ferric reducing antioxidant power of young (YL) and old (OL) grapevine leaves without (PAR, open bars) and with (PAR+UV, grey bars) supplemental UV irradiation (n= 4-9).

Figure 6.
(A) Hydroxyl radical and (B) singlet oxygen scavenging capacities of young (YL) and old (OL) grapevine leaves without (PAR, open bars) and with (PAR+UV, grey bars) supplemental UV irradiation (n= 3, hydroxyl radical data and (n= 3-9, singlet oxygen data).

Figure 7.
Correlations between various parameters. Data points represent average values with standard deviations re-plotted from Figs.1-5, from measurements of young (YL, circles) and old (OL, squares) grapevine leaves without (PAR, open symbols) and with (PAR+UV, closed symbols) supplemental UV irradiation. Dashed lines show weighted linear least squares fits (see Materials and Methods for details) on the following data sets: (A) effective photochemical yield Y(II)-55 and maximum photochemical yield F_v/F_m, R^2 = 0.847; (B) Y(II)-55 and anthocyanin content, R^2 = 0.762; (C) total phenolics content and TEAC, R^2 = 0.934; (D) UV-B absorbing pigments and TEAC, R^2 = 0.973; (E) hydroxyl radical scavenging capacity and TEAC, R^2 = 0.822; (F) singlet oxygen scavenging capacity and TEAC, R^2 = 0.953.

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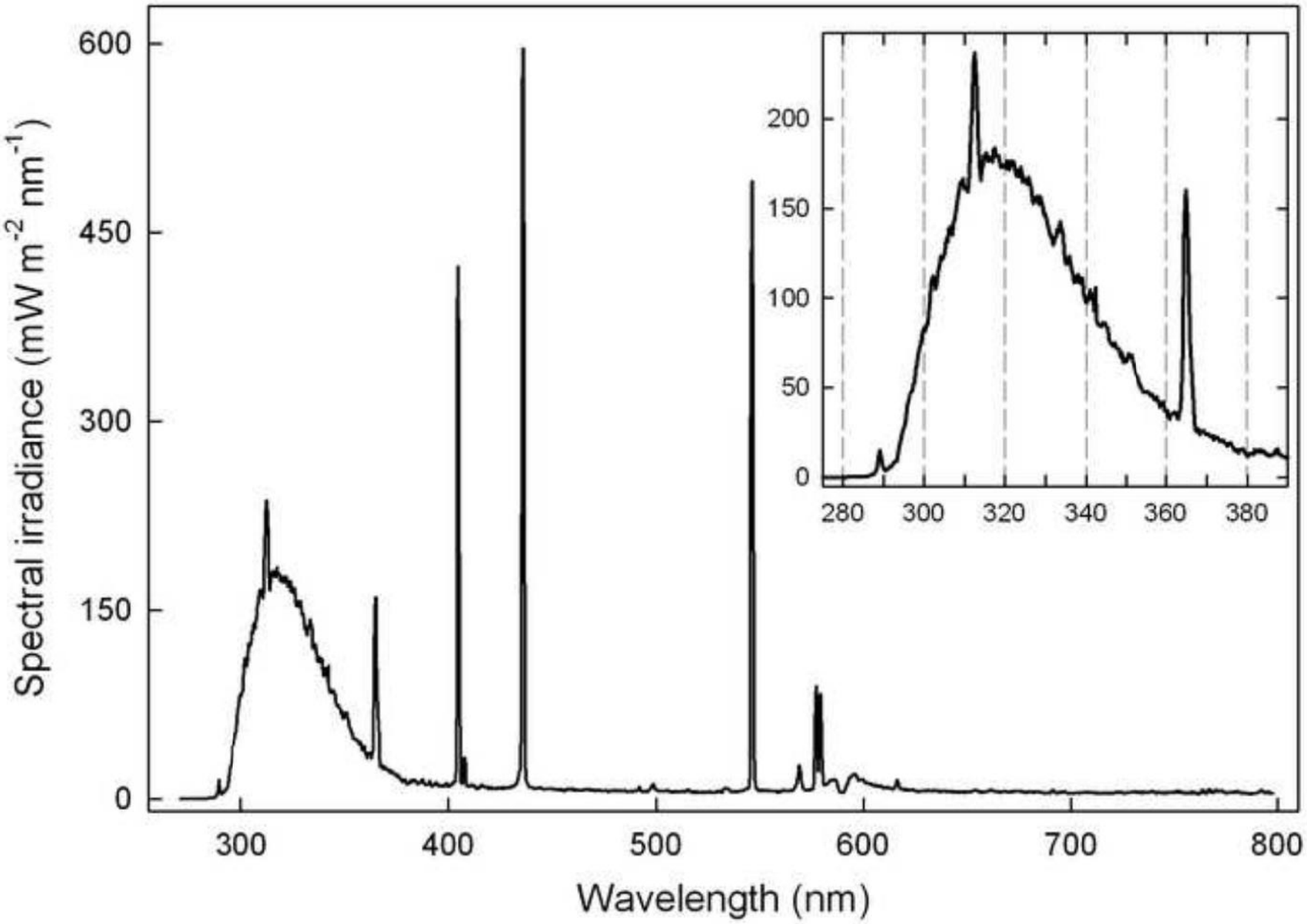

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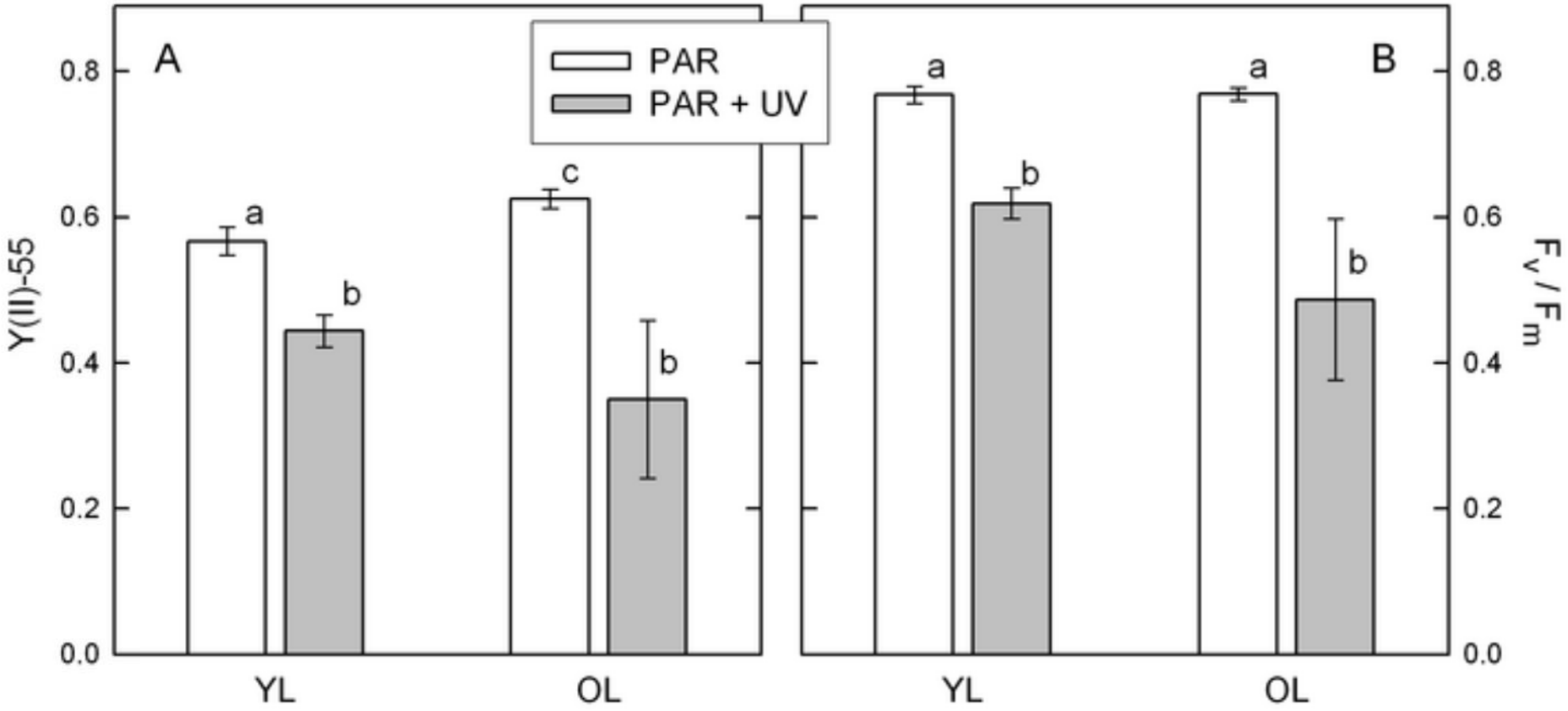

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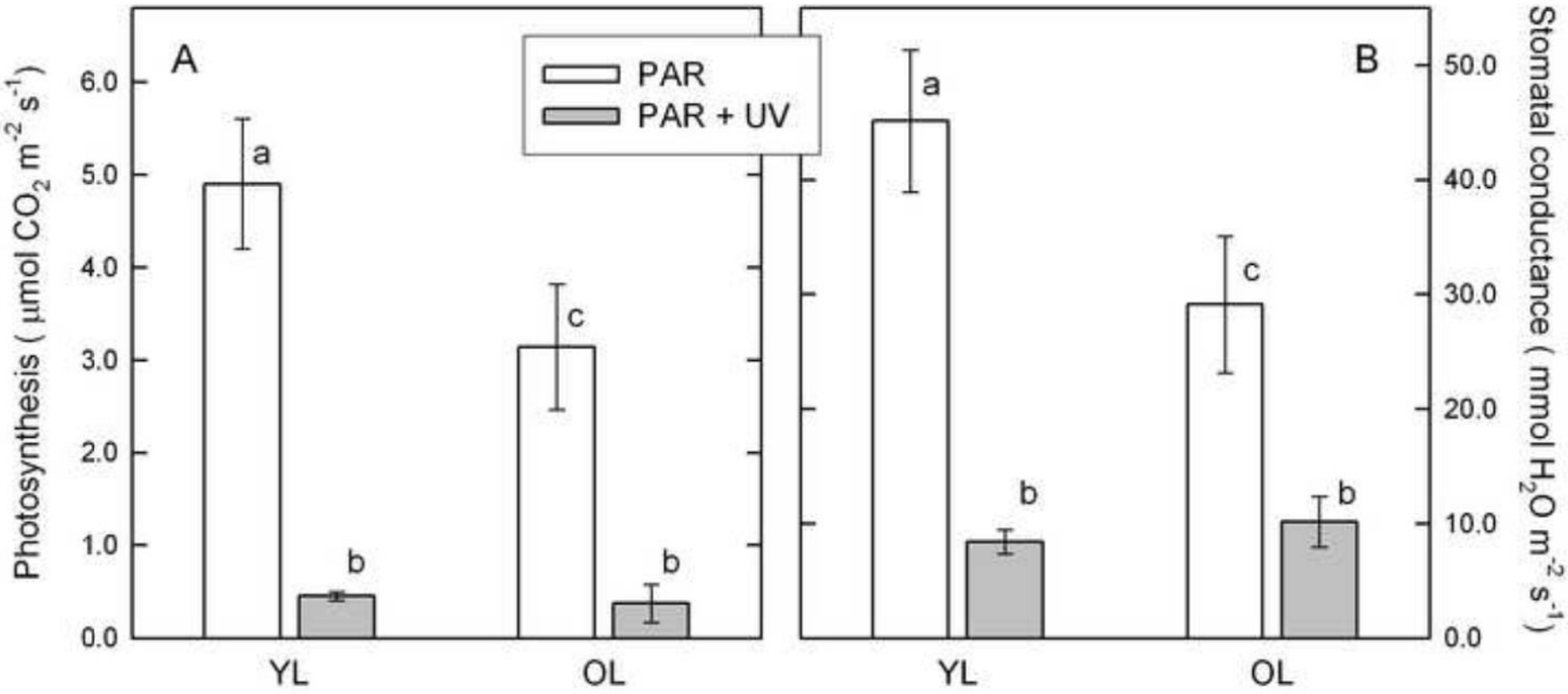

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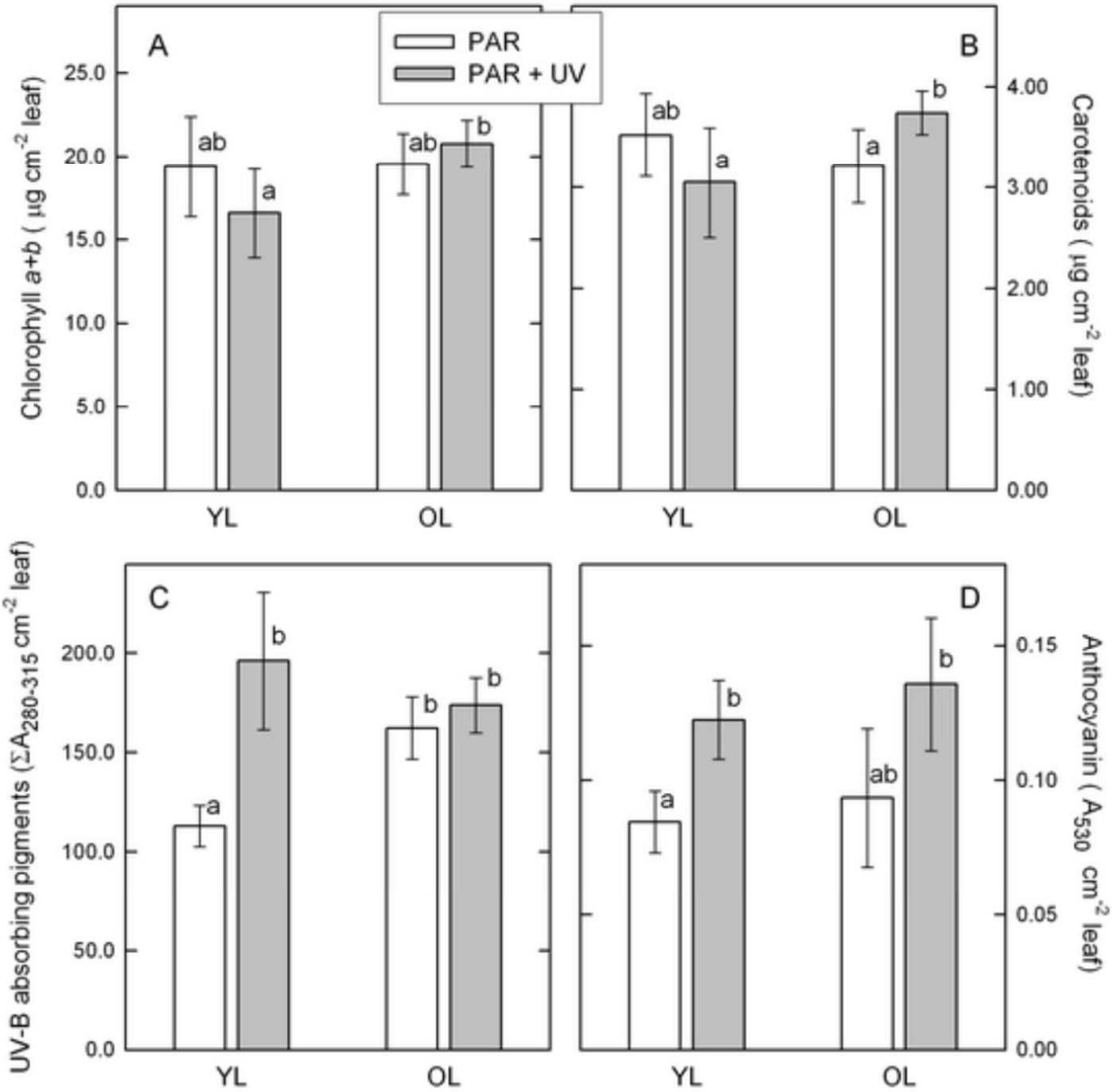

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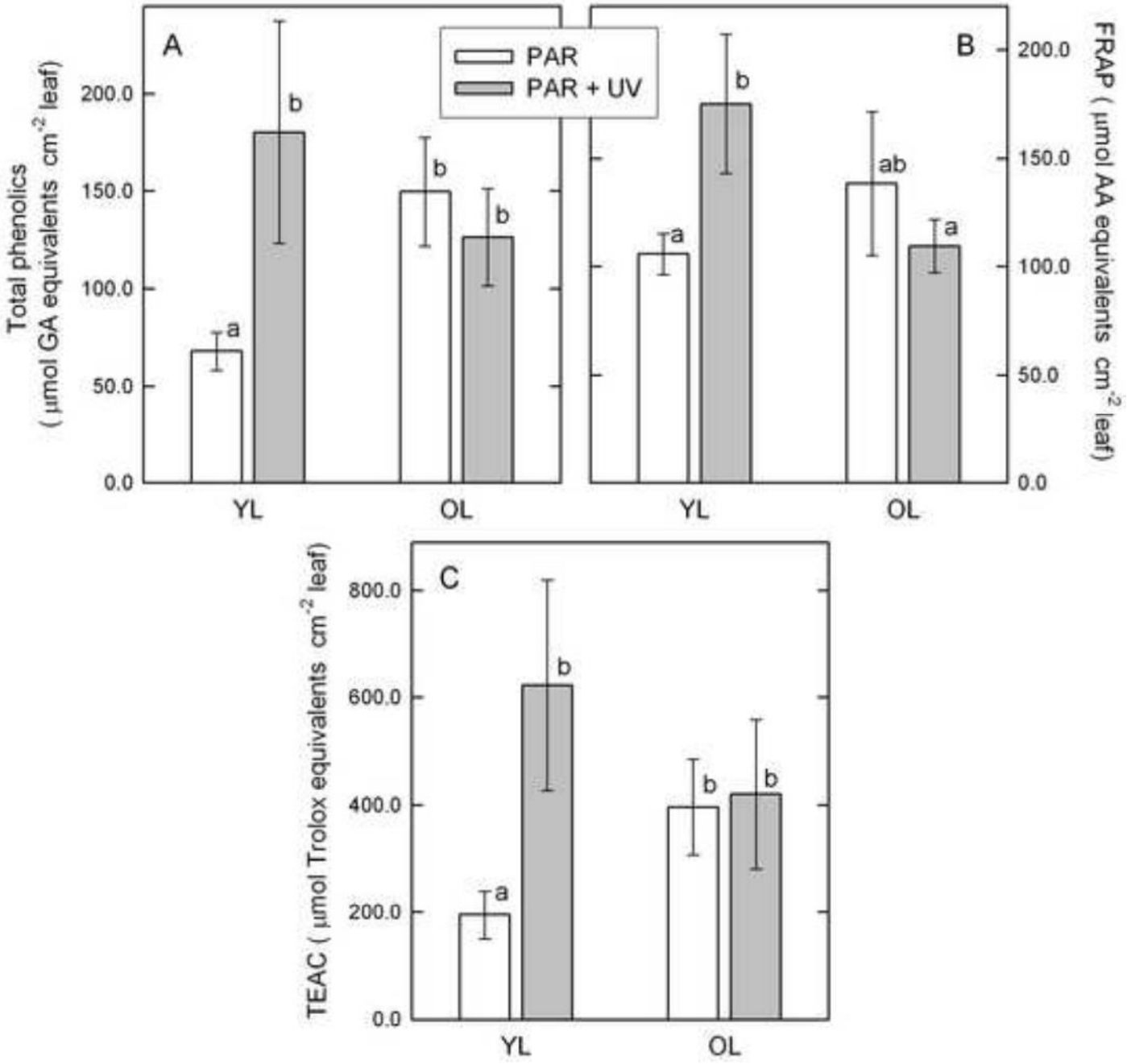

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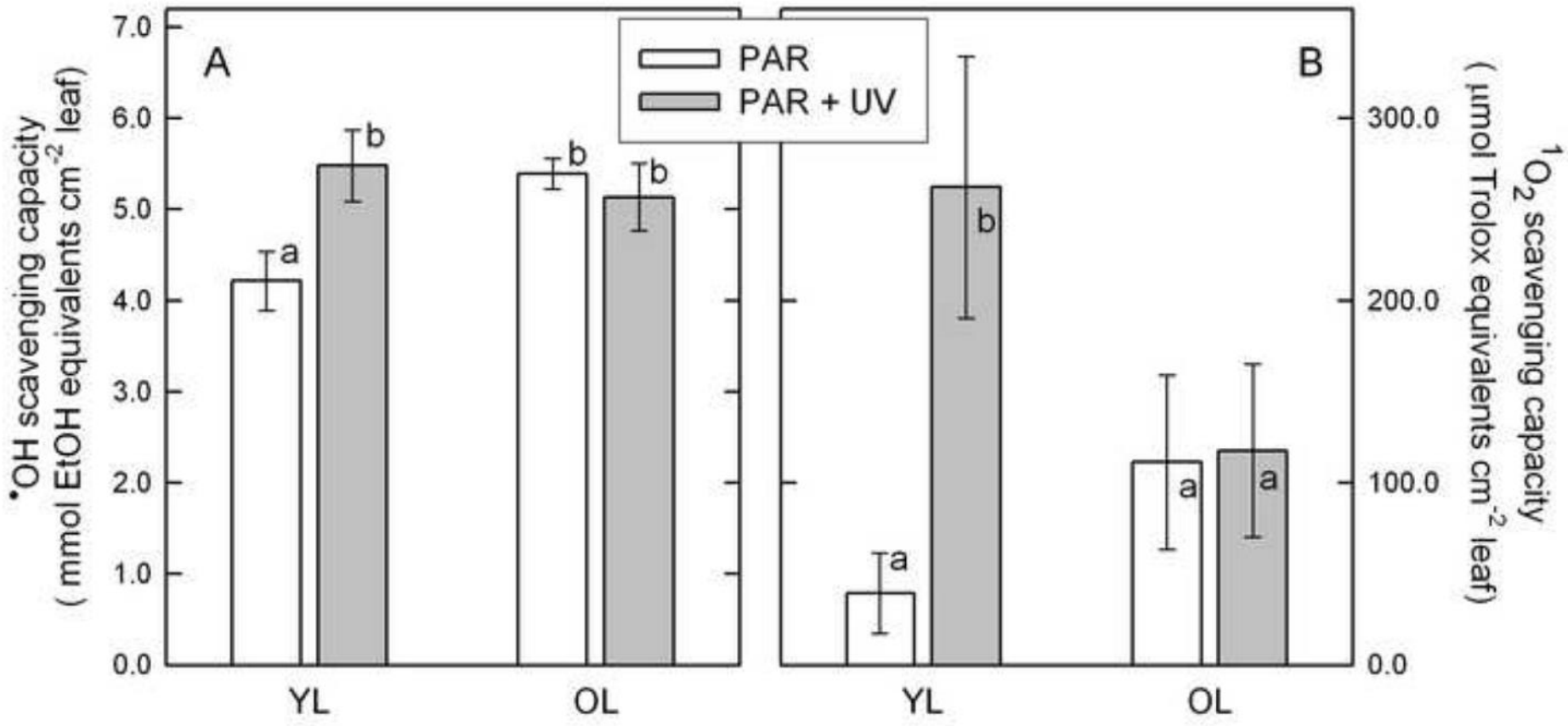

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