Plant Physiology and Biochemistry 134 (2019) 9–19

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

UV-B strengthens antioxidant responses to drought in *Nicotiana benthamiana* leaves not only as supplementary irradiation but also as pre-treatment☆

Anikó Mátai, Dóra Nagy, Éva Hideg*

Department of Plant Biology, University of Pécs, Hungary

ARTICLE INFO

Keywords:
Ultraviolet radiation
Drought
Antioxidant capacity
ROS
Flavonoid index
Peroxidase
SOD
Stress interaction

ABSTRACT

Potentials of UV-B (280–315 nm) radiation to alleviate effects of water deficit were studied using *Nicotiana benthamiana* plants in growth chambers. 10-days of limited watering resulted in 40% loss of soil water content as compared to well-watered controls. This drought was applied in three different ways: (i) in itself, (ii) after 4-days exposure of 6.9 kJ m^{-2} d^{-1} biologically effective supplementary UV-B radiation as pre-treatment, or (iii) in parallel with 6.9 kJ m^{-2} d^{-1} biologically effective supplementary UV-B. Responses were examined in two leaf groups: fully developed mature leaves (ML) and young leaves emerging during the 10-day treatment (YL). ML responded to UV-B or drought as single factor treatments with 7–14% loss of photochemical yield, while YL photochemistry was not decreased under the same conditions. The parallel two-factor treatment had no aggravating effect but alleviated drought-induced loss of leaf photochemistry in ML. Several positive single factor effects of drought or UV-B on antioxidants remained significant in the two-factor treatment both in ML and YL. Effects of the two factors applied in parallel were additive (equal to the sum of the effects caused by single factors separately) on total antioxidant capacities and singlet oxygen neutralizing; and synergistic (larger than the sum of single factor effects) on the flavonoid index in ML. A sequential application of UV-B and drought had additive positive effects on antioxidant capacity and flavonoid index of ML suggesting lasting effects of UV-B pre-treatment.

1. Introduction

High acute doses of UV-B (280–315 nm) radiation are potentially cytotoxic via the production of reactive oxygen species (ROS) and may also damage cellular components directly (Jansen et al., 1998). Stress signalling pathways are also activated and the extent of cellular damage and plant survival are determined by the relation of pro-oxidants and antioxidants. On the other hand, environmentally relevant UV-B doses have been repeatedly shown to be less detrimental to plant performance than previously thought (reviewed in Hideg et al., 2013). Plant responses to low UV-B doses were shown to be acclimatory including photoreceptor mediated changes in morphology and metabolites (Jenkins, 2014). Physiological adjustments in plants grown under UV-B include increased antioxidant enzyme activities and higher amounts of non-enzymatic antioxidants (Czégény et al., 2016a). These are also the characteristics of acclimative responses to several other stressors, suggesting the potential of UV-B to enhance survival under biotic or abiotic stress conditions. For example, UV-B radiation was suggested to act as a signal to initiate plant defence from photo-oxidative stress in Arabidopsis (Jansen et al., 2012) and in linden leaves (Majer et al., 2014). Further, moderate doses of UV-B were found to increase the cold tolerance of rhododendron (Chalker-Scott and Scott, 2004) and the heat tolerance of cucumber (Teklemariam and Blake, 2003). On the other hand, Kovács et al. (2014) showed that the same UV-B treatment can

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); AsA, L-ascorbic acid; DPBF, 2,5-diphenyl-3,4-benzofuran; FRAP, ferric reducing potential; Fv/Fm, maximum quantum efficiency of PS II; HRP, horseradish peroxidase; ML, mature leaves; PAR, photosynthetically active radiation; PARD, plants drought exposed under PAR; PARW, well-watered control plants kept under PAR; pUVW, UV-B treated well-watered plants; pUVD, plants exposed to both UV-B and drought; ROS, reactive oxygen species; SOD, superoxide dismutase; sUVW, UV-B pre-treated plants; sUVD, UV-B pre-treated then drought exposed plants; TAC, total antioxidant capacity; TEAC, Trolox equivalent antioxidant capacity; TPA, 1,4-benzenedicarboxylic acid; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; UV, ultraviolet radiation; UV-B, 280–315 nm UV; YL, young leaves; Y(II), light acclimated effective quantum yield of PSII; Y(NO), non-regulated non-photochemical quenching of PS II; Y(NPQ), regulated non-photochemical quenching of PS II

☆ This article is part of the Special Issue "Interactive effects of UV-B radiation in a complex environment" published at the journal Plant Physiology and Biochemistry 134, 2019.

* Corresponding author. Department of Plant Biology, Institute of Biology, University of Pécs, Ifjúság u. 6, H-7624, Pécs, Hungary
E-mail address: ehideg@gamma.ttk.pte.hu (É. Hideg).

https://doi.org/10.1016/j.plaphy.2018.09.014
Received 13 April 2018; Received in revised form 9 September 2018; Accepted 11 September 2018
Available online 12 September 2018
0981-9428/

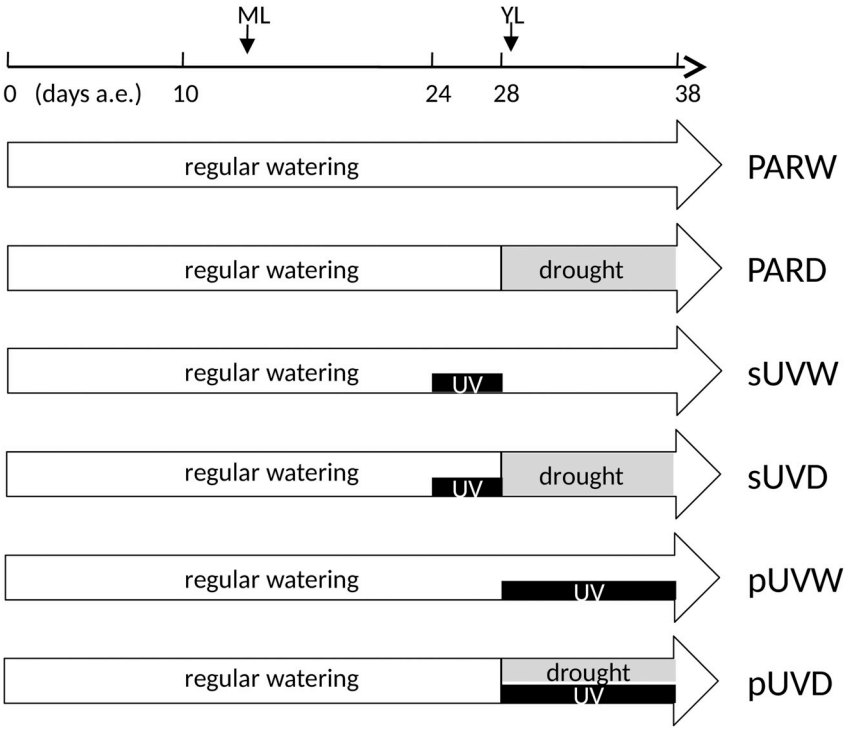

Fig. 1. Scheme of the applied treatments and plant group identifiers.
All treatments were carried out under PAR. Supplementary UV-B radiation was applied either before (sUVD) or in parallel to (pUVD) a 10-day drought treatment. Control experiments were carried out using one factor only, either drought (PARD) or UV-B (sUVW, pUVW); and growth conditions were maintained for plants in the PARW group. ML and YL labelled arrows indicate the time when mature and young leaves emerged, respectively. See Section 2.1 for details of treatment conditions.

have either a positive or negative impact on wheat leaves depending on the type of the additional abiotic stress factor.

Several studies explored the possible link between plant responses to UV-B and drought tolerance (reviewed in Bandurska et al., 2013). It was suggested that UV-B radiation could enhance drought tolerance through photomorphogenic effects (Gitz and Liu-Gitz, 2003), via the accumulation of foliar flavonoids and phenols (Ren et al., 2007), by increasing of leaf salicylic acid contents (Bandurska and Cieślak, 2013; Kovács et al., 2014), or via decreasing stomatal conductance and enhancing proline synthesis (Poulson et al., 2006). Because both UV-B and drought as single factors were found to stimulate antioxidant enzyme activities, it is plausible to assume an overlapping in signalling pathways. Nevertheless, responses to a combined application of the two factors were found to depend on a number of factors, including the plant species, the developmental stage, the applied UV-B doses as well as the duration and severity of drought stress. Under field conditions, the impact of UV-B may be overshadowed by the effects of water deficit (Martínez-Lüscher et al., 2015, grapevine studies) or may only be significant under low water availability (Bernal et al., 2015, laurel). In another study, UV-B ameliorated drought effects in plants suffering from strong water deficit but rendered well-watered plants "slightly disadvantaged" (Robson et al., 2015, silver birch). Different genotypes of the same species may react differently, for example a combined application of drought and UV-B in growth chambers brought out strong adverse effects on wheat seedlings of a susceptible variety, but more positive effects on a tolerant genotype (He et al., 2011). Poplar plants originating from low altitudes suffered from an additive effect of the two stresses, while the high altitude genotype already adapted to higher levels of UV-B exhibited greater tolerance (Ren et al., 2007).

Results of two factors drought and UV-B experiments are contradictory, possibly due to differences in experimental conditions and the studied species. For example, one factor was found to reduce the antioxidant stimulating effects caused by simultaneous application of the other factor in cucumber leaves (Kubis and Rybus-Zajac, 2008) but caused no pronounced additive effect in wheat (Kovács et al., 2014) or pea (Alexieva et al., 2001), whereas the two factors resulted in increased activities as compared to single factors in another wheat experiment (Tian and Lei, 2007).

In the work presented here we examined antioxidant responses of tobacco (*Nicotiana benthamiana*) plants to low, acclimative UV-B doses and drought achieved by limited watering. Conditions were chosen to evoke a mild stress and not extreme damage as single factors, i.e. to result in only a small loss of photochemical efficiency and enhance antioxidant defences as single factors. Studying the interaction of the two factors we had three major aims. Firstly, to analyse whether a UV-B pre-treatment modified concomitant responses to drought. In addition to study how long plants maintained UV-B-induced increased antioxidant defences, this point has relevance to the exploitation of UV-B to improve transplantation and transportation tolerance (Wargent et al., 2011). Secondly, we intended to compare contributions of antioxidant enzymes and non-enzymatic ROS specific defence in response to the single factors and their sequential or parallel application. Lastly, we compared antioxidant responses of mature leaves present throughout the experiments to those of young leaves emerging during the treatments.

2. Materials and methods

2.1. Plant growth and treatment conditions

Nicotiana benthamiana plants were grown on standard soil in growth chambers (Sanyo MLR-352H-PE, Panasonic Healthcare Co., Ltd., Oizumi, Japan) under 175 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), long day conditions (16 h/8 h light/dark, 25 °C/20 °C) and 70% relative air humidity.

Drought treatment started four weeks after emergence. Pots were weighed at 100% soil water content and were transferred to lower, 50% relative air humidity keeping all other conditions the same. A 40% loss of soil water content (estimated from a 40% reduction in pot weight) was achieved by complete water withdrawal in 2–3 days and was maintained by limited watering for the rest of the treatment, a total of 10 days. Exposure to UV-B was applied from Q-Panel UVB-313EL tubes (Q-Lab Ltd., Bolton, UK) using a cellulose diacetate filter (Courtaulds Chemicals, Derby, UK). The applied UV was centered at 318 nm and corresponded to 6.9 kJ m^{-2} d^{-1} biologically effective radiation as calculated using the Biological Spectral Weighting Function (Flint and

Caldwell, 2003). UV-B treatment was performed in two different experiments: (i) applied for 4 days before drought as sequential treatment (sUVD), or (ii) present during the 10 days drought treatment as parallel factor (pUVD) (Fig. 1.). Both combinations of drought and UV-B treatments had parallel plant groups kept in the absence of UV-B and/or drought. Controls for the sequential experiment (sUVD) were either drought-exposed without the UV-B pre-treatment (PARD) or were watered throughout the experiment (sUVW plants). To study the combined action of the two factors, pUVD plants were compared with the group subjected to limited watering under PAR only (PARD plants), with untreated plants (PARW) and to another group exposed to ten days of UV only (pUVW). All groups consisted of five plants (n = 5).

It should be noted that the UV-B pre-treatment applied in sequential two-factor experiment was shorter than the one applied in parallel with drought. This is due to the fact that a 10-day UV-B pre-treatment followed by a 10-day drought would have been too long to avoid the interference of flowering related metabolic changes.

From each plant two different leaves were examined: (i) one that was fully developed when treatments started (mature leaves, ML), and (ii) another that was in a bud stage at the start of the experiment, emerged during treatments and was still not fully developed by end of the experiment (young leaves, YL) (Fig. 1.). Consecutively, ML were approximately two weeks older than YL, and YL of sUVW and sUVD plants were not exposed to UV-B irradiation directly.

2.2. Non-invasive leaf measurements

Chlorophyll and flavonoid contents of leaves were estimated using a DUALEX® Scientific (FORCE-A, Orsay, France) leaf clip equipment. Chlorophyll and flavonoid indexes were measured at the adaxial leaf side (Goulas et al., 2004). Following these measurements, plants were dark adapted for 30 min before variable chlorophyll fluorescence parameters were assessed using the MAXI-version of Imaging-PAM (Heinz Walz GmbH, Effeltrich, Germany). After 30 min, minimum (F_0) and maximum (F_m) fluorescence yields were measured before and after a saturating light pulse, respectively. This was followed by actinic irradiation with blue light corresponding to 230 μmol m^{-2} s^{-1} PAR for 1 min and the determination of fluorescence quantum yields F and F_m' using a saturating pulse. PSII maximum quantum efficiency (F_v/F_m), and the light acclimated yield of photochemical conversion, Y(II) = (F_m'-F)/F_m', were calculated, together with quantum yields of regulated, Y(NPQ) = F/F_m' - F/F_m, and non-regulated, Y(NO) = F/F_m, non-photochemical energy quenching according to Klughammer and Schreiber (2008).

2.3. Preparation of leaf extracts

After measuring plant and leaf weights, mature (ML) and young (YL) leaves were ground in liquid nitrogen using mortar and pestle, then ice cold potassium-phosphate buffer (50 mM, pH 7.5) containing 0.3 mM EDTA. Samples were centrifuged twice at 32,300 × g 20 min, 4 °C (Hitachi CR22GIII, Hitachi High-Technologies Corporation, Japan). Total soluble protein content was measured using the standard Bradford assay (Bradford, 1976) based on 620 nm absorption measured with a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) using bovine serum albumin for calibration. Aliquots of samples were stored in the above potassium-phosphate buffer at −20 °C until further use.

2.4. Total antioxidant capacities

2.4.1. TEAC

Total antioxidant capacities of leaf extracts were determined by measuring the reduction of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$). The assay was carried out as described earlier (Majer and Hideg, 2012). A mixture of 0.1 mM ABTS, 0.0125 mM HRP (horseradish peroxidase) and 1 mM H_2O_2 in phosphate buffer (50 mM, pH 6.0) was incubated for 15 min to ensure the oxidation of ABTS to $ABTS^{\cdot+}$. As modification of the original protocol, further oxidation of ABTS was stopped and the blue colour of the TAC reagent was stabilized by the addition of 25% (v/v%) ethanol. Antioxidant capacities were assessed by allowing leaf extracts (5 μL in 200 μL TAC reagent) to neutralize the ABTS cation radical for 3 min. Absorption change was followed at 651 nm with a plate reader (Section 2.3.), and total antioxidant capacities were expressed as mg Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) equivalents per mg soluble protein.

2.4.2. FRAP

Electron transfer based total antioxidant capacities of samples were also assessed as ferric reducing capacities following the absorption change of ferrous TPTZ (2,4,6-tris(2-pyridyl)-s-triazine) complex at 620 nm (Szőllősi and Szőllősi-Varga, 2002). The assay reagent contained 2.5 mL 10 mM TPTZ solution (dissolved in 40 mM HCl), 2.5 mL 20 mM $FeCl_3$ solution (dissolved in water) and 25 mL sodium-acetate buffer (300 mM, pH 3.6). After adding 5 μL leaf extract, the mixture was incubated at room temperature for 30 min. Absorptions were measured with a plate reader (Section 2.3.), and FRAP values were expressed as mg ascorbic acid (AsA) equivalents per mg soluble protein.

2.5. Total peroxidase activity

Total peroxidase activity was determined with *o*-phenylenediamine (OPD) (Fornera and Walde, 2010) which is an artificial peroxidase substrate and produces a soluble orange-brown product during enzyme activity. The assay contained 50 mM OPD and 360 μM H_2O_2 in 50 mM phosphate citrate buffer (pH 5.0). Increase in absorption at 450 nm was recorded for 2 min using a Shimadzu UV-1800 spectrophotometer. HRP (Sigma-Aldrich) was used for calibration and peroxidase activities of samples were expressed as unit peroxidase per μg soluble protein.

2.6. SOD activity

Superoxide dismutase (SOD) activity was measured as the inhibition of formazan generation from the reaction of superoxide radical and nitro blue tetrazolium (NBT). The reaction mixture contained 12 μM NBT and 10 μM riboflavin in 50 mM potassium-phosphate buffer (pH 7.5) (Song et al., 2007). Absorption at 540 nm was recorded twice, immediately after mixing assay components and after 5 min illumination with 1000 μmol m^{-2} s^{-1} PAR (LED Light Source SL 3500, Photon Systems Instruments, Drasov, Czech Republic) using a plate reader (Section 2.3.). Standard superoxide dismutase (Sigma-Aldrich) was used for calibration and SOD activities of samples were expressed as unit SOD per μg soluble protein.

2.7. Singlet oxygen neutralizing antioxidant capacity

The oxidation of 2,5-diphenyl-3,4-benzofuran (DPBF) by $^1O_2$ generated from 50 μmol m^{-2} s^{-1} red (600–650 nm) light illuminated methylene blue is decreased by $^1O_2$ reactive antioxidants (Majer et al., 2014). One mL reaction mixture in 1 cm optical path cuvette contained 20 μM methylene blue and 120 μM DPBF in 40/60 v/v% methanol/water. DPBF oxidation was measured as decrease in absorption at 410 nm using a spectrophotometer (Section 2.5.). $^1O_2$ antioxidant capacities were expressed as mM AsA equivalents per mg soluble protein.

2.8. Non-enzymatic hydrogen peroxide neutralizing antioxidant capacity

The applied assay is based on the photometric detection of iodine (I_2) from the reaction between H_2O_2 and potassium iodide (KI), and the ability of H_2O_2 reactive compounds to limit this reaction as described earlier (Csepregi and Hideg, 2016). The 200 μL reaction mixture

contained 25 μM H_2O_2, and 595 μM KI in potassium-phosphate buffer (100 mM, pH 7.0) and 20% v/v% ethanol to inhibit enzymatic capacities. Absorption at 405 nm was recorded twice, immediately and 3 min after mixing assay components, using a plate reader (Section 2.3.). Non-enzymatic H_2O_2 antioxidant capacities of leaf extracts were given as mM AsA equivalents per mg soluble protein.

2.9. Hydroxyl radical neutralizing antioxidant capacity

Hydroxyl radicals oxidize terephthalic acid (TPA, 1,4-benzenedicarboxylic acid) into fluorescent 2-hydroxyl-terephthalate. The assay is based on the ability of antioxidant containing samples to decrease this fluorescence via neutralizing hydroxyl radicals (Šnyrychová and Hideg, 2007). The increase in hydroxyl-terephthalate (HTPA) fluorescence intensity was followed at 420 nm in response to 315 nm excitation using a Hitachi F7000 fluorimeter. Hydroxyl radical antioxidant capacities were characterized by amounts of plant samples required to decrease HTPA fluorescence by 50% as described earlier and were given as μM ethanol equivalents per μg soluble protein.

2.10. Statistical analyses

All treatment groups contained 5 plants as biological repetitions (n = 5). In order to study the effects of single factors, such as drought (a PARW-PARD comparison), sequential (PARW-sUVW) or parallel (PARW-pUVW) UV-B treatment, differences between group means were assessed with two-sample Student's t-tests for either equal or unequal variances, depending on results of F-tests. Significantly different ($p < 0.05$) means are marked with asterisks in Table 1 and Fig. 2. In order to study the effect of combined treatments, data were analysed in four groups: (i) PARW, PARD, sUVW, sUVD treatments in ML, (ii) the same in YL, (iii) PARW, PARD, pUVW, pUVD treatments in ML, and (iv) the same in YL. In each group, for each variable, two-factor ANOVA was used to test three null hypotheses: (1) light conditions (PAR or UV) have no effect (2) water conditions (+/− drought) have no effect, and (3) there is no interaction between light and water conditions as factors. Rejections of the null hypotheses, if these were verified with Tukey's post hoc tests are characterized with p values in Tables 2 and 3, and with asterisk or hash tag symbols in Fig. 3. When the two main effects, (drought and UV) were characterized by $p < 0.05$ and post-hoc tests identified a clear order of means (either as UV means always above PAR means or as D means always above W means), the main effects were regarded as significant, even with a $p < 0.05$ factor interaction was present.

Calculations were carried out using either the MS Excel Analysis ToolPak (Version, 2007, Microsoft Corporation, Redmond, WA, USA) or the PAST software (Hammer et al., 2001). Graphs were created using SigmaPlot (Version 12.0, Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Differences between untreated YL and ML

Developmental age has been shown to affect antioxidant capacities and stress responses, for example in *Vitis vinifera* (Majer and Hideg, 2012) or *Arabidopsis thaliana* (Csepregi et al., 2017) leaves. A comparison of developing (YL) and mature leaves (ML) of untreated (PARW) *N. benthamiana* plants in Table 1 shows that in the experiment reported here YL had lower weight and chlorophyll content than ML. In the absence of stress factors, there were no significant differences between flavonoid indexes of YL and ML. YL had ca. 10% lower maximum PSII quantum efficiency than ML, but showed no significant differences in either light acclimated leaf photochemistry or non-photochemical quenching. YL had lower total antioxidant capacities, peroxidase enzyme activity, and contained less $^1O_2$ or $^{\bullet}OH$ scavenging compounds than ML (Table 1). Percentage differences in amounts and activities *in planta* may be modulated by different extraction yields from older, harder leaves than from developing ones. Nevertheless, we can conclude that with the exception of SOD and non-enzymatic H_2O_2 scavenging YL had much lower antioxidant capacities than ML.

3.2. Effects of single factor (UV−B or drought) treatments in ML and YL

Comparisons of PARW and pUVW plants showed that a 10-days of supplementary UV-B resulted in a small (4–7%) decrease in leaf photochemical yields Fv/Fm and Y(II). This was rather due to re-allocation of absorbed quanta than to PSII damage, as indicated by the marked (25%) increase in non-photochemical quenching Y(NPQ) (Table 1, Fig. 2A). pUVW plants showed documented features of UV-acclimation, such as decreased leaf weight, increased flavonoid contents and higher

Table 1
Mature (ML) and young (YL) leaf responses to one of the following treatments: drought (PARD), 4d UV-B followed by 10d PAR (sUVW), or 10d UV-B (pUVW).

	PARW ML	Single factor comparisons (*t*-test)						
		compared to PARW ML			compared to PARW YL			
		PARD ML	sUVW ML	pUVW ML	PARW YL	PARD YL	sUVW YL	pUVW YL
Plant weight	28.47 ± 6.14	16.91 ± 2.44*	32.23 ± 5.01	27.73 ± 6.95				
Leaf weight	2.85 ± 0.24	1.62 ± 0.15*	3.04 ± 0.72	2.20 ± 0.24*	1.15 ± 0.22*	0.81 ± 0.20*	1.27 ± 0.23	0.96 ± 0.18
Chlorophyll	25.38 ± 1.38	28.73 ± 0.84*	26.78 ± 2.64	28.02 ± 5.18	20.44 ± 0.89*	28.74 ± 2.88*	22.42 ± 1.80	21.50 ± 4.31
Fv/Fm	0.858 ± 0.021	0.874 ± 0.005	0.880 ± 0.003	0.810 ± 0.025*	0.765 ± 0.045*	0.868 ± 0.007*	0.783 ± 0.011	0.754 ± 0.031
Y(II)	0.337 ± 0.017	0.290 ± 0.028*	0.341 ± 0.038	0.315 ± 0.008*	0.321 ± 0.016	0.334 ± 0.012	0.312 ± 0.004	0.309 ± 0.015
Y(NPQ)	0.214 ± 0.027	0.356 ± 0.026*	0.185 ± 0.020	0.265 ± 0.026*	0.241 ± 0.018	0.308 ± 0.032*	0.236 ± 0.017	0.268 ± 0.040
Y(NO)	0.449 ± 0.026	0.354 ± 0.006*	0.506 ± 0.026	0.420 ± 0.024	0.438 ± 0.006	0.358 ± 0.032*	0.467 ± 0.036	0.423 ± 0.028
Flavonoid	0.14 ± 0.01	0.20 ± 0.03*	0.25 ± 0.03*	0.48 ± 0.04*	0.16 ± 0.03	0.27 ± 0.02*	0.15 ± 0.01	0.69 ± 0.03*
TEAC	4.88 ± 0.65	8.41 ± 0.88*	6.12 ± 0.92*	17.68 ± 0.25*	0.83 ± 0.10*	0.74 ± 0.11	0.79 ± 0.09	1.68 ± 0.24*
FRAP	0.36 ± 0.04	0.50 ± 0.07*	0.49 ± 0.02*	1.04 ± 0.05*	0.09 ± 0.02*	0.08 ± 0.01	0.09 ± 0.01	0.23 ± 0.01*
SOD	8.60 ± 2.28	10.42 ± 0.60	9.57 ± 0.47	13.54 ± 1.61*	5.93 ± 1.01	7.31 ± 1.09	4.64 ± 0.12	6.32 ± 0.68
Peroxidase	0.39 ± 0.09	0.59 ± 0.08*	0.45 ± 0.06	0.63 ± 0.07*	0.10 ± 0.02*	0.13 ± 0.03	0.10 ± 0.01	0.12 ± 0.03
H_2O_2 scavenging	10.95 ± 1.32	40.99 ± 4.08*	9.41 ± 0.58	7.59 ± 1.03*	13.21 ± 1.24	10.59 ± 1.32	15.72 ± 3.59	13.78 ± 1.32
$^1O_2$ scavenging	0.44 ± 0.09	0.95 ± 0.06*	0.52 ± 0.09	0.66 ± 0.04*	0.12 ± 0.03*	0.16 ± 0.03	0.15 ± 0.04	0.18 ± 0.04*
$^{\bullet}OH$ scavenging	0.23 ± 0.03	0.27 ± 0.03	0.27 ± 0.02	0.37 ± 0.03*	0.026 ± 0.006*	0.028 ± 0.003	0.013 ± 0.003*	0.082 ± 0.006*

Parameters are expressed as: Plant weight (g), Leaf weight (g), TEAC (mg Trolox equivalent/mg soluble protein), FRAP (mg AsA equivalent/mg soluble protein), SOD (U/μg soluble protein), Peroxidase (U/μg soluble protein), H_2O_2 scavenging (mM AsA equivalent/mg soluble protein), $^1O_2$ scavenging (mM AsA equivalent/mg soluble protein), •OH scavenging (μM ethanol equivalent/μg soluble protein). Results are shown as means ± standard deviations, n = 5. Asterisks indicate significant ($p < 0.05$, Student's t-test) difference between two means.

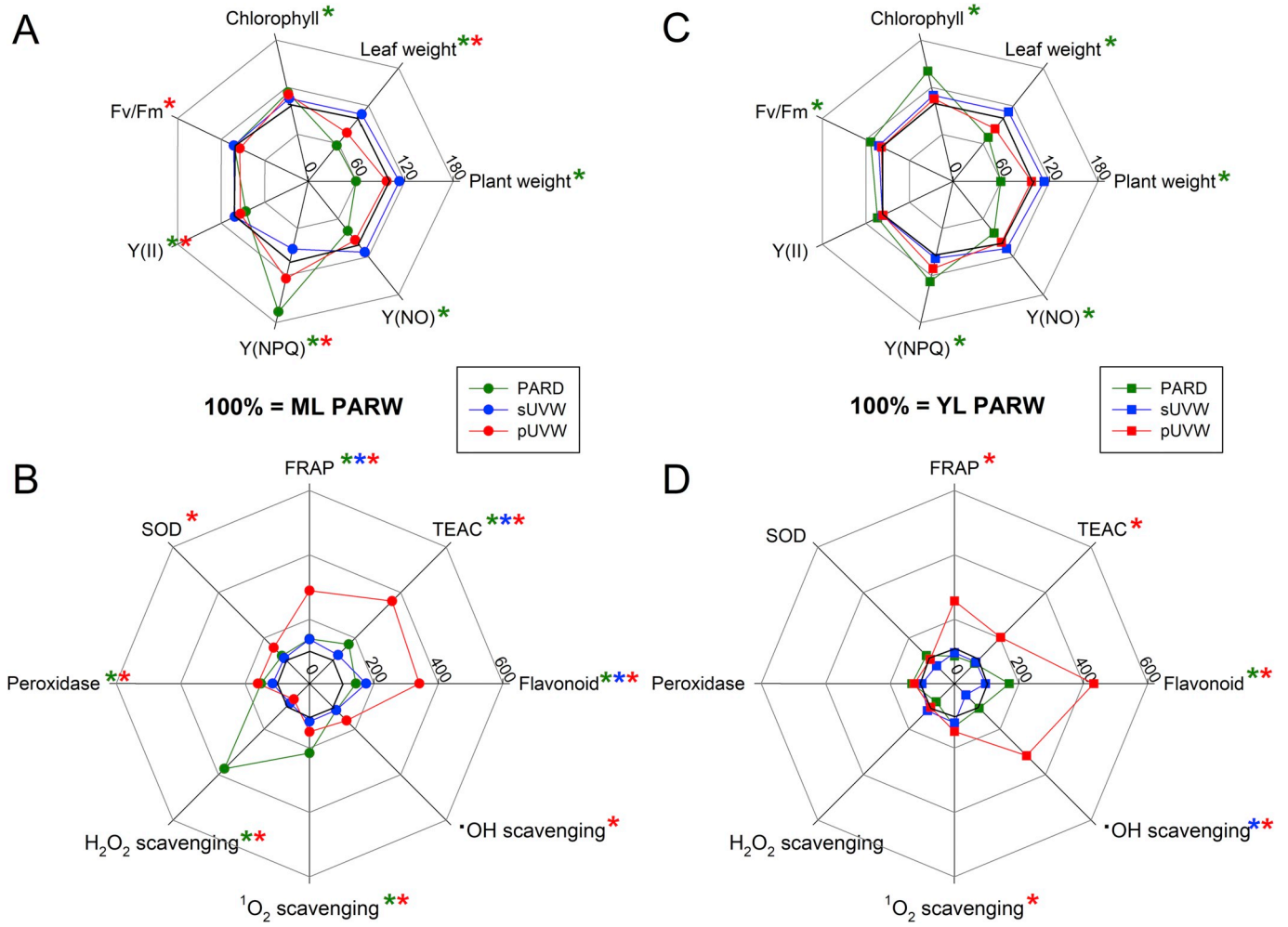

Fig. 2. Overview of mature (ML) and young (YL) leaf responses to single factor treatments.
(A) and (B): Full circles represent means (n = 5) as % of untreated PARW ML means (shown as 100% in black). (C) and (D): Full squares represent means (n = 5) as % of untreated PARW YL means (shown as 100% in black). Green, blue and red correspond to 10-days drought (PARD), 4-days UV-B followed by 10 days under control conditions (sUVW), and 10 days UV-B (pUVW), respectively. Asterisks mark significant ($p < 0.05$) differences from PARW ML (A and B panels) or from PARW YL (C and D panels) as calculated with Student's t-tests (see text and Table 1 for details).

antioxidant activities (Jansen et al., 1998) than PARW controls. Enzymatic antioxidant defence was equally stimulated as peroxidase and as SOD activity, contrary to our earlier results on *Nicotiana tabacum* leaves showing higher peroxidase than SOD activation by UV-B (Czégény et al., 2016b). Also, *N. benthamiana* leaves of the present study showed a smaller increase in peroxidase activity (160% of control, Table 2.) in response to UV-B than *N. tabacum* leaves (900%, Czégény et al., 2016a). In addition to differences between responses of the two species, the application of lower UV-B doses in the present than in the previous study may explain discrepancies. Non-enzymatic total antioxidant capacities were approximately 3-times higher in pUVW plants than in PARW controls (Table 1, Fig. 2B). Responses of ROS specific capacities included more efficient higher hydroxyl radical neutralizing, in accordance with our earlier reports (Majer et al., 2014; Czégény et al., 2016a), as well as higher $^1O_2$ scavenging, that is reported here for the first time as a response to supplementary UV-B (Fig. 2B). Surprisingly, non-enzymatic H_2O_2 neutralizing was lower in pUVW leaves than in PARW controls, despite higher contents of flavonoids (Fig. 2B) with good H_2O_2 antioxidant capacities (Csepregi and Hideg, 2018). The reason of this UV-B response of *N. benthamiana* leaves is yet unknown and it is to be confirmed in other species in a comparative study of enzymatic and non-enzymatic H_2O_2 neutralizing responses.

N. benthamiana plants exposed to 10-days of limited watering (PARD) had lower above ground weight than watered controls (PARW) (Fig. 2A). However, responses to drought were largely acclimatory. The maximum efficiency of photochemical yield Fv/Fm was maintained, but the actual quantum yield Y(II) was 14% lower in PARD than in PARW plants. Similarly to the effect of UV-B, drought resulted in higher regulated non-photochemical energy dissipation Y(NPQ) (Fig. 2A). Unlike the UV-B treatment that had no significant effect on non-regulated non-photochemical quenching, Y(NO) decreased in response to drought. Although a higher Y(NPQ) signified enhanced photo-protection, possibly preventing PSII-derived ROS production (Schreiber and Klughammer, 2008), PARD plants had significantly higher $^1O_2$ scavenging capacities than PARW controls (Fig. 2B). Both enzymatic and non-enzymatic defence against H_2O_2 was increased under drought, although the H_2O_2 yielding SOD activity did not increase. ML of drought treated PARD plants had higher flavonoid contents and non-enzymatic total antioxidant capacities than PARW controls, although not as high as UV-B exposed pUVW leaves (Fig. 2B).

YL responses to UV-B as single factor treatment were at several points distinct from those of ML. Contrary to ML responses, UV-B alone had no effect on YL weight or photochemistry (Table 1, Fig. 2C). On the other hand, YL, just like ML responded to UV-B with increased leaf flavonoid content and enhanced antioxidant capacities (Table 1, Fig. 2D). In YL, the latter was restricted to non-enzymatic total

Table 2A
Mature leaf (ML) responses to combined drought and UV-B pre-treatments.

	PARW ML	PARD ML	sUVW ML	sUVD ML	Two-factor ANOVA			
					Drought effect? D vs. W	UV effect? sUV vs. PAR	Interaction?	Differences of means verified in post-hoc tests
Plant weight	28.47 ± 6.14	16.91 ± 2.44	32.23 ± 5.01	20.01 ± 3.16	negative $p < 10^{-4}$			PARW > PARD, sUVW > sUVD
Leaf weight	2.85 ± 0.24	1.62 ± 0.15	3.04 ± 0.72	1.87 ± 0.36	negative $p < 10^{-4}$			PARW > PARD, sUVW > sUVD
Chlorophyll	25.38 ± 1.38	28.73 ± 0.84	26.78 ± 2.64	24.72 ± 3.12				PARD > sUVD
Fv/Fm	0.858 ± 0.021	0.874 ± 0.005	0.880 ± 0.003	0.849 ± 0.012				PARD > sUVD, PARW < sUVW
Y(II)	0.337 ± 0.017	0.290 ± 0.028	0.341 ± 0.038	0.318 ± 0.030				PARW > PARD
Y(NPQ)	0.214 ± 0.027	0.356 ± 0.026	0.185 ± 0.020	0.219 ± 0.018	positive $p < 10^{-4}$	negative $p < 10^{-4}$	yes $p < 10^{-4}$	PARW < PARD, sUVW < sUVD PARW > sUVW, PARD > sUVD
Y(NO)	0.449 ± 0.026	0.354 ± 0.006	0.506 ± 0.026	0.471 ± 0.018	negative $p < 10^{-4}$	positive $p < 10^{-4}$	yes $p = 7.7\ 10^{-4}$	PARW > PARD, sUVW > sUVD PARW < sUVW, PARD < sUVD
Flavonoid	0.14 ± 0.01	0.20 ± 0.03	0.25 ± 0.03	0.33 ± 0.02	positive $p < 10^{-4}$	positive $p < 10^{-4}$	no $p = 0.149$	PARW < PARD, sUVW < sUVD PARW < sUVW, PARD < sUVD
TEAC	4.88 ± 0.65	8.41 ± 0.88	6.12 ± 0.92	7.50 ± 0.75	positive $p < 10^{-4}$			PARW < PARD, sUVW < sUVD PARW < sUVW
FRAP	0.36 ± 0.04	0.50 ± 0.07	0.49 ± 0.02	0.57 ± 0.03	positive $p < 10^{-4}$	positive $p < 10^{-4}$	no $p = 0.053$	PARW < PARD, sUVW < sUVD PARW < sUVW, PARD < sUVD
SOD	8.60 ± 2.28	10.42 ± 0.60	9.57 ± 0.47	12.11 ± 2.37				sUVW < sUVD
Peroxidase	0.39 ± 0.09	0.59 ± 0.08	0.45 ± 0.06	0.53 ± 0.11				PARW < PARD
H_2O_2 scavenging	10.95 ± 1.32	40.99 ± 4.08	9.41 ± 0.58	13.41 ± 2.56	positive $p < 10^{-4}$			PARW < PARD, sUVW < sUVD PARD > sUVD
$^1O_2$ scavenging	0.44 ± 0.09	0.95 ± 0.06	0.52 ± 0.09	0.58 ± 0.06				PARW < PARD PARD > sUVD
$^{\cdot}OH$ scavenging	0.23 ± 0.03	0.27 ± 0.03	0.27 ± 0.02	1.22 ± 0.09				sUVW < sUVD PARD > sUVD

Units are the same as in Table 1. Results of two-factor ANOVA analysis: drought and UV effects found significant in post-hoc tests are characterized by *p* values. When both factors were found significant, their interaction was also characterized with a *p* value.

Table 2B
Mature leaf (ML) responses to drought and parallel UV-B treatments.

	PARW ML	PARD ML	pUVW ML	pUVD ML	Two-factor ANOVA			
					Drought effect? D vs. W	UV effect? pUV vs. PAR	Interaction?	Differences of means verified in post-hoc tests
Plant weight	28.47 ± 6.14	16.91 ± 2.44	27.73 ± 6.95	16.83 ± 3.05	negative $p < 10^{-4}$			PARW > PARD, pUVW > pUVD PARW > pUVW
Leaf weight	2.85 ± 0.24	1.62 ± 0.15	2.20 ± 0.24	1.57 ± 0.35	negative $p < 10^{-4}$			PARW > PARD, pUVW > pUVD PARW > pUVW
Chlorophyll	25.38 ± 1.38	28.73 ± 0.84	28.02 ± 5.18	30.68 ± 1.90				–
Fv/Fm	0.858 ± 0.021	0.874 ± 0.005	0.810 ± 0.025	0.864 ± 0.009				pUVW < pUVD PARW < pUVD
Y(II)	0.337 ± 0.017	0.290 ± 0.028	0.315 ± 0.008	0.316 ± 0.030				PARW < PARD
Y(NPQ)	0.214 ± 0.027	0.356 ± 0.026	0.265 ± 0.026	0.336 ± 0.035	positive $p < 10^{-4}$			PARW < PARD, pUVW < pUVD PARW < pUVW
Y(NO)	0.449 ± 0.026	0.354 ± 0.006	0.420 ± 0.024	0.348 ± 0.022	negative $p < 10^{-4}$			PARW > PARD, pUVW > pUVD PARW > pUVW
Flavonoid	0.14 ± 0.01	0.20 ± 0.03	0.48 ± 0.04	0.70 ± 0.01	positive $p < 10^{-4}$	positive $p < 10^{-4}$	yes $p = 0.039$	PARW < PARD, pUVW < pUVD PARW < pUVW, PARD < pUVD
TEAC	4.88 ± 0.65	8.41 ± 0.88	17.68 ± 0.25	22.28 ± 1.25	positive $p < 10^{-4}$	positive $p < 10^{-4}$	yes $p = 0.045$	PARW < PARD, pUVW < pUVD PARW < pUVW, PARD < pUVD
FRAP	0.36 ± 0.04	0.50 ± 0.07	1.04 ± 0.05	1.21 ± 0.16	positive $p < 10^{-4}$	positive $p < 10^{-4}$	no $p = 0.576$	PARW < PARD, pUVW < pUVD PARW < pUVW, PARD < pUVD
SOD	8.60 ± 2.28	10.42 ± 0.60	13.54 ± 1.61	29.33 ± 8.32		positive $p < 10^{-4}$		pUVW < pUVD PARW < pUVW, PARD < pUVD
Peroxidase	0.39 ± 0.09	0.59 ± 0.08	0.63 ± 0.07	0.73 ± 0.17		positive $p < 10^{-4}$		PARW < PARD PARW < pUVW, PARD < pUVD
H_2O_2 scavenging	10.95 ± 1.32	40.99 ± 4.08	7.59 ± 1.03	39.64 ± 1.02	positive $p < 10^{-4}$			PARW < PARD, pUVW < pUVD PARW < pUVW
$^1O_2$ scavenging	0.44 ± 0.09	0.95 ± 0.06	0.66 ± 0.04	1.06 ± 0.09	positive $p < 10^{-4}$	positive $p < 10^{-4}$	yes $p = 0.024$	PARW < PARD, pUVW < pUVD PARW < pUVW, PARD < pUVD
$^{\cdot}OH$ scavenging	0.23 ± 0.03	0.27 ± 0.03	0.37 ± 0.03	0.55 ± 0.034		positive $p < 10^{-4}$		pUVW < pUVD PARW < pUVW, PARD < pUVD

Units are the same as in Table 1. Results of two-factor ANOVA analysis: drought and UV effects found significant in post-hoc tests are characterized by *p* values. When both factors were found significant, their interaction was also characterized with a *p* value.

Table 3A
Young leaf (YL) responses to combined drought and UV-B pre-treatments.

	PARW YL	PARD YL	sUVW YL	sUVD YL	Two-factor ANOVA			
					Drought effect? D vs. W	UV effect? sUV vs. PAR	Interaction?	Differences of means verified in post-hoc tests
Leaf weight	1.15 ± 0.22	0.81 ± 0.20	1.27 ± 0.23	0.86 ± 0.13	negative $p < 10^{-4}$			PARW > PARD, sUVW > sUVD
Chlorophyll	20.44 ± 0.89	28.74 ± 2.88	22.42 ± 1.80	24.49 ± 1.33				PARW < PARD PARD > sUVD
Fv/Fm	0.765 ± 0.045	0.868 ± 0.007	0.783 ± 0.011	0.793 ± 0.001				PARW < PARD PARD > sUVD
Y(II)	0.321 ± 0.016	0.334 ± 0.012	0.312 ± 0.004	0.363 ± 0.007				sUVW < sUVD PARD > sUVD
Y(NPQ)	0.241 ± 0.018	0.308 ± 0.032	0.236 ± 0.017	0.216 ± 0.002				PARW < PARD PARD > sUVD
Y(NO)	0.438 ± 0.006	0.358 ± 0.032	0.467 ± 0.036	0.416 ± 0.013	negative $p < 10^{-4}$			PARW > PARD, sUVW > sUVD PARD < sUVD
Flavonoid	0.16 ± 0.03	0.27 ± 0.02	0.15 ± 0.01	0.24 ± 0.03	positive $p < 10^{-4}$			PARW < PARD, sUVW < sUVD
TEAC	0.83 ± 0.10	0.74 ± 0.11	0.79 ± 0.09	0.95 ± 0.13				PARW < PARD PARD < sUVD
FRAP	0.09 ± 0.02	0.08 ± 0.01	0.09 ± 0.01	0.11 ± 0.02				sUVW < sUVD PARD < sUVD
SOD	5.93 ± 1.01	7.31 ± 1.09	4.64 ± 0.12	6.08 ± 0.65				sUVW < sUVD PARW > sUVW
Peroxidase	0.10 ± 0.02	0.13 ± 0.03	0.10 ± 0.01	0.12 ± 0.02				–
H_2O_2 scavenging	13.21 ± 1.24	10.59 ± 1.32	15.72 ± 3.59	12.73 ± 3.53				–
$^1O_2$ scavenging	0.12 ± 0.03	0.16 ± 0.03	0.15 ± 0.04	0.24 ± 0.04				sUVW < sUVD PARD < sUVD
$^{\cdot}OH$ scavenging	0.026 ± 0.006	0.028 ± 0.003	0.013 ± 0.003	0.026 ± 0.001				sUVW < sUVD PARW > sUVW

Units are the same as in Table 1. Results of two-factor ANOVA analysis: drought and UV effects found significant in post-hoc tests are characterized by *p* values. When both factors were found significant, their interaction was also characterized with a *p* value.

Table 3B
Young leaf (YL) responses to drought and parallel UV-B treatments.

	PARW YL	PARD YL	pUVW YL	pUVD YL	Two-factor ANOVA			
					Drought effect? D vs. W	UV effect? pUV vs. PAR	Interaction?	Differences of means verified in post-hoc tests
Leaf weight	1.15 ± 0.22	0.81 ± 0.20	0.96 ± 0.18	0.76 ± 0.21				PARW > PARD
Chlorophyll	20.44 ± 0.89	28.74 ± 2.88	21.50 ± 4.31	30.62 ± 1.61	positive $p < 10^{-4}$			PARW < PARD, pUVW < pUVD
Fv/Fm	0.765 ± 0.045	0.868 ± 0.007	0.754 ± 0.031	0.861 ± 0.011				PARW < PARD
Y(II)	0.321 ± 0.016	0.334 ± 0.012	0.309 ± 0.015	0.331 ± 0.022				–
Y(NPQ)	0.241 ± 0.018	0.308 ± 0.032	0.268 ± 0.040	0.317 ± 0.023	positive $p < 3.8\ 10^{-4}$			PARW < PARD, pUVW < pUVD
Y(NO)	0.438 ± 0.006	0.358 ± 0.032	0.423 ± 0.028	0.353 ± 0.033	negative $p < 10^{-4}$			PARW > PARD, pUVW > pUVD
Flavonoid	0.16 ± 0.03	0.27 ± 0.02	0.69 ± 0.03	0.93 ± 0.04	positive $p < 10^{-4}$	positive $p < 10^{-4}$	yes $p = 0.042$	PARW < PARD, pUVW < pUVD PARW < pUVW, PARD < pUVD
TEAC	0.83 ± 0.10	0.74 ± 0.11	1.68 ± 0.24	2.40 ± 0.28		positive $p < 10^{-4}$		pUVW < pUVD PARW < pUVW, PARD < pUVD
FRAP	0.09 ± 0.02	0.08 ± 0.01	0.23 ± 0.01	0.27 ± 0.02		positive $p < 10^{-4}$		pUVW < pUVD PARW < pUVW, PARD < pUVD
SOD	5.93 ± 1.01	7.31 ± 1.09	6.32 ± 0.68	12.30 ± 2.83				pUVW < pUVD PARD < pUVD
Peroxidase	0.10 ± 0.02	0.13 ± 0.03	0.12 ± 0.03	0.15 ± 0.03				–
H_2O_2 scavenging	13.21 ± 1.24	10.59 ± 1.32	13.78 ± 1.32	19.27 ± 2.83				PARW > PARD, pUVW < pUVD PARD < pUVD
$^1O_2$ scavenging	0.12 ± 0.03	0.16 ± 0.03	0.18 ± 0.04	0.22 ± 0.03		positive $p = 2.92\ 10^{-4}$		PARW < pUVW, PARD < pUVD
$^{\cdot}OH$ scavenging	0.026 ± 0.006	0.028 ± 0.003	0.082 ± 0.006	0.104 ± 0.001		positive $p < 10^{-4}$		pUVW < pUVD PARW < pUVW, PARD < pUVD

Units are the same as in Table 1. Results of two-factor ANOVA analysis: drought and UV effects found significant in post-hoc tests are characterized by *p* values. When both factors were found significant, their interaction was also characterized with a *p* value.

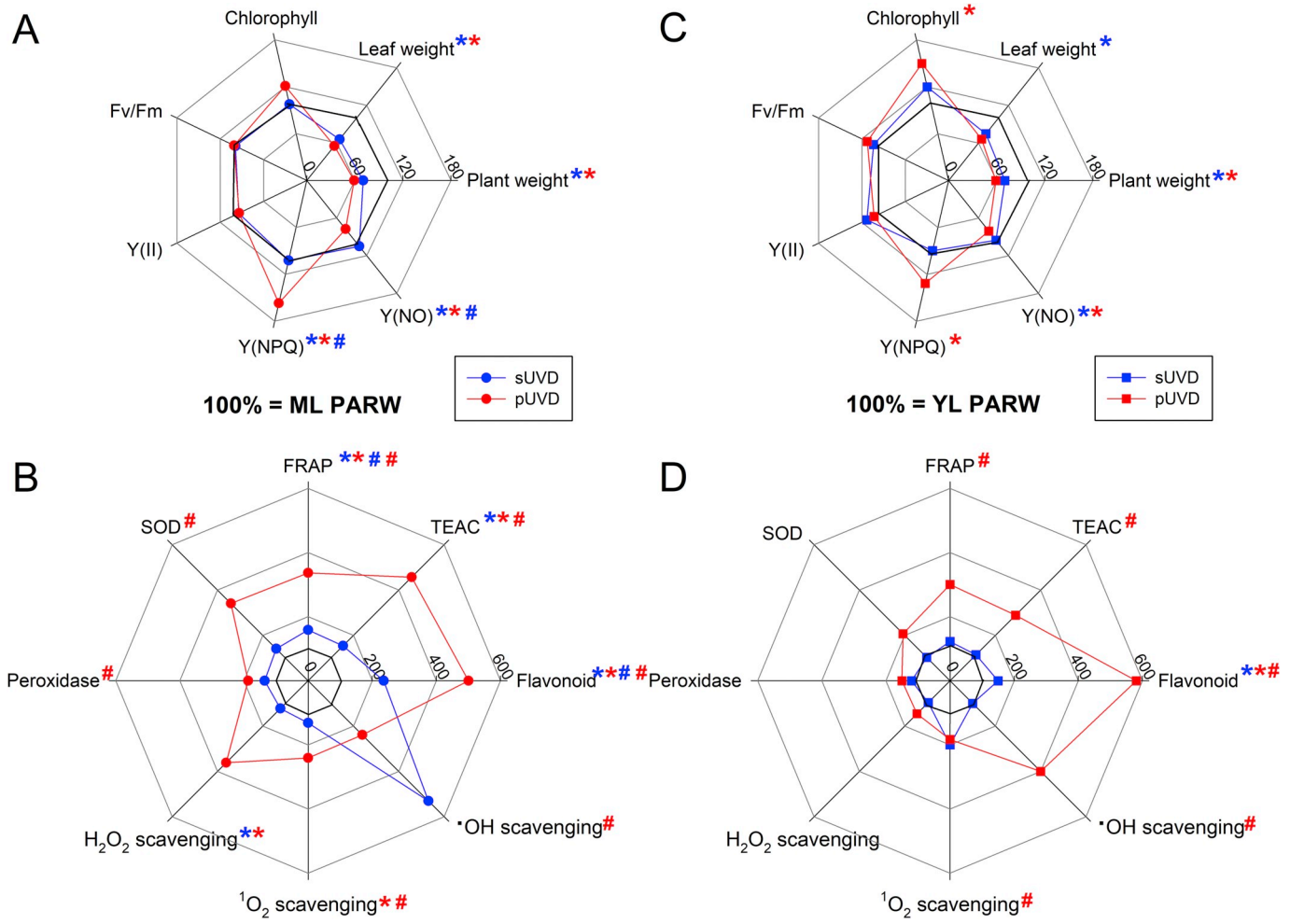

Fig. 3. Overview of mature (ML) and young (YL) leaf responses to two-factor treatments.
(A) and (B): Full circles represent means (n = 5) as % of untreated PARW ML means (shown as 100% in black). (C) and (D): Full squares represent means (n = 5) as % of untreated PARW YL means (shown as 100% in black). Blue and red correspond to 4-days UV-B followed by 10 days drought (sUVD), and 10 days UV-B and drought in parallel (pUVD), respectively. Asterisks and hash-tags mark significant ($p < 0.05$) drought and UV-B main effects, respectively, as calculated with two-factor ANOVA (see text, and Tables 2 and 3 for details).

capacities FRAP and TEAC, and to $^1O_2$ and $^{\bullet}OH$ scavenging. Neither enzymatic nor non-enzymatic H_2O_2 neutralization was affected in UV-B acclimated YL, similarly to SOD activity (Table 1, Fig. 2D). Unlike ML, YL that emerged during the drought treatment and had not been directly exposed to UV-B showed no significant antioxidant increases when assayed 10 days after the 4-day UV-B irradiation. The only exception was in $^{\bullet}OH$ scavenging capacity that was 50% lower, sUVW < PARW (Table 1, Fig. 2D).

Drought as single factor had no effect on YL antioxidant parameters (Table 1, Fig. 2D). Similarly to ML, leaf weight decreased, but chlorophyll content and Fv/Fm even increased in response to drought. Unlike in ML, light acclimated photochemical yield was unaffected by the drought treatment. Non-photochemical quenching responses were the same as in ML (Table 1, Fig. 2C).

3.3. Two-factor (UV–B and drought) experiments

Combined effects of drought and UV-B were analysed using two-factor ANOVA and results are reported in Tables 2A and 2B (ML) and Tables 3A and 3B (YL) together with those of post-hoc tests. Effects of sequential application, UV-B pre-treatment followed by drought, and parallel application of the two factors are discussed separately. For the ease of comparison, means and standard deviations of data sets used for the analysis are given here, even though it leads to inevitable overlapping with Table 1. A graphical comparison of all results from Tables 2A, 2B, 3A and 3B is offered in Fig. 3. The effect of a factor was considered significant, when two conditions were met: (i) the ANOVA test gave $p < 0.05$ for the effect and (ii) post-hoc tests verified an effect regardless of the presence or absence of the other factor. Factor interactions were only explored when both factors were found significant based on the above criteria.

3.3.1. A sequential application of two factors: effects of UV-B pre-treatment on drought responses in ML

Before effects of the UV-B pre-treatment on subsequent drought were studied, the first question to be examined was whether plants maintained UV-induced metabolic changes for a prolonged time after the cessation of the UV-B treatment. A comparison of PARW and sUVW plants showed that leaf flavonoid indexes and total antioxidant capacities (TEAC and FRAP) were significantly higher even 10 days after the end of the UV-B pre-treatment. No such differences were detected in either enzymatic (SOD, peroxidase) or non-enzymatic ROS specific antioxidant (Table 1, Fig. 2B).

In the two-factor treatment, UV-B remained a significant positive factor for flavonoid index and FRAP when it was followed by drought (PARD < sUVD), without interacting with the positive effect of drought itself (PARW < PARD) that was also maintained in UV-B pre-treated leaves (sUVW < sUVD) (Table 2A, Fig. 3B). For TEAC, UV-B

pre-treatment showed no effect in drought exposed leaves (PARD = sUVD) and thus drought remained the only significant factor (PARW < PARD, sUVW < sUVD). From among the stimulating effects of drought on various ROS neutralizing capacities as single factor (Table 1, Fig. 2B), only a positive effect on non-enzymatic H_2O_2 scavenging was maintained in the two-factor experiment (Table 2A, Fig. 3B).

A comparison of plants exposed to drought without (PARD) or after the UV pre-treatment (sUVD) in Table 2A shows that the negative effect of drought on leaf photochemistry as single factor was removed by the UV-B pre-treatment. In the two-factor experiment drought and UV-B pre-treatment showed opposite main effects on non-photochemical quenching parameters: drought increased Y(NPQ) and decreased Y (NO), similarly to its single factor effect, while UV-B pre-treatment decreased Y(NPQ) and increased Y(NO). Although these main effects were characterized with very low *p* values, strong factor interactions and pair-wise comparisons of means in post-hoc tests (Table 2A) question the interpretation of main effects and thus these were not regarded (and not marked in Fig. 3A) as significant. Negative effects of drought on leaf and whole plant size were maintained, without any additional effect of UV-B (Table 2A, Fig. 3A).

3.3.2. A parallel application of two factors: effects of UV-B and drought in ML

As expected, the parallel application of the two factors brought more interactions in effects than the sequential experiment. Although the application of either UV-B or drought for 10 days as single factor imposed resulted in decreased photochemical yields (Table 1, Fig. 2A), a simultaneous presence of the two factors had no effect on Y(II) (Table 2B, Fig. 3A). Changes in non-photochemical quenching routes indicated active, drought-driven defence: lower Y(NO) and higher Y (NPQ); while the negative effect of UV-B as single factor on Y(NPQ) was not significant in the two parallel factors experiment (Table 2B., Fig. 3A). The negative effect of drought as single factor on leaf and whole plant size remained significant when drought was combined with UV-B, similarly to the sequential application of UV-B and drought (Section 3.2.1.). On the other hand, the negative effect of 10 days UV-B as single factor on leaf weight was not observed in the two-factor experiment (Table 2B, Fig. 3A). When simultaneously exposed to drought and UV-B, leaf flavonoid indexes were higher than in response to either treatment alone, and values followed a PARW < PARD < pUVW < pUVD order. A similar pattern was observed in the two total antioxidant capacities: an additive defence in both TEAC and FRAP, as well as in $^1O_2$ scavenging (Table 2B). Enzymatic ROS neutralization, POD and SOD activities were UV-B-driven with both factors present, and positive effects of drought as single factor on these enzymes were not significant. The observed, yet unexplained decrease in non-enzymatic H_2O_2 scavenging under UV-B was overridden by the application of drought in the combined treatment, and only the latter factor remained significant (Table 2B, Fig. 2A).

3.3.3. A sequential application of two factors: effects of UV-B pre-treatment on drought responses in YL

In our experiment, YL emerged after the UV-B pre-treatment and sUVW leaves showed no differences from untreated PARW controls when assayed 10 days after the last day of UV-B treatment (Table 1). In accordance with this lack of single factor UV-B effect, with the exception of one parameter, the significant main effect in the two-factor experiment was drought only. Drought main effects included: lower leaf weight, smaller Y(NO), and higher flavonoid content. Four parameters, that were increased by drought as single factor (chlorophyll content, Fv/Fm, Y(II) and Y(NPQ), Table 1) were no longer significantly affected by drought in the two-factor experiment (Table 3A).

3.3.4. A parallel application of two factors: effects of UV-B and drought in YL

Similarly to ML, flavonoid index means in YL followed a PARW < PARD < pUVW < pUVD order and thus effects of both drought and UV-B were regarded as significant, despite of the significant factor interaction. In the two-factor experiment, the effect of drought on leaf weight, and photochemical yields Fv/Fm and Y(II) was no longer present, but was maintained on non-photochemical quenching parameters Y(NPQ) and Y(NO) (Table 3B, Fig. 3C). Antioxidant parameters of YL were unaffected by drought as single treatment (Table 1) and remained so even in the presence of both drought and UV-B (Table 3B). TEAC, FRAP, hydroxyl radical and $^1O_2$ neutralizing capacities were increased under UV-B applied as single treatment (Table 1) and were also positively affected by UV-B only in the two-factor experiment (Table 3B).

4. Discussion

Many stresses are known to act via enhanced production of ROS that pose a threat to cells but may also signal for the activation of stress-response and defence pathways including higher antioxidant activities (Mittler, 2002). Regarding the limited chemical variety of ROS, the consistency of principal ROS neutralizing enzymes, and the broad reactivity of non-enzymatic antioxidants; it is inevitable that defence pathways against distinct stressors overlap. We chose two environmentally relevant abiotic factors, UV-B and drought caused by limited water supply. When UV-B and drought were combined, several effects of these treatments remained significant as main effects in the two-factor experiment. Such maintained effects include a negative effect of drought on plant and ML leaf weights, and various antioxidant capacities. Lost single effects, i.e. those significant as single factors but not as main ones in the two-factor experiment, were with one exception drought effects; and the majority of these affected chlorophyll content and various photochemical yields. Drought in itself decreased ML and YL fresh weights. UV-B treatment as single factor showed the well established negative effect on ML leaf fresh weight (Jansen et al., 1998). On the other hand, UV-B alone had no effect on the fresh weight of YL, which emerged during the 10 days of this treatment, indicating a network complex, a whole plant level UV-B response that is to be investigated in the future. When UV-B and drought were combined, either in this order as sequential treatment or in parallel, the only significant leaf fresh weight limiting factor was drought in both ML an YL. Single factor drought effects on non-photochemical quenching parameters resulting in increased Y(NPQ) and decreased Y(NO) were maintained in the two-factor experiment, both in ML and YL. On the other hand, neither ML nor YL photochemical yields were significantly affected in the two-factor combined treatment. These results indicate that the plants' response was rather acclimation to the new conditions than stress, and the work was focused on changes in various leaf antioxidant capacities.

In ML, all studied antioxidant parameters increased in response to at least one of the treatments. Increased antioxidant activities or capacities are common acclimative stress responses, and the novelty of our study is in exploring interactions between UV-B and drought effects. Several components of antioxidant defence gave positive responses to one treatment only, either to UV-B (SOD activity and hydroxyl radical scavenging) or to drought (non-enzymatic H_2O_2 scavenging) when applied as single factors, and all these effects remained significant in the two-factor treatment. Four among the seven assayed parameters (peroxidase enzyme activities, total antioxidant capacities TEAC and FRAP, and singlet oxygen neutralizing) increased in ML in response to both drought and UV-B as single treatments. UV-B and drought were applied together, peroxidase activity was only drought and not UV-B driven. On the other hand, but both drought and UV-B remained significant positive factors for TEAC, FRAP and singlet oxygen neutralizing. Moreover, positive effects of drought and UV-B on these three

antioxidant parameters were additive in ML. For example the sum of the +72 ± 18% change in TEAC by drought as single factor and the +262 ± 14% change by UV-B as single factor is +334 ± 23% (using the propagation of uncertainty to calculate the standard deviation for the sum of means), matching the observed percentage change in TEAC in the two-factor treatment, +356 ± 18%. The same calculation for FRAP means gave +227 ± 23% and +235 ± 24% for the sum of single factor effects and for the two-factor effect, respectively. Corresponding changes in singlet oxygen neutralizing were characterized by +166 ± 28% and +140 ± 20%.

Younger leaves are less sensitive to water stress than older ones due to differences in their osmoregulation (Chaves et al., 2003). In our experiment, YL photochemical yields were less affected by drought alone, contrary to the small but significant, 13 ± 9% loss observed in ML. Unlike ML, YL antioxidants did not respond to drought as single treatment. Peroxidase or SOD enzyme activities were not responsive to UV-B either. Similarly to ML, positive antioxidant responses of YL to UV-B as single factor included TEAC and FRAP, as well as hydroxyl radical and $^1O_2$ scavenging, and these were maintained as positive main effects in the parallel drought and UV-B treatment. In accordance with the lack of drought response, no additive antioxidant response was found in the two-factor treatment.

A response observed both in of YL and in ML to the parallel application of drought and UV-B was the synergistic increase in flavonoid index. This parameter increased in response to either treatment as single factor. The two-factor effect was +397 ± 7% when the mean flavonoid index of pUVD ML was compared to those of PARW ML, markedly larger than the sum of single factor effects, +284 ± 22%. In YL, the corresponding values were +482 ± 22% and +402 ± 22%, respectively, showing the same super-additivity. Although several flavonoids display high total antioxidant capacities (Csepregi et al., 2016), corresponding *in planta* antioxidant functions remain to be proven. A strong implication for such function is the similar additivity of flavonoid index and antioxidant responses in our experiment. There is no consensus on whether UV-A absorbance of the adaxial epidermis measured as the flavonoid index is a proportional representative total of flavonoid contents (Agati et al., 2002; Goulas et al., 2004; Bilger et al., 2007), and this most likely depends on plant species and growth conditions. It should be noted that in our experiment, H_2O_2 or $^1O_2$ scavenging capacities of ML did not show the same additivity as the flavonoid index, although flavonoids have high reactivity to both ROS (Csepregi and Hideg, 2018). Consequently, while a contribution of flavonoids to leaf total antioxidant capacities is plausible, contributions of other, UV-B or drought inducible non-enzymatic antioxidants is also likely.

Hydrogen peroxide is considered a signalling messenger in plants (Mittler, 2002) and whenever UV-B is included among the studied factors, changes in H_2O_2 neutralization and production are of special interest (Czégény et al., 2016b). In ML, parallel application of UV-B and drought resulted in higher H_2O_2 yielding SOD activity as a UV-B-driven effect. Resulting higher H_2O_2 concentrations were in turn lowered by higher peroxidase activity (a UV-B-driven effect) and stronger non-enzymatic H_2O_2 scavenging (a drought-driven effect). The same conditions induced neither higher SOD activity nor increased H_2O_2 neutralization in YL. Increased hydroxyl radical scavenging was, however, present in both ML and YL as a UV-B dependent effect. The latter result is in line with the suggested pivotal role of hydroxyl radicals in damage by either severe drought (Price et al., 1989) or high UV-B doses (Hideg and Vass, 1996): the plants' defence system against this ROS was alerted to a high level by the appearance of two potential stress factor, even though the UV-B and drought conditions applied in the present work were milder than in the above mentioned earlier studies.

An interesting new aspect of the present work is that UV-B as pre-treatment had a long-term positive effect on leaf flavonoid index and antioxidant capacity assessed as FRAP in ML. When UV-B pre-treatment was followed by drought in the sequential two-factor treatment, UV-B was maintained as significant positive factor. Moreover, effects of UV-B pre-treatment and drought were additive on both FRAP and flavonoid index, although not synergistic as observed in the parallel treatment. In the sequential treatment the sum of single factor effects on flavonoid index was, +119 ± 20% and the two-factor treatment resulted in +136 ± 14%. Corresponding values for FRAP were +76 ± 22% and +57 ± 10%, respectively. UV-B irradiation during early development has been suggested to enhance photo-protection in *Lactuca sativa* plants (Wargent et al., 2011), and the present study demonstrates the capability of UV-B pre-treatment to augment drought inducible increase in leaf antioxidant capacity and flavonoid index. However, the relevance of this model experiment to outdoor conditions is yet to be tested. The above described positive effect of UV-B as pre-treatment appears direct and non-systemic as it was observed in ML only. YL that emerged after the UV-B pre-treatment showed no UV-B-driven antioxidant response in the sequential two-factor treatment, and their flavonoid index was increased by drought only.

Contributions

ÉH and AM conceived the original idea. AM performed the majority of experiments with the participation of DN. ÉH and AM carried out statistical analyses, and designed tables and figures together. All authors discussed the results, and the manuscript was written by ÉH with the contribution of AM.

Acknowledgements

The project was supported by the European Union, co-financed by the European Social Fund Grant no.: EFOP-3.6.1.-16-2016-00004 entitled by Comprehensive Development for Implementing Smart Specialization Strategies at the University of Pécs; and by the Higher Education Institutional Excellence Programme of the Ministry of Human Capacities in Hungary, within the framework of the 20765-3/2018/FEKUTSTRAT 'Innovation for sustainable and healthy living and environment' thematic programme of the University of Pécs.

References

Agati, G., Galardi, C., Gravano, E., Romani, A., Tattini, M., 2002. Flavonoid distribution in tissues of *Phillyrea latifolia* L. leaves as estimated by microspectrofluorometry and multispectral fluorescence microimaging. Photochem. Photobiol. 76, 350–360.

Alexieva, V., Sergiev, I., Mapelli, S., Karanov, E., 2001. The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ. 24, 1337–1344.

Bandurska, H., Niedziela, J., Chadzinikolau, T., 2013. Separate and combined responses to water deficit and UV-B radiation. Plant Sci. 213, 98–105.

Bandurska, H., Cieślak, M., 2013. The interactive effect of water deficit and UV-B radiation on salicylic acid accumulation in barley roots and leaves. Environ. Exp. Bot. 94, 9–18.

Bernal, M., Verdaguer, D., Badosa, J., Abadía, A., Llusià, J., Peñuelas, J., Núñez-Olivera, E., Llorens, L., 2015. Effects of enhanced UV radiation and water availability on performance, biomass production and photoprotective mechanisms of *Laurus nobilis* seedlings. Environ. Exp. Bot. 109, 264–275.

Bilger, W., Rolland, M., Nybakken, L., 2007. UV screening in higher plants induced by low temperature in the absence of UV-B radiation. Photochem. Photobiol. Sci. 6, 190–195.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation ofmicrogram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chalker-Scott, L., Scott, J.D., 2004. Elevated ultraviolet-B radiation induces cross-protection to cold in leaves of rhododendron under field conditions. Photochem. Photobiol. 79, 199–204.

Chaves, M.M., Maroco, J.P., Pereira, J.S., 2003. Understanding plant response to drought-from genes to the whole plant. Funct. Plant Biol. 30, 239–264.

Csepregi, K., Hideg, É., 2016. A novel procedure to assess the non-enzymatic hydrogen-peroxide antioxidant capacity of metabolites with high UV absorption. Acta Biol. Hung. 67, 447–450.

Csepregi, K., Neugart, S., Schreiner, M., Hideg, É., 2016. Comparative evaluation of total antioxidant capacities of plant polyphenols. Molecules 21, 208.

Csepregi, K., Coffey, A., Cunningham, N., Hideg, É., Jansen, M.A.K., 2017. Developmental age and UV-B exposure co-determine antioxidant capacity and flavonol accumulation in Arabidopsis leaves. Environ. Exp. Bot. 140, 19–25.

Csepregi, K., Hideg, É., 2018. Phenolic compound diversity explored in the context of

photo-oxidative stress protection. Phytochem. Anal. 29, 129–136.
Czégény, Gy, Le Martret, B., Pávkovics, D., Dix, P.J., Hideg, É., 2016a. Elevated ROS-scavenging enzymes contribute to acclimation to UV-B exposure in transplastomic tobacco plants, reducing the role of plastid peroxidases. J. Plant Physiol. 201, 95–100.
Czégény, Gy, Mátai, A., Hideg, É., 2016b. UV-B effects on leaves – oxidative stress and acclimation in controlled environments. Plant Sci. 248, 57–63.
Flint, S.D., Caldwell, M.M., 2003. A biological spectral weighting function for ozone depletion research with higher plants. Physiol. Plantarum 117, 137–144.
Fornera, S., Walde, P., 2010. Spectrophotometric quantification of horseradish peroxidase with *o*-phenylenediamine. Anal. Biochem. 407, 293–295.
Gitz, D.C., Liu-Gitz, L., 2003. How do UV photomorphogenic responses confer water stress tolerance? Photochem. Photobiol. 78, 529–534.
Goulas, Y., Cerovic, Z.G., Cartelat, A., Moya, I., 2004. Dualex: a new instrument for field measurements of epidermal ultraviolet absorbance by chlorophyll fluorescence. Appl. Optic. 43, 4488–4496.
Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4, 9.
He, L., Jia, X., Gao, Z., Li, R., 2011. Genotype-dependent responses of wheat (*Triticum aestivum* L.) seedlings to drought, UV-B radiation and their combined stresses. Afr. J. Biotechnol. 10, 4046–4056.
Hideg, É., Vass, I., 1996. UV-B induced free radical production in plant leaves and isolated thylakoid membranes. Plant Sci. 115, 251–260.
Hideg, É., Jansen, M.A.K., Strid, Å., 2013. UV-B, ROS and stress; inseparable companions or loosely linked associates? Trends Plant Sci. 18, 107–115.
Jansen, M.A.K., Gaba, V., Greenberg, B.M., 1998. Higher plants and UV-B radiation balancing damage, repair and acclimation. Trends Plant Sci. 3, 131–135.
Jansen, M.A.K., Coffey, A.M., Prinsen, E., 2012. UVB induced morphogenesis; four players or a quartet? Plant Signal. Behav. 7, 1185–1187.
Jenkins, G.I., 2014. The UV-B photoreceptor UVR8: from structure to physiology. Plant Cell 26, 21–37.
Klughammer, C., Schreiber, U., 2008. Complementary PS II quantum yields calculated from simple fluorescence parameters measured by PAM fluorometry and the Saturation Pulse method. PAM Appl. Notes 1, 27–35.
Kovács, V., Gondor, O.K., Szalai, G., Majláth, I., Janda, T., Pál, M., 2014. UV-B radiation modifies the acclimation processes to drought or cadmium in wheat. Environ. Exp. Bot. 100, 122–131.
Kubis, J., Rybus-Zajac, M., 2008. Drought and excess UV-B irradiation differentially alter the antioxidant system in cucumber leaves. Acta Biol. Cracov. Ser. Bot. 50, 35–41.
Majer, P., Hideg, É., 2012. Developmental stage is an important factor that determines the antioxidant responses of young and old grapevine leaves under UV irradiation in a green-house. Plant Physiol. Biochem. 50, 15–23.
Majer, P., Neugart, S., Krumbein, A., Schreiner, M., Hideg, É., 2014. Singlet oxygen scavenging by leaf flavonoids contributes to sunlight acclimation in *Tilia platyphyllos*. Environ. Exp. Bot. 100, 1–9.
Martínez-Lüscher, J., Morales, F., Delrot, S., Sánchez-Díaz, M., Gomès, E., Aguirreolea, J., Pascual, I., 2015. Characterization of the adaptive response of grapevine (cv. Tempranillo) to UV-B radiation under water deficit conditions. Plant Sci. 232, 13–22.
Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405–410.
Price, A.H., Atherton, N.M., Hendry, G.A., 1989. Plants under drought-stress generate activated oxygen. Free Radic. Res. Commun. 8, 61–66.
Poulson, M.E., Torres Boeger, M.R., Donahue, R.A., 2006. Response of photosynthesis to high light and drought for *Arabidopsis,thaliana* grown under a UV-B enhanced light,regime. Photosynth. Res. 90, 79–90.
Ren, J., Dai, W., Xuan, Z., Yao, Y., Korpelainen, H., Li, C., 2007. The effect of drought and enhanced UV-B radiation on the growth and physiological traits of two contrasting poplar species. For. Ecol. Manag. 239, 112–119.
Robson, T.M., Hartikainen, S.M., Aphalo, P.J., 2015. How does solar ultraviolet-B radiation improve drought tolerance of silver birch (*Betula pendula* Roth.) seedlings? Plant Cell Environ. 38, 953–967.
Schreiber, U., Klughammer, C., 2008. Non-photochemical fluorescence quenching and quantum yields in PS I and PS II: analysis of heat-induced limitations using Maxi-ImagingPAM and Dual-PAM-100. PAM Appl. Notes 1, 15–18.
Šnyrychová, I., Hideg, É., 2007. The first application of terephthalate fluorescence for highly selective detection of hydroxyl radicals in thylakoid membranes. Funct. Plant Biol. 34, 1105–1111.
Song, N.H., Yin, X.L., Chen, G.F., Yang, H., 2007. Biological responses of wheat (*Triticum aestivum*) plants to the herbicide chlorotoluron in soils. Chemosphere 68, 1779–1787.
Szőllősi, R., Szőllősi-Varga, I., 2002. Total antioxidant power in some species of *Labiatae*, adaptation of FRAP method. Acta Biol. Szeged. 46, 125–127.
Teklemariam, T., Blake, T.J., 2003. Effects of UVB preconditioning on heat tolerance of cucumber (*Cucumis sativus* L.). Environ. Exp. Bot. 50, 169–182.
Tian, X.R., Lei, Y.B., 2007. Physiological responses of wheat seedlings to drought and UV-B radiation. Effect of exogenous sodium nitroprusside application. Russ. J. Plant Physiol. 54, 676–682.
Wargent, J.J., Elfadly, E.M., Moore, J.P., Paul, N.D., 2011. Increased Exposure to UV-B Radiation during Early Development Leads to Enhanced Photoprotection and Improved Long-term Performance in Lactuca sativa, vol. 34. pp. 1401–1413.