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UV-B radiation modifies the acclimation processes to drought or cadmium in wheat

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Abstract

Under natural conditions plants are often subjected to multiple stress factors. The main aim of the present work was to reveal how UV-B radiation affects acclimation to other abiotic stressors. Wheat seedlings grown under normal light conditions or normal light supplemented with UV-B radiation were exposed to drought or Cd stress and were screened for changes in the contents of salicylic acid and its putative precursor *ortho*-hydroxy-cinnamic acid, and in the activity of the key synthesis enzyme, phenylalanine ammonia lyase. Certain other protective mechanisms, such as antioxidant enzyme activities and polyamines, were also investigated. PEG treatment under UV-B radiation did not cause wilting, but resulted in more pronounced salicylic acid accumulation, which may provide protection against drought stress in wheat plants. In contrast, the high level of salicylic acid accumulation in Cd-treated plants was not further enhanced by UV-B stress, but resulted in pronounced oxidative stress and the activation of antioxidant systems and polyamine synthesis. Changes in the levels of phenolic compounds are accompanied by increased phenylalanine ammonia lyase activity in the roots, but not in the leaves. The similar pattern observed for stress-induced changes in salicylic acid and *ortho*-hydroxy-cinnamic acid contents suggested that salicylic acid may play a decisive role via *ortho*-hydroxy-cinnamic acid. The results indicated that UV-B radiation might have either a positive or negative impact under the same conditions in wheat, depending on the type of secondary abiotic stress factor. The protective or damaging effects observed may be related to changes in the levels of phenolic compounds.

Keywords: cadmium, drought, oxidative stress, salicylic acid, UV-B radiation, wheat

Running title: UV-B radiation modifies the acclimation processes

Abbreviations: APX: ascorbate peroxidase; CAT: catalase; $\Delta F/F_m'$: actual quantum efficiency of photosystem II; GR: glutathione reductase; GST: glutathione-S-transferase; G-POD: guaiacol peroxidase; MDA: malondialdehyde; *o*HCA: *ortho*-hydroxy-cinnamic acid; PAL: phenylalanine ammonia lyase; PAs: polyamines; PUT: putrescine; SA: salicylic acid; SPD: spermidine; SPN: spermine.

1. Introduction

Plants are exposed to many environmental stresses, which are further aggravated by the effects of global climate change. In response to abiotic stress various biochemical and physiological changes are induced in plants leading to the ability of plants to cope with stress. The simultaneous exposure of plant to different abiotic stress conditions may results in the coactivation of different stress response pathways, which might have a synergistic or antagonistic effect on each other (Fraire-Velázquez et al., 2011; Peleg and Blumwald, 2011). Furthermore, when the plant exposed firstly to a single stress agent, is capable to increasing its resistance to a subsequent stress factor, leading to the so called cross-acclimation (Çakirlar et al., 2011).

Water deficit is a multidimensional stress affecting plants at various levels of their organization, not only manifested at the morphological level but also at the physiological level [e.g. decrease in water potential and photosynthetic rate (Ashraf and Harris, 2013)] and at the biochemical and molecular level (as formation of radical scavenging compounds, accumulation of compatible organic solutes, changes in endogenous phytohormone contents and lipid composition) (Yordanov et al., 2000; Aimar et al., 2011).

The effects of heavy metals on plant species have been well studied (Pál et al., 2006a; Yadav 2010), as a quite complex phenomenon can evoke several parallel and consecutive changes and events. In responses to oxidative stress caused by Cd, the members of the antioxidant defence mechanisms are influenced (such as antioxidant enzymes, proline and polyamines) (Lin et al., 2007; Hegedűs et al., 2001; Ekmekci et al., 2008; Pál et al., 2006b).

Salicylic acid (SA), an endogenous plant growth regulator, participates in many physiological and metabolic reactions (Yusuf et al., 2013; Janda et al., 2012). Endogenous SA level rised in several species when they are exposed to stress conditions. For example, the endogenous SA content exhibited a concentration-dependent increase in maize plants treated

with Cd (Pál et al. 2005); or the endogenous SA accumulation during drought stress has also been demonstrated on several occasions (Munné-Bosch and Penuelas 2003; Bandurska and Stroinski 2005; Abreu and Munné-Bosch 2008; Aimar et al. 2011). It was found that exogenous SA enhances the chilling tolerance of various plant species and this enhanced tolerance is accompanied by the increased activity of certain antioxidant enzymes (Horváth et al., 2007), furthermore endogenous SA was reported to protect rice plants from oxidative damage caused by aging as well as biotic and abiotic stress by modulating the redox balance (Yang et al., 2004). Exogenous SA also affects the polyamine metabolism in various plants (Németh et al. 2002; Szepesi et al. 2011); and it was found that the different concentrations of SA had different effects on polyamine metabolism (Wang et al., 2011). It was suggested that relationship exists between endogenous SA and PAs contents and antioxidant activities (Pál et al., 2013a); and even between these protective compounds and the sensitivity or resistance of plants to various biotic stresses (Liu et al. 2007; Talieva and Kondrat'eva, 2002). However, it is still not clear how endogenous SA and PAs contents influence each other.

UV radiation is traditionally divided into UV-A (320–400 nm), UV-B (280–320 nm) and UV-C (200–280 nm) wavelength ranges, which have increasing levels of energy and harmful effects. The ozone layer depletion and increase of the ozone hole increase the UV-B radiation, which could change the adaptive mechanisms of plants to known stressor factors. Although the effects UV-B radiation exhibit variations in the higher plant species, there are three potential targets of UV-B radiation in plant cells, namely the genetic system (Agrawal et al., 2009), the photosynthetic system (Majer and Hideg, 2012) and membrane lipids (An et al., 2000). At lower doses UV stress induces changes in morphology, gene expression and plant metabolism, mainly through the stimulation of the antioxidant system leading to acclimation (Kakani et al., 2003).

As under natural conditions plants are often subjected to multiple stress factor, the impact of a particular stress can be elevated by a simultaneous action of other stress. The main aims of the present study were to reveal (1) how UV-B treatment can affect alone and in combination with drought or Cd the synthesis of SA, a signalling molecule playing role in the acclimation processes to various types of stressors, and (2) what relationship may be exist between the changes in the SA content and the alteration, caused by UV-B radiation in the effect of Cd or drought stress on some physiological parameters and certain stress responses, which can be important in defence mechanism in wheat plants. In order to achieve our goals wheat seedlings grown under normal light condition or normal light supplemented with UV-B radiation at the same time were screened for changes in SA content, with the amount of the putative precursor *ortho*-hydroxy-cinnamic acid (*o*HCA) and the activity of the key enzyme of the phenyl-propanoid pathway, phenylalanine ammonia lyase (PAL), certain other protective compounds, antioxidant enzyme activities, contents of proline and polyamines were also investigated.

2. Materials and methods

2.1. Plant material and growth conditions

Winter wheat (*Triticum aestivum* L. Mv Emese) variety from MTA ATK, Martonvásár, Hungary was used for the experiments. Seeds were germinated for 3 days at 22 °C, then seedlings were grown on modified Hoagland solution (Pál et al., 2005) for 2 weeks at 20/18°C with 16/8-h light/dark periodicity in a Conviron G-48 plant growth chamber (Controlled Environments Ltd, Winnipeg, Canada) in the phytotron of the Agricultural Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Martonvásár, Hungary at the photosynthetic photon flux density (PPFD) of 250 μmol m^{-2} s^{-1}. Half of the plants were grown at normal light (CL) and the others grown at normal light combined with

UV-B radiation (UV) which was performed by 7 UV-B Narrowband TL 100W/01 lamps from Philips (with maximal radiation at 311nm). Dose of UV-B radiation was 38 μWatt/cm^2 in the case of control plants, while 430 μWatt /cm^2 in the case of UV-B-treated plants. At two-week-old stage seedlings were divided into three groups under both light condition. First part of plants was the control (C), second part of the plants was treated with 50 μM $Cd(NO_3)_2$ for 7 days (Cd) and the third part of the plants was treated with 15% polyethylene glycol (PEG-6000) for 5 days (PEG). The 1st, 2nd and 3rd leaves and roots of control, Cd-treated and PEG-treated plants were sampled at the end of the experiment. The concentrations and duration of Cd and PEG treatments were determined based on the results of phenotypic test from preliminary experiments.

2.2. Leaf rolling score

Leaf rolling is often interpreted as a strong manifestation of leaf response to water deficit. Plants showing leaf rolling at early stage of stress appears to have poor drought tolerance. Leaf rolling is scored on a scale from 1 to 5, where 1 is slightly rolled and 5 is tightly rolled (O'Toole and Cruz, 1980). Visual leaf rollings score were performed on the 5th days of PEG treatment.

2.3. Determination of chlorophyll content

The total chlorophyll content was measured on the third leaves using a SPAD-502 chlorophyll meter (Minolta Camera Co., Ltd, Japan). The SPAD-502 meter measures the transmittance of red (650 nm) and infrared (940 nm) radiation through the leaf, and calculates a relative SPAD meter value.

2.4. Chlorophyll fluorescence induction measurement

The quantum yield of photosystem II (PSII) indicated by the ΔF/Fm' [(Fm'- Fs)/ Fm'] chlorophyll fluorescence induction parameter where Fm' and Fs represent the maximum and steady-state chlorophyll fluorescence levels in the light-adapted state, respectively) was measured on fully expanded leaves using a pulse amplitude modulated fluorometer (PAM-2000, Walz, Effeltrich, Germany) as described by Janda et al. (1994). In order to compare the values the measuring conditions was the same. During the fluorescence measurement the PPFD was 250 μmol m^{-2} s^{-1} as during the plant growth, and the UV-B Narrowband TL 100W/01 lamps were switched off.

2.5. Determination of proline content

0.5 g of the samples was homogenized in a mortar with quartz sand and 4 ml distilled water. The mortar was rinsed with another dose of 4 ml distilled water. Centrifugation was carried out at 10 000g for 10 min. The supernatant was made up to 10 ml with distilled water and 2.5 ml of this dilution was used for proline quantification according to Bates *et al*. (1973). The proline content was expressed as μg g^{-1} fresh plant weight (FW), was derived from a standard curve obtained by diluting 250 μg ml^{-1} L-proline stock solution (Reanal, Budapest, Hungary) to concentrations of 0.5, 0.75, 1.0, 1.25 and 1.5 μg ml^{-1}.

2.6. Estimation of lipid peroxidation

The lipid peroxidation analysis was based on malondialdehyde (MDA) levels. 0.2 g of tissue was ground in 600 μl 0.1% (w/v) TCA, then centrifuged at 12 000*g* for 10 min. 300 μl of the supernatant was mixed with 2 ml of 0.5% (w/v) TBA in 20% (w/v) TCA and incubated at 90°C for 30 min. The MDA concentration was measured spectrophotometrically at 532 nm, with the subtraction of non-specific absorption at 600 nm.

The concentration of lipid peroxides, together with the oxidatively modified proteins were thus quantified in terms of the MDA level using an extinction coefficient of 155 mM^{-1} cm^{-1}, and expressed as nM g^{-1} fresh weight (Thomas et al. 2004).

2.7. SA extraction and analytical procedure

SA was measured according to Meuwly and Métraux (1993) by grinding 1 g of plant tissue in liquid nitrogen in a mortar and pestle, in the presence of 0.5 g quartz sand. The tissue powder was transferred to a centrifugation tube and mixed with 2 ml of 70% methanol containing 250 ng *ortho*-anisic acid (*o*ANI) (used as internal standard) and 25 µg *para*-hydroxy-benzoic acid (*p*HBA) (used as extraction carrier). The extract was centrifuged at 10 000g for 20 min. The pellet was resuspended in 2 ml 90% methanol, vortexed and centrifuged as above. The methanol content was evaporated from the mixed supernatants at room temperature under a vacuum. After adding 1 ml of 5% (w/v) TCA to the residual aqueous phase, the mixture was centrifuged in an Eppendorf centrifuge at 15 000g for 10 min. The supernatant was gently partitioned twice against 2 ml of a 1:1 (v/v) mixture of ethyl acetate/cyclohexane. The upper organic layers contained the free phenolic portion. The aqueous phases containing the methanol-soluble bound phenolics were acid hydrolysation by adding 1.3 ml 8 N HCl to the aqueous phase and incubating for 60 min at 80 °C before partitioning twice as above. Just prior to the HPLC analysis, the samples were resuspended in 1000 µl of the HPLC starting mobile phase. After separation on a reverse phase column (ABZ+, 150x4.5mm, 5µm, Supelco, Bellefonte, USA) SA and *o*HCA were quantified fluorimetrically (W474 scanning fluorescence detector, Waters, USA), with the excitation at 317 nm and emission at 436 nm for *o*HCA, followed by excitation at 305 nm and emission at 407 nm for SA.

2.8. Measurement of phenylalanine ammonia lyase (EC 4.3.1.5) activity

1 g of plant leaves and roots were homogenised at 4 C° in a mortar and pestle in 4 ml of 50 mM Tris-HCl (pH 8.8) puffer containing 5mM β-mercaptoethanol and 4% (w/v) polyvinylpolypyrrolidone. The homogenate was centrifuged at 10 000g for 10 min and supernatant was used for enzymatic assay. The reaction mixture consisted of 2.75 ml 50 mM L-phenylalanine in 50 mM Tris-HCl (pH 8.8) puffer and 250 µl supernatant. The reaction was started with the plant extract and incubated on 37 °C. After 1 h the reaction was stopped with 10% TCA. After centrifugation at 10 000g for 5 min PAL activity was determined spectrophotometrically by measuring the increase in A_{290} due to the formation of *trans*-cinnamic acid against a blank without substrate according to (Gao *et al*., 2008). One unit of enzyme activity was defined as the amount of enzyme causing the increase in absorbance of 0.01 min^{-1}. PAL activity was expressed as enzyme units per gram fresh weight (U g^{-1} FW).

2.9. Enzyme assays

For the analysis of antioxidant enzyme activity, 0.5 g tissue was homogenized in 2.5 ml ice-cold Tris-HCl buffer (0.5 M, pH 7.5) containing 3 mM $MgCl_2$ and 1 mM EDTA.

The catalase (CAT; EC 1.11.1.6.) activity of the extract was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm. The reaction mixture contained 0.44 M Tris-HCl buffer (pH 7.4), 0.0375% H_2O_2 and enzyme extract.

The ascorbate peroxidase (APX; EC 1.11.1.11.) activity was determined in the presence of 0.2 M Tris-HCl buffer (pH 7.8) and 5.625 mM ascorbic acid. The reaction was started with 0.042% H_2O_2. The decrease in absorbance at 290 nm was monitored.

The guaiacol peroxidase (G-POD; EC 1.11.1.7.) activity was measured at 470 nm as described by Ádám *et al*. (1995). The reaction mixture consisted of 88 mM Na-acetate buffer (pH 5.5), 0.88 mM guaiacol, 0.0375% H_2O_2 and enzyme extract.

The glutathione reductase (GR; EC 1.6.4.2.) activity was determined at 412 nm according to Smith *et al*. (1988). The reaction mixture contained 75 mM Na-phosphate buffer (pH 7.5), 0.15 mM diethylenetriamine-pentaacetic acid, 0.75 mM 5,5'-dithiobis(2-nitrobenzoic acid), 0.1 mM NADPH, 0.5 mM oxidized glutathione and 50 µl plant extract in a total volume of 1 ml.

The glutathione-S-transferase (GST; EC 2.5.1.18.) activity was measured by monitoring changes in the absorbance at 340 nm in a mixture containing 72.7 mM Na-phosphate buffer (pH 6.5), 3.6 mM reduced glutathione, 1 mM 1-chloro-2,4-dinitrobenzene and enzyme extract (Mannervik and Guthenberg, 1981).

The enzyme activities were determined photometrically with a UV-visible recording spectrophotometer (UV–VIS 160A, Shimadzu Corp. Kyoto, Japan).

2.10. Polyamine analysis

Polyamine (PA) analysis was carried out as described by Németh et al. (2002). Two 0.2g of plant tissue were homogenized with 1 ml 0.2 M ice-cold perchloric acid and were allowed to stand for 20 min on ice. The extract was centrifuged at 10 000g for 20 min and the supernatant was used. The PAs were analysed as dansylated derivatives via HPLC using a W2690 separation module and a W474 scanning fluorescence detector with excitation at 340 nm and emission at 515 nm (Waters, Milford, MA, USA). Conjugated and bound forms of PAs were measured after 1 hour of acid hydrolization at 96 °C.

2.11. Statistical analysis

The results are the means of at least ten repetitions for the chlorophyll induction and chlorophyll content, and of 5 repetitions for the enzyme activity and the HPLC analysis for

each treatment. Changes in these parameters were compared to the same day of control. The data were statistically evaluated using the standard deviation and t-test methods.

3. Results

3.1. Changes in stress markers caused by Cd or drought stress with or without UV-B radiation

Compared to control plants grown under control light conditions, UV-B radiation alone for 21 days caused visible shoot growth retardation, while the development of roots was not affected. Cd stress in combination with UV-B stress resulted in similar phenotypic appearance as it was found in the case of UV-B stress alone, but yellowish area on the leaves could also observed. While neither PEG nor UV-B treatment affected the chlorophyll content, Cd decreased it in plants grown under both normal light conditions and UV-B radiation (Fig. 1). The ΔF/Fm' chlorophyll-*a* fluorescence induction parameter showed, that none of the treatments used in the present experiment caused significant change in the quantum efficiency of Photosystem 2 (data not shown). However, after 5 days of PEG treatment under control light conditions wilting was observed, while when it was combined with UV-B light besides the UV-B induced reduction in growth, wilting was not observed. According to the leaf rolling scale (O'Toole & Cruz, 1980) the wilting score was 5 for PEG-treated plants, and 1 for UV PEG-treated plants. In parallel with this, drought stress with or without UV-B stress caused large increment in the proline content of the roots (Fig. 2B). Besides these, the levels of MDA in the leaves significantly increased after Cd, PEG and UV-B treatment alone, but the highest increment was observed in the case of UV-B combined with Cd stress (Fig. 3). The MDA content in the roots increased slightly, but statistically significantly in plants treated with Cd or PEG in combination with UV-B radiation (Fig.3).

3.2. Effects of UV-B radiation alone or in combination with Cd or PEG on SA and oHCA contents

In 21 day-old plants the total *o*HCA content was about two orders of magnitude higher than the total SA content (Fig.4). The free form of *o*HCA (f*o*HCA) was under the detection limit in the roots. Changes in free SA (fSA) and bound SA (bSA) contents in the leaves and the roots, furthermore in the amounts of f*o*HCA and bound *o*HCA (b*o*HCA) in the leaves showed similar pattern after the treatments applied in this experiment. The 50 μM Cd treatment alone significantly increased the total SA content in the leaves, but when this treatment was applied in combination with UV-B (UV Cd), the UV-B stress could not cause further increase compared to the Cd treatment alone (Fig. 4A). The pattern of the changes observed in the amount of root total SA was similar to the leaves, but in this case besides Cd treatment, both PEG or UV-B alone and combination of them could induce statistically significant increases compared to the control (Fig. 4B). Interestingly, the combined UV PEG treatment caused further increase in root SA content compared to the PEG induced changes, but this increase was not enough, similarly as it was found in the leaves, to result in significant difference from UV-B treatment alone. The highest increase in the SA content was observed in the case of combined UV Cd treatment. In contrast to the bound form the f*o*HCA content did not show statistically significant changes, in the leaves. The Cd or UV-B stress alone, and the combined treatment of them (UV Cd) increased the total leaf *o*HCA level with higher increment in the case of UV-B alone, which could not be further enhanced by Cd stress in UV-B treated plants (Fig.4C). The drought stress alone could not influence the root total *o*HCA, but when it was applied on plant grown under UV-B radiation increased it, though the increment was lower than it was found it the case of UV-B stress alone (Fig. 4C). All of the applied treatments (Cd, PEG, UV-B) increased the level of b*o*HCA in the roots and these

increases were more pronounced in the case of combined treatments (UV Cd and UV PEG) (Fig. 4D).

3.3. Changes in PAL activity

The initial activity of PAL in the roots was approximately 2.5-fold higher compared to the leaves (Fig. 5). In the leaf PAL activity only PEG treatment could cause a slight but statistically significant increase compared to control, while in the roots all the treatment increased it to varying degrees. Cd treatment caused lower increment in the root PAL activity compared to the PEG or UV-B alone. The PAL activity in the roots of plants grown under additional UV-B light conditions increased compared to the control, but when UV-B radiation was followed by Cd or PEG treatment it could not lead further increase (Fig. 5).

3.4. Effects of UV-B radiation on Cd- or PEG-induced changes in the antioxidant enzyme activities

Neither of the treatment could influence the GR activity in the leaves of 21 day-old wheat seedlings. PEG treatment alone and in combination with UV-B stress increased significantly the root GR activity. The highest increment was observed in UV PEG treatment (Fig. 6A). 50 μM Cd treatment or UV-B radiation alone could not cause statistically significant changes; however, the combined application of them resulted in increased GR activity of the roots.

Only UV-B radiation combined with 50 μM Cd treatment caused significant decrease in the GST activity in the leaves of wheat plants. In contrast to these Cd, PEG treatment alone could increase, UV-B radiation could not affect, while when followed by either Cd or PEG treatment, it could also increase the GST activity in the roots, but only similar manner as without UV-B stress (Fig. 6B).

Although the CAT activity in the leaves of plants grown under UV-B stress condition was significantly higher compared to the control plants, but when UV-B radiation combined with Cd could not change, and when followed by PEG treatment caused similar increase as it was observed in the case of UV-B stress alone (Fig. 6C). UV-B radiation, Cd and PEG neither alone nor in combination could affect CAT activity in the roots of wheat plants.

The APX activity in the leaves did not change significantly under either of the applied treatments, and only UV-B stress followed by Cd or PEG treatment resulted in significantly increased APX activity in the roots (Fig. 6D).

Interestingly in the leaves of plants grown under UV-B radiation the G-POD activity was significantly lower compared to the control plants. Although when UV-B stress was followed by PEG treatment G-POD activity increased, but still remained below the values of the controls (Fig. 4E). The G-POD activity in roots significantly increased after drought stress. UV-B radiation could not influence it, while when it was combined with Cd of PEG it also statistically significantly increased (Fig. 6E).

3.5. Polyamines

An analysis was made of the levels of free, conjugated (associated with small molecules) and bound forms (associated with macromolecules) of the polyamines, namely agmatine, cadaverine (CAD), putrescine (PUT), spermidine (SPD) and spermine (SPN). The agmatine content was below the detection limit in all the samples analysed (data not shown). The free and conjugated forms of the other polyamines had the same order of magnitude, while the bound form was an order of magnitude less (Fig. 7-8). The SPD content was the most abundant in all the three forms.

Cd, PEG and UV-B stress caused different changes in the levels of polyamines in leaves of wheat plants (Fig. 7). The free PUT (fPUT) content increased by both Cd and PEG

treatments, but UV-B stress could not influence it (Fig. 7A). Cd treatment applied on plant grown under UV-B radiation caused lower increase in fPUT compared to Cd treatment alone, while UV PEG treatment was found to induce similar increase as it was observed in the case of CL PEG or UV C treatment alone. Only the Cd treatment could increase significantly the free CAD (fCAD) level, while only UV-B treatment alone could case slight increase in free SPD (fSPD) content in the leaves. The amount of free SPN (fSPN) decreased after Cd or PEG treatments; however, when these treatments applied on UV-B stressed plants caused less pronounced decreases (Fig. 7A). Changes in the conjugated forms showed similar pattern as free polyamine levels in the leaves (Fig. 7B). The leaf conjugated PUT (cPUT) content increased in Cd-treated plants grown under normal light conditions, but in UV Cd treated these increase was lower. Similarly, as it was found in the case of fCAD, conjugated CAD (cCAD) level only increased in Cd-treated plants, while in contrast to fSPD level, the conjugated SPD (cSPD) content decreased by PEG treatment. The conjugated SPN (cSPN) level decreased by Cd stress and this decrease was lower when Cd was combined with UV-B stress. Other treatment could hardly influence the cSPN content (Fig. 7B). Changes in the amount of bound polyamines showed slightly different pattern compared to the free and conjugated forms (Fig. 7C). The bound PUT (bPUT) level slightly decreased after PEG, UV Cd and UV PEG treatments. The bound CAD (bCAD) level increased in after both PEG and UV PEG treatments. The bound SPD (bSPD) content showed increase in Cd- and UV-B-treated plant, while the highest accumulation was found in the combination of these treatments (Fig. 7C). The amount of bound SPN (bSPN) significantly decreased after Cd stress both in the leaves of plants grown under normal light and UV-B supplemented conditions.

Quite different pattern of polyamines was observed in the roots compared to the leaves (Fig. 8). The fPUT content significantly decreased by PEG or UV-B stress, while the

combination of them caused lower decrease compared to the control (Fig. 8A). Cd treatment applied on plant grown under UV-B radiation caused increase in fPUT compared to Cd treatment alone. Cd treatment could increase significantly the fCAD level, but its combination with UV-B induced more pronounced accumulation of fCAD. UV-B alone could cause slight increase, while its combination with Cd doubled the fSPD content it in the roots. The amount of fSPN could hardly affected by the applied treatments (Fig. 8A). Changes in the conjugated polyamine contents showed similar pattern, as it was found in the case of free forms (Fig. 8B). The root cPUT content decreased in PEG-treated plants both under under normal light conditions and under UV-B radiation (Fig 8B). The cCAD level only slightly increased in Cd-treated plants, but pronounced increase was found after the combined treatment of UV-B and Cd. The cSPD content slightly decreased by Cd treatment, but the other treatment could not influence it. Similarly, neither of the treatment could affect the cSPN level (Fig. 8B). The bPUT level significantly increased in the roots of Cd- and UV Cd-treated plants, while bSPN level only increased in the roots of plants grown under UV-B radition combined with Cd treatment (Fig. 8C).

4. Discussion

Several reports on different plant species demonstrated the direct and indirect effects of supplemental and ambient UV-B radiation on plant growth and metabolism with morphological, physiological and biochemical changes (Zlatev et al., 2012). Investigation on the interaction between UV-B stresses and other stress factors may help to understand plant adaptation to changing environmental condition. In the present study the main aims were to reveal how influence continuous, supplementary UV-B radiation, enhance or reduce the effect of Cd or drought stress, and to investigate the putative role of changes in the synthesis and metabolism of SA, a signalling molecule playing role in the acclimation processes to various

types of stressors, in these processes. Another point of interest was that how UV-B radiation can influence certain other SA-related stress responses, which can be important in defence mechanism, induced by drought or Cd treatment in wheat plants. Nevertheless, there are no reports about the combined effect of drought or Cd treatments with UV-B radiation on the level and synthesis of SA in wheat plants under the same experimental conditions.

Changes in chlorophyll and proline contents were reported in response to individual UV-B treatment and combined with heavy metal or drought stress in several plant species, but the effect of UV-B depended on the intensity and duration of it, furthermore the order of the applied stress factors also influenced the results (Alexieva et al., 2003; Mishra and Agrawal, 2006; Singh et al., 2009). In the present experiment in plants grown under UV-B radiation growth retardation was observed and Cd stress resulted in enhanced oxidative stress, which was manifested in pronounced yellowing area on the leaves, especially on leaf tips of the plants; while UV-B pre-treatment interestingly alleviated the wilting induced by PEG treatment. Cd treatment alone and with UV-B significantly decreased the total chlorophyll content, while the proline level significantly increased only after PEG treatment in the roots both under normal light conditions and under UV-B supplemented conditions. Cd and PEG treatment, even UV-B stress alone significantly increased the lipid peroxidation in the leaves, while in the roots only combined treatments (UV Cd and UV PEG) resulted in increased MDA level. These result indicated that Cd, PEG and UV-B treatment could cause injury in wheat plants, but the mode of action of these treatments are different.

SA was shown to accumulate during Cd treatment in barley (Metwally et al., 2003) or maize (Pál et al., 2005). In turn although the protective effect of exogenous SA against heavy metal stress was also demonstrated in maize (Krantev et al., 2008) or pea (Popova et al., 2009), SA treatment itself may also cause oxidative stress and damage to the root system (Pál et al., 2002). Because of these contradictory results the mode of action of SA is still unclear.

In the present study the changes in total SA contents in the leaves and roots showed similar pattern, and it can be concluded, that Cd has greater effect on SA level than PEG or UV-B alone; furthermore, UV-B light could not further enhance the Cd-induced SA accumulation. UV-B could elevate SA content more effectively than PEG treatment, but combined treatment of them only caused similar increase as UV-B alone. In contrast to these, UV-B radiation alone caused higher increase in the level of leaf *o*HCA than Cd treatment alone, while combined treatment of UV-B and Cd could not elevate statistically significantly the effect of Cd. Interestingly, although drought stress alone could not influence, the combined UV+PEG treatment could result in increased level of the leaf total *o*HCA, compared to the control, but still remained under the UV-B stress induced value. In turn, in the roots UV+PEG treatment was the most effective, as resulted in the highest b*o*HCA accumulation. Similarly to these earlier endogenous SA levels were found to increase in the leaves of maize seedlings with the degree of Cd stress, which may be associated with the oxidative stress observed in the leaves of Cd-treated plants; furthermore, among the phenolic compounds the highest accumulation was found in the case of bound *o*HCA in the leaves of maize plants during Cd stress (Pál et al., 2005).

Although, endogenous SA accumulation during drought stress has been demonstrated on several occasions (Bandurska and Stroinski, 2005; Abreu and Munné-Bosch, 2008), in the present study it was found that PEG treatment did not induce the accumulation of these phenolic compounds, but together with UV-B radiation both SA and *o*HCA levels in the leaves and roots significantly increased. Increased total SA content was also reported in the leaves and roots of barley, when drought stress preceded UV-B radiation (Bandurska and Cieslak, 2012). The increased SA content may be responsible for the observed alleviating effect of UV-B light on wilting induced by PEG treatment. This observation is in accordance with a recent study, where it was demonstrated that deficiency of SIZ1 gene, which encodes

an *Arabidopsis* SUMO E3 ligase, caused reduced stomatal aperture and enhanced drought tolerance via controlling SA-induced accumulation of reactive oxygen species (Miura et al., 2013). As the control of stomatal aperture is important for efficiency and regulation of water use and for the response to drought, UV-B stress-induced SA accumulation could have positive effect in PEG-treated plants. Furthermore, since *o*HCA has also been demonstrated to have antioxidant properties (Foley et al. 1999), these results suggest that the increase in the *o*HCA content was also induced independently from the SA biosynthesis, and may play a role in the antioxidative response to Cd, drought and UV-B stress.

The activity of PAL, which is a crucial enzyme in plant metabolism, synthesis of lignins, flavonoids, anthocyanins and simple phenolic acids, was stimulated by UV-B radiation (Józwiak-Żurek et al., 2011), Cd stress (Pawlak-Sprada et al., 2011) or PEG treatment (Shehab et al., 2010). Interestingly, PAL activity in the leaves showed slight changes, while in the roots all treatments could increase it, especially in the case of PEG, UV-B and UV PEG, but additive effect of combined treatment could not be observed. These results are in accordance with the changes in SA and *o*HCA contents in the roots, but the higher in SA and *o*HCA contents in the leaves of Cd-treated plants compared to PEG or UV-B treated ones, were not accompanied with higher PAL activities, which could not explain these differences. In barley it was found that the accumulation of SA induced by drought or UV-B was accompanied by increase in PAL and benzoic acid hydroxylase enzyme activities (Bandurska and Cieslak, 2012). Moreover the similar pattern in stress induced changes of SA and *o*HCA contents suggested that SA via *o*HCA may play decisive role, similarly as it was found in rice (Pál et al., 2013a).

In the present study the applied treatments activated various acclimation systems such as antioxidant enzymes or polyamines at varying degree; and leaves and roots also responded differently. Additive effect of UV-B and Cd resulted in the increment of the GR and APX

activities and even the levels of polyamine of the roots, which could be related with the increased levels of phenolic compounds in the roots and manifested in the observed negative effect UV-B radiation on Cd-treated plant. Recently it was demonstrated, that correlation exists between SA content, antioxidant enzymes and the levels of polyamines (Pál et al., 2013b). Combined treatments with supplemental UV-B and Cd resulted in more pronounced changes in antioxidant enzyme activities and the levels of antioxidant compounds in pea (Agrawal and Mishra, 2009). Combination of UV-B and PEG treatment did not cause any pronounced additive effect on the antioxidant enzyme activities or polyamine contents, indicating that although both UV-B and PEG treatments induced stress, combination of them could not result in enhanced damage of wheat plants. Exogenous SA treatment was found to delay leaf rolling by inducing antioxidant enzymes and modulating osmoprotectant content under PEG induced osmotic stress (Demiralay et al., 2013; Marcinska et al., 2013). However, in the present study changes in antioxidant defence mechanisms could not be related to the observed changes in the levels of phenolic compounds. Similarly to the present results in some cases UV radiation with heavy metal or drought stress has synergistic or antagonistic effect on antioxidant system (Mishra et al., 2006; Alexieva et al., 2003), basically depending on the method, condition and intensity of UV-B treatment.

In conclusion, UV-B radiation may have either a positive or negative impact under the same conditions in wheat, depending on the type of secondary abiotic stress factor (Fig. 9). The protective or damaging effects observed may be related to the changes observed in the levels of phenolic compounds. Differences in the role and mode of action of SA in the case of Cd or PEG stress could explain the different upshot of the increased SA content in plants grown under normal light supplemented with UV-B radiation. The fact that PEG treatment under UV-B radiation did not cause wilting may due to the more pronounced SA accumulation in the combined treatment, which provides protection against drought stress in

wheat plants. In contrast, the very high level of SA accumulation in Cd-treated plants, which was not further enhanced by UV-B stress, resulted in pronounced oxidative stress, and the activation of antioxidant systems and other defence compounds, such as polyamines. Although changes in PAL activity in the roots was accompanied by changes in the root levels of SA and *o*HCA, the levels of these compounds in the leaves increased even when greater PAL activity was not induced.

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Legends to Figures

Figure 1. Effect of 50 μM Cd stress after 7 days, 15% PEG treatment after 5 days on total chlorophyll content in leaves of wheat seedlings grown under normal light condition or under UV-B radiation. Data presented as mean ± SD (*n*=10). Values carrying different letters were significantly different at $p < 0.05$ within the given genotypes.

Figure 2. Changes in proline content after 7 days of 50 μM Cd stress, after 5 days of 15% PEG treatment in the leaves (A) and roots (B) of wheat seedlings grown under normal light condition or under UV-B radiation. Data presented as mean ± SD (*n*=3). Values carrying different letters were significantly different at $p < 0.05$ within the given genotypes.

Figure 3. Changes in malondialdehyde (MDA) content after 7 days of 50 μM Cd stress, after 5 days of 15% PEG treatment in the leaves and roots of wheat seedlings grown under normal light condition or under UV-B radiation. Data presented as mean ± SD (*n*=3). Values carrying different letters were significantly different at $p < 0.05$ within the given genotypes.

Figure 4. The 7 days of 50 μM Cd stress- or 5 days of 15% PEG treatment-induced changes in the salicylic acid contents (SA) of the leaves (A) and the roots (B), and *ortho*-hydroxycinnamic acid contents (*o*HCA) in the leaves (C) and roots (D) of wheat seedlings grown under normal light condition or under UV-B radiation. The black columns represent the free form, while the white columns indicate the bound forms of the phenolic compounds. Data presented as mean ± SD (*n*=3). Values carrying different letters were significantly different at $p < 0.05$ within the given genotypes.

Figure 5. Changes in phenylalanine ammonia-lyase activity after 7 days of 50 μM Cd stress, after 5 days of 15% PEG treatment in the leaves and roots of wheat seedlings grown under

normal light condition or under UV-B radiation. Data presented as mean ± SD (*n*=3). Values carrying different letters were significantly different at $p < 0.05$ within the given genotypes.

Figure 6. Effect of 50 μM Cd stress after 7 days, 15% PEG treatment after 5 days on the glutathione reductase (A), gluthatione-S-transferase (B), catalase (C), ascorbate peroxidase (D) and guaiacol peroxidase (E) activities the leaves and roots of wheat seedlings grown under normal light condition or under UV-B radiation. Data presented as mean ± SD (*n*=3). Values carrying different letters were significantly different at $p < 0.05$ within the given genotypes.

Figure 7. Accumulation of polyamines, namely putrescine (PUT), cadaverin (CAD), spermidine (SPD) and spermine (SPN) in free (f), conjugated (c) and bound (b) forms, after 7 days of 50 μM Cd stress, after 5 days of 15% PEG treatment in the leaves of wheat seedlings grown under normal light condition or under UV-B radiation. Data presented as mean ± SD (*n*=3). Values carrying different letters were significantly different at $p < 0.05$ within the given genotypes.

Figure 8. Accumulation of polyamines, namely putrescine (PUT), cadaverin (CAD), spermidine (SPD) and spermine (SPN) in free (f), conjugated (c) and bound (b) forms, after 7 days of 50 μM Cd stress, after 5 days of 15% PEG treatment in the roots of wheat seedlings grown under normal light condition or under UV-B radiation. Data presented as mean ± SD (*n*=3). Values carrying different letters were significantly different at $p < 0.05$ within the given genotypes.

Figure 9. Schematic diagram of the combined effects of cadmium or drought and UV-B. Dotted lines indicate combined treatment effects. For details, see text.

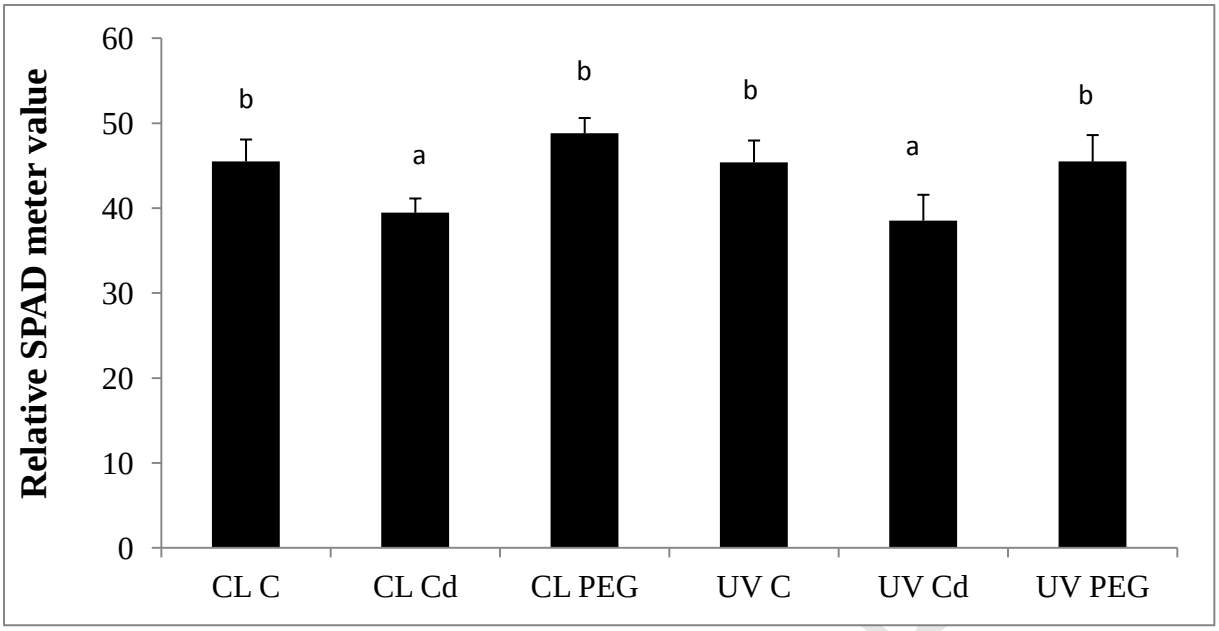
Highlights

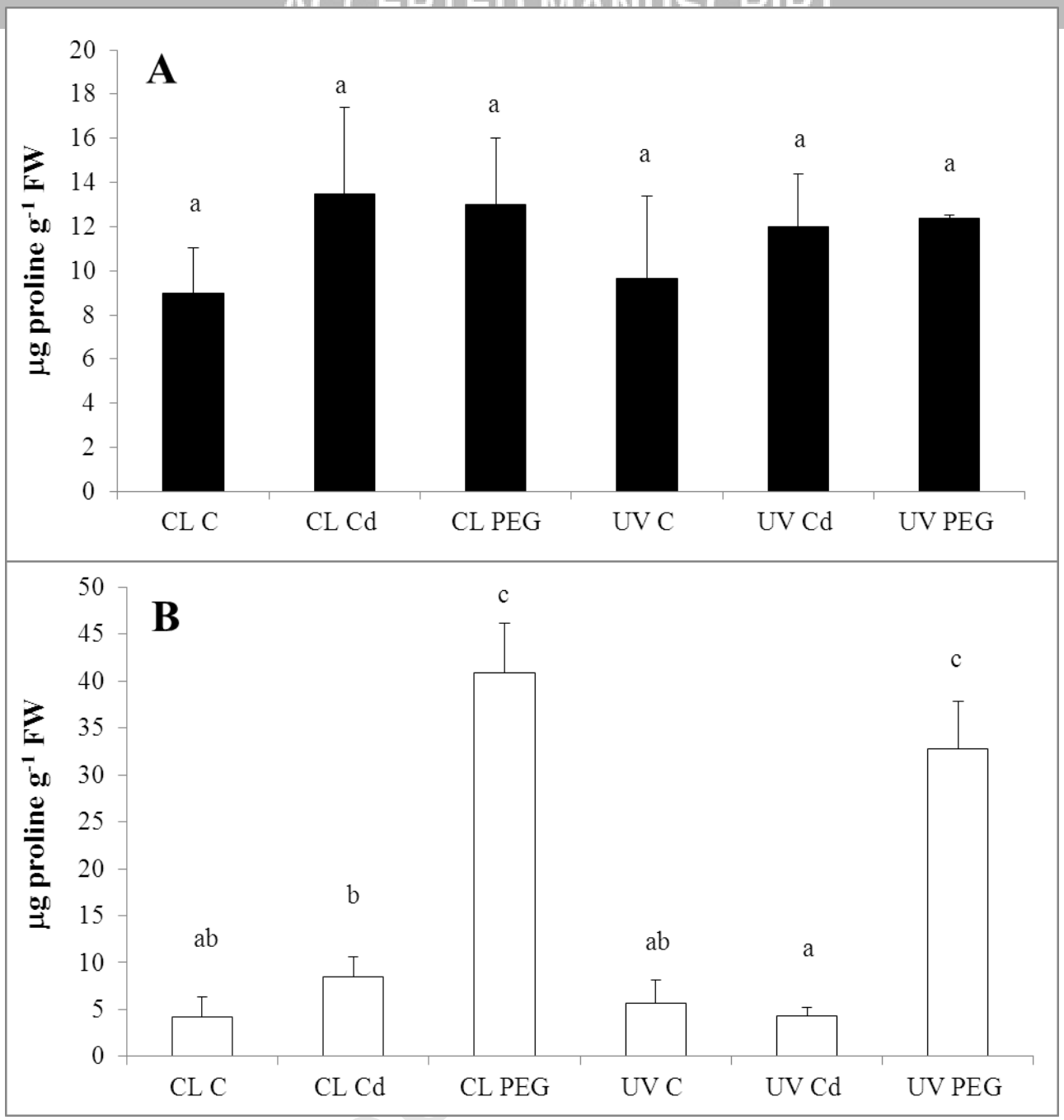
UV-B has different impact on drought and Cd stress responses.

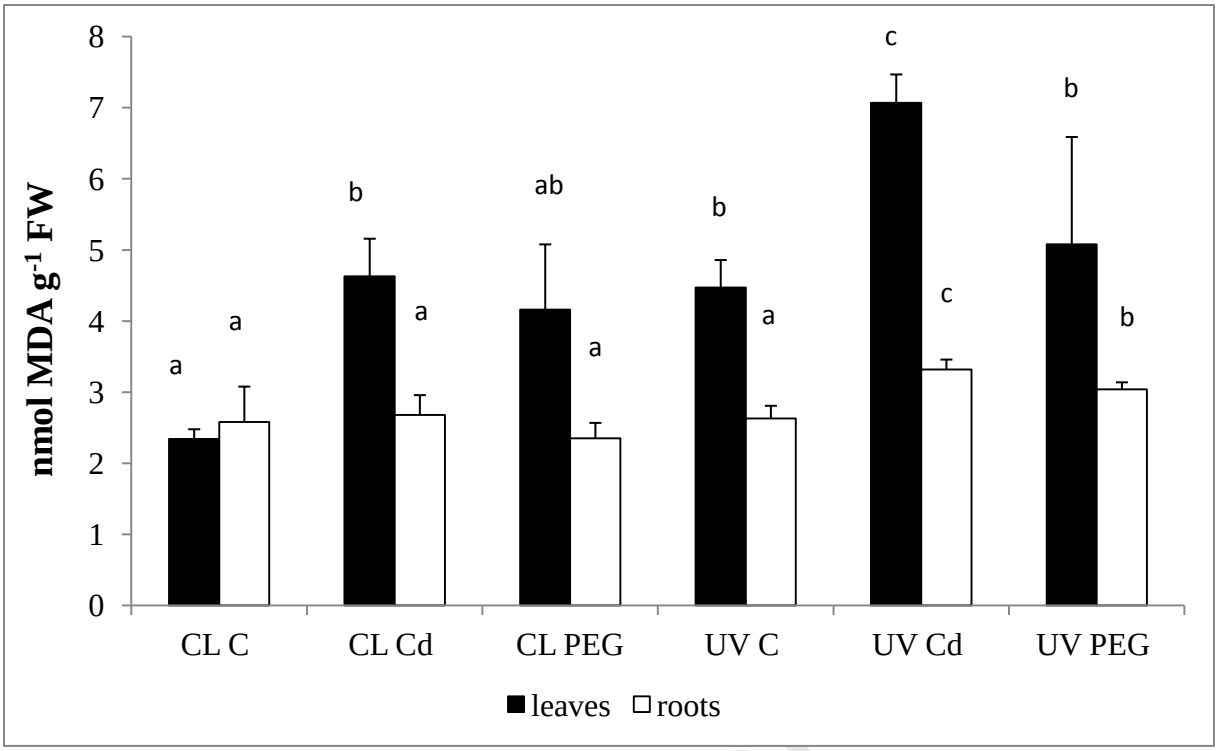
Protective effect of UV-B against drought may be in relation with salicylic acid.

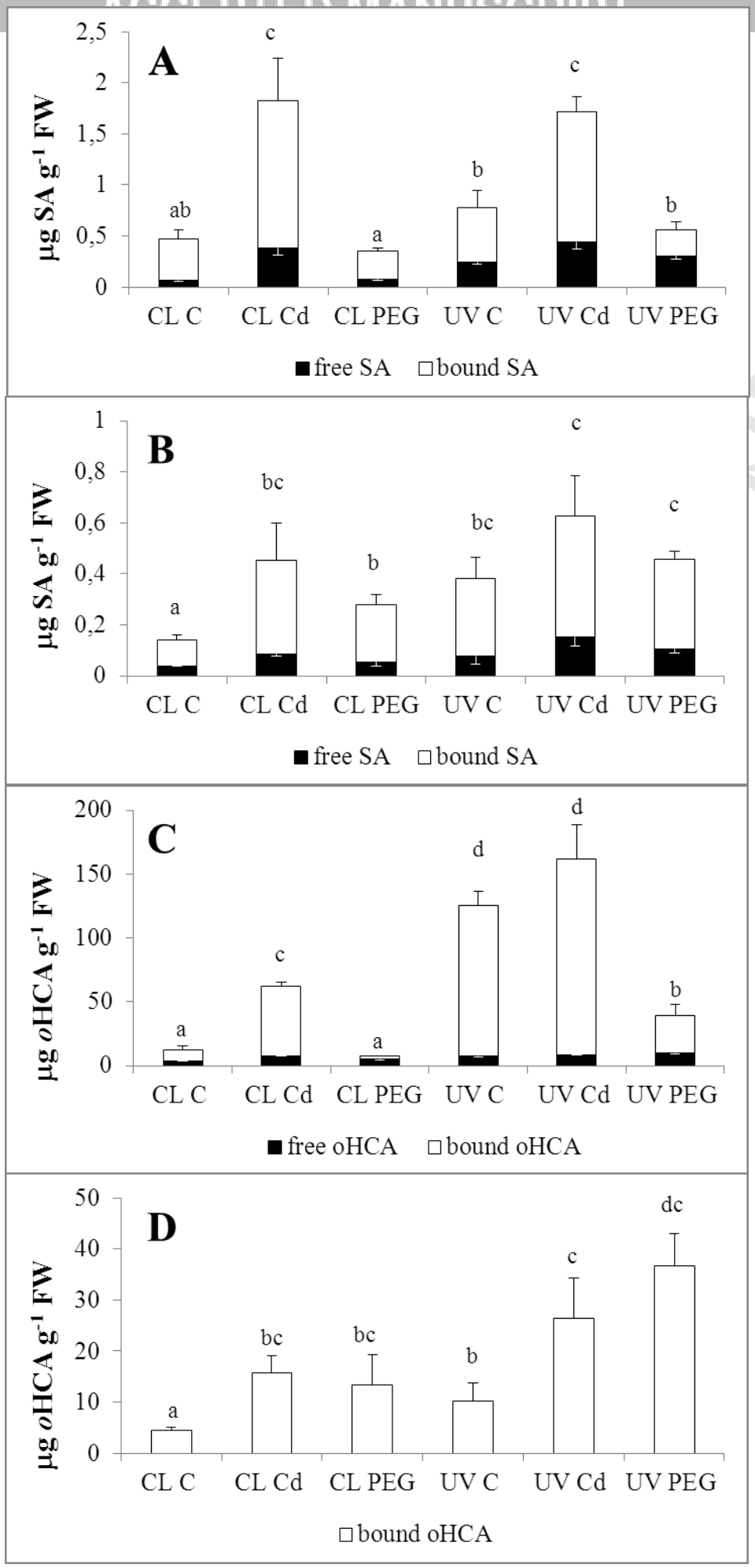
UV-B specifically influences drought or Cd-induced antioxidant/polyamine responses.

Changes in *ortho*-hydroxy-cinnamic acid content are also UV-B dependent.





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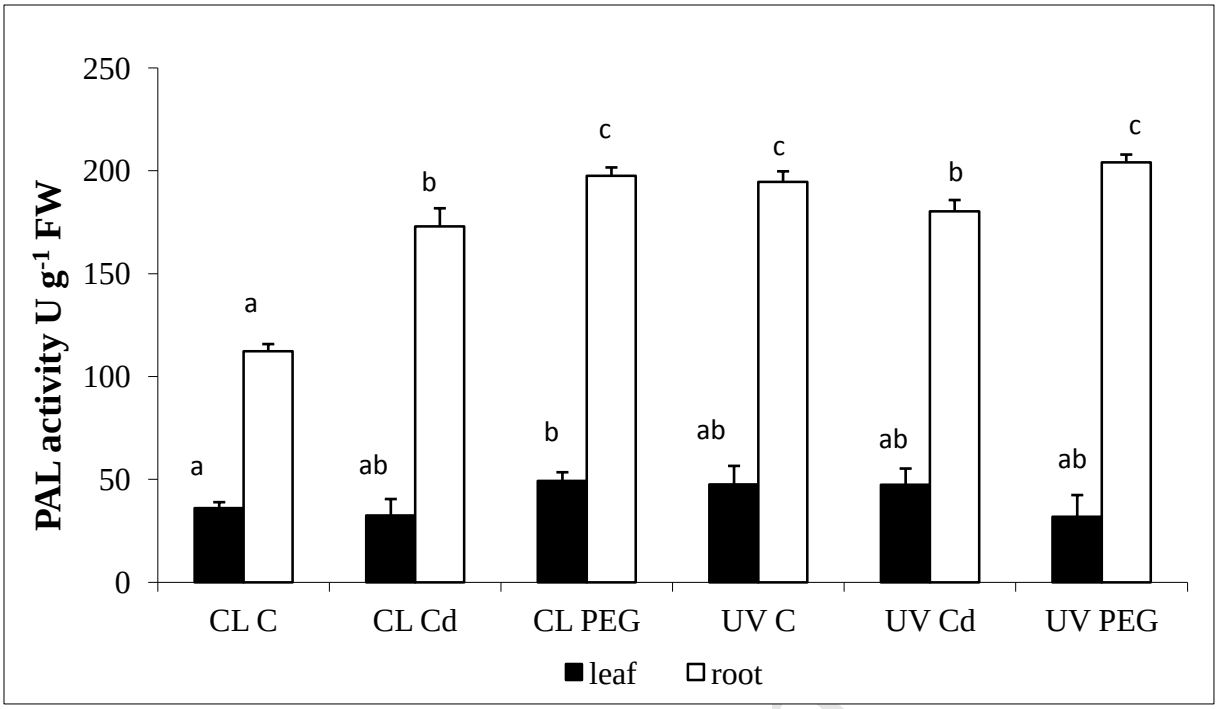

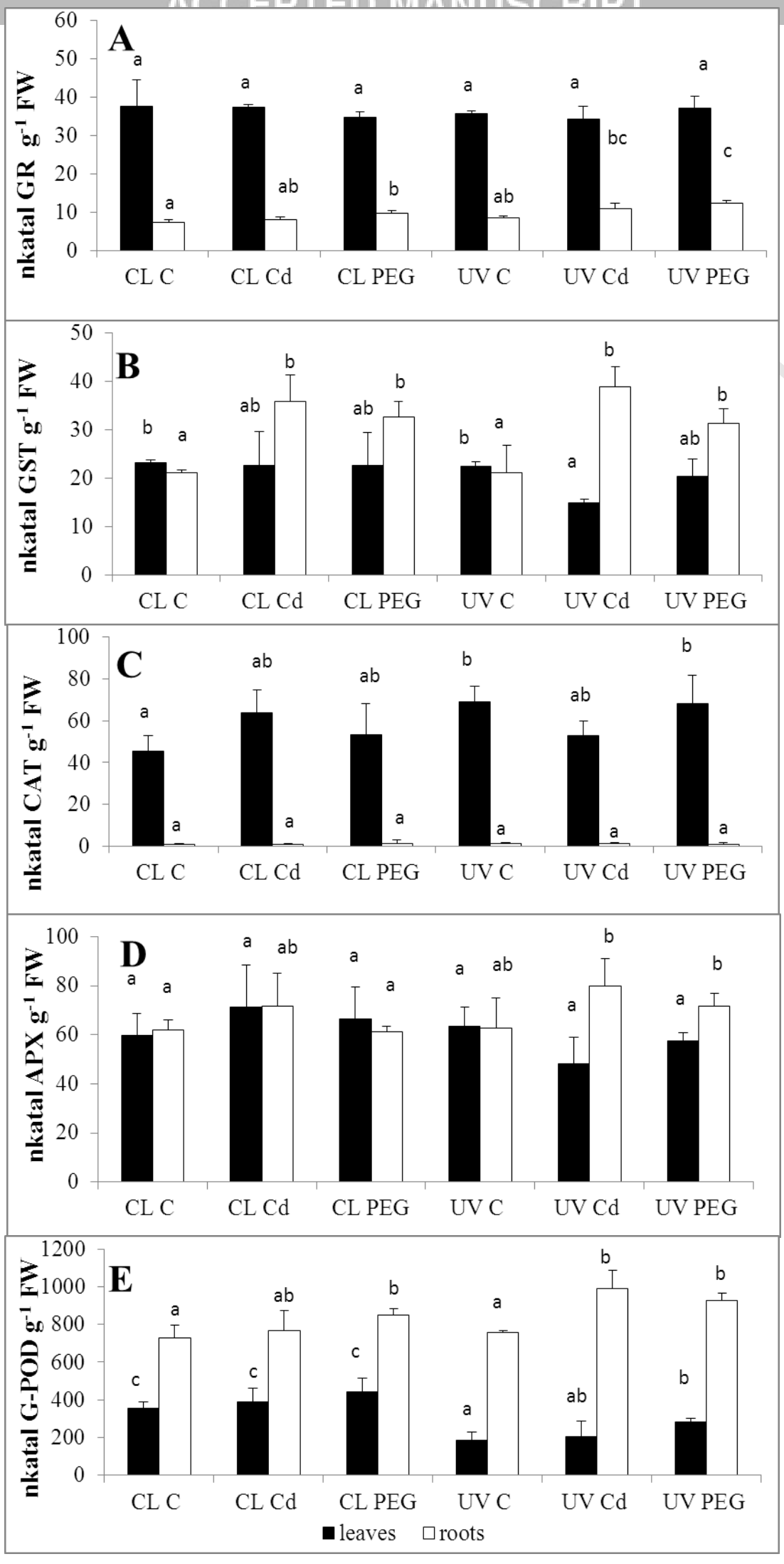

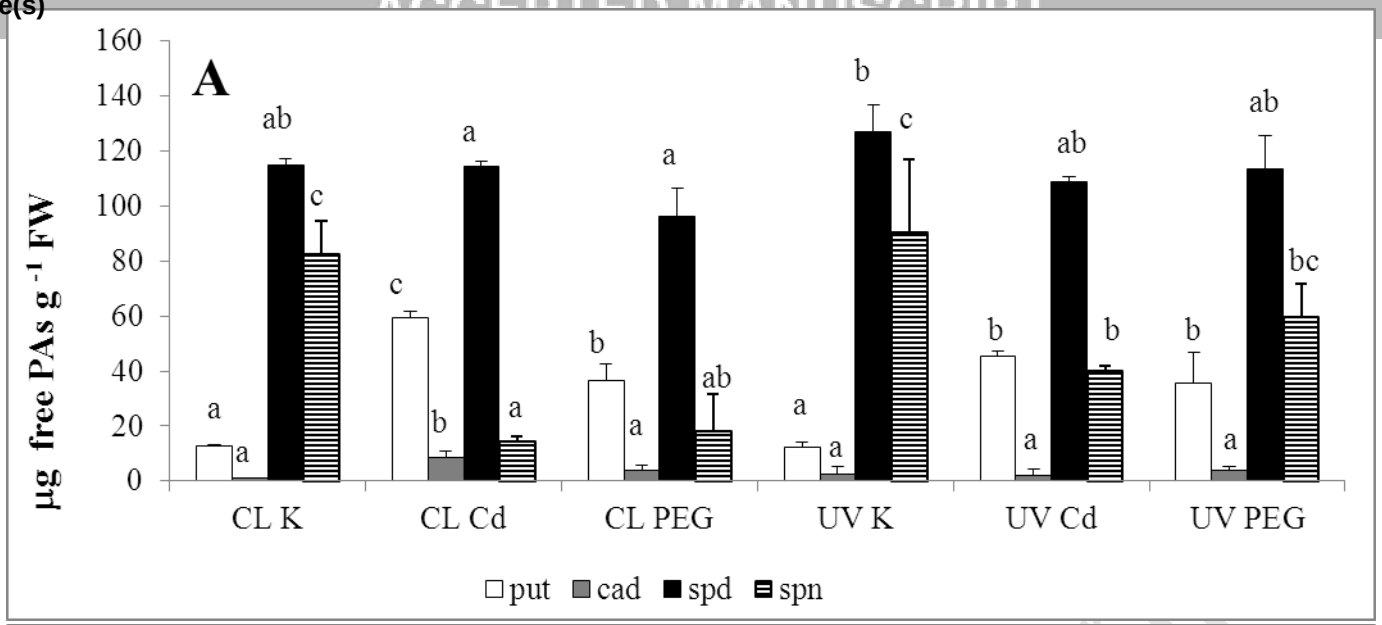

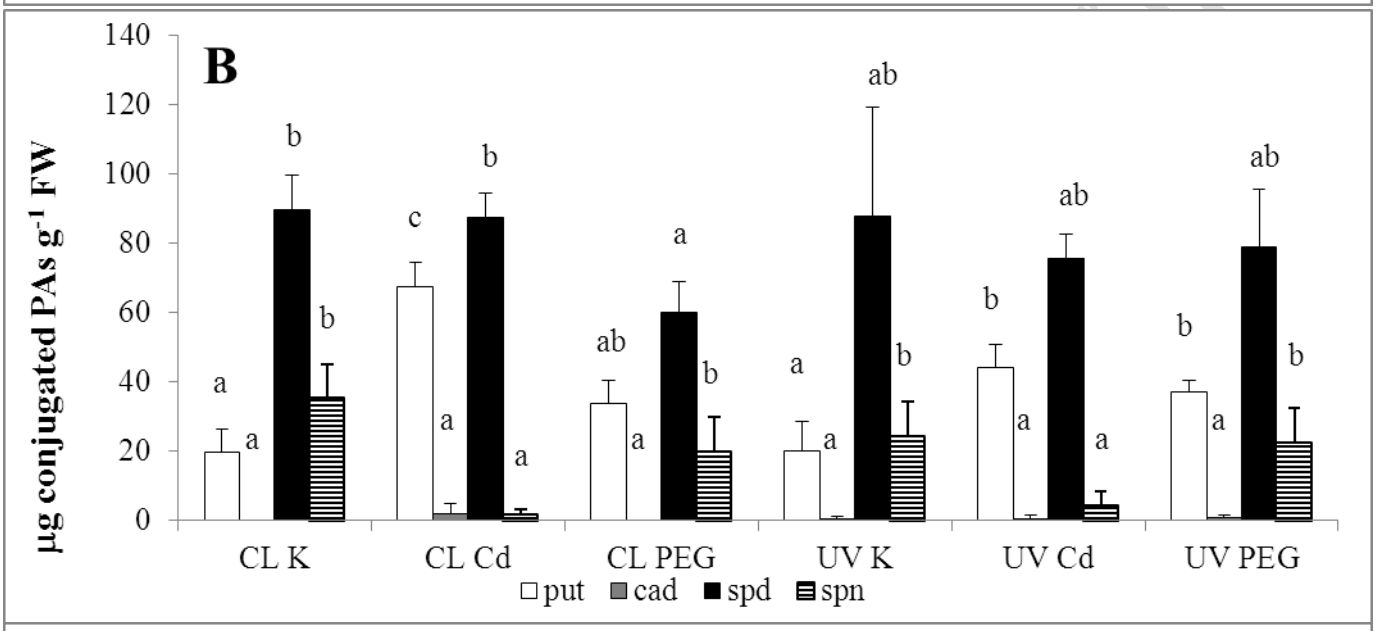

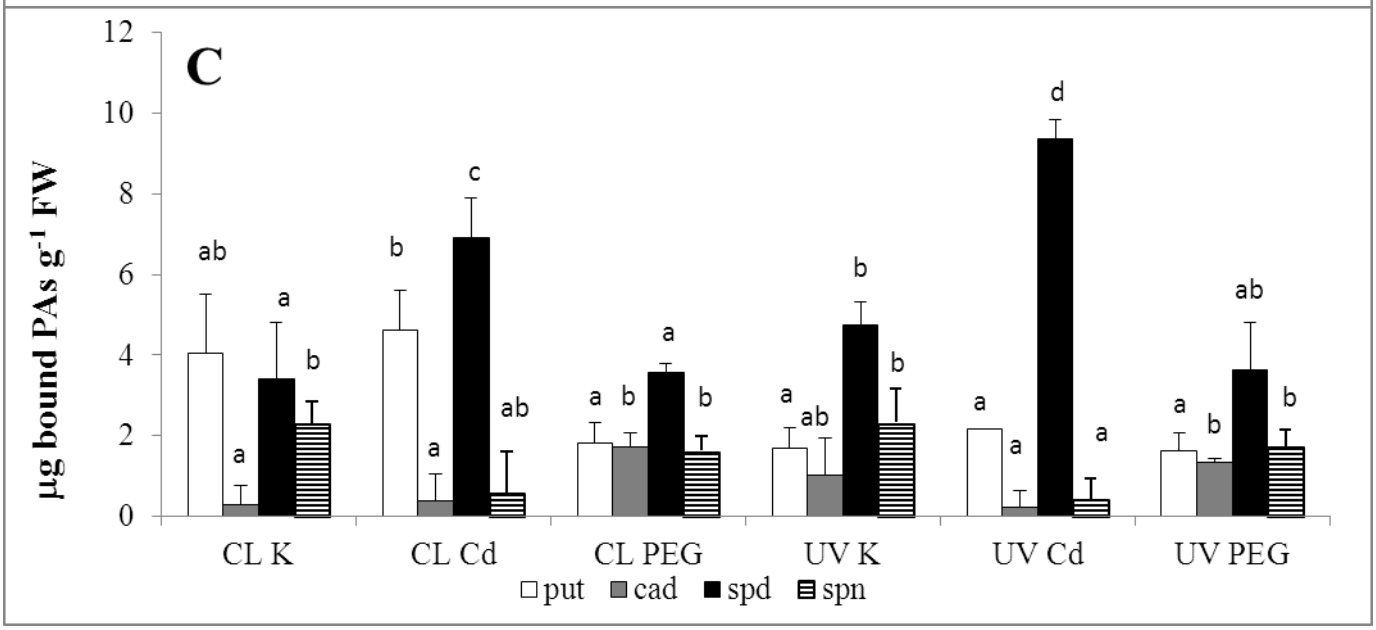

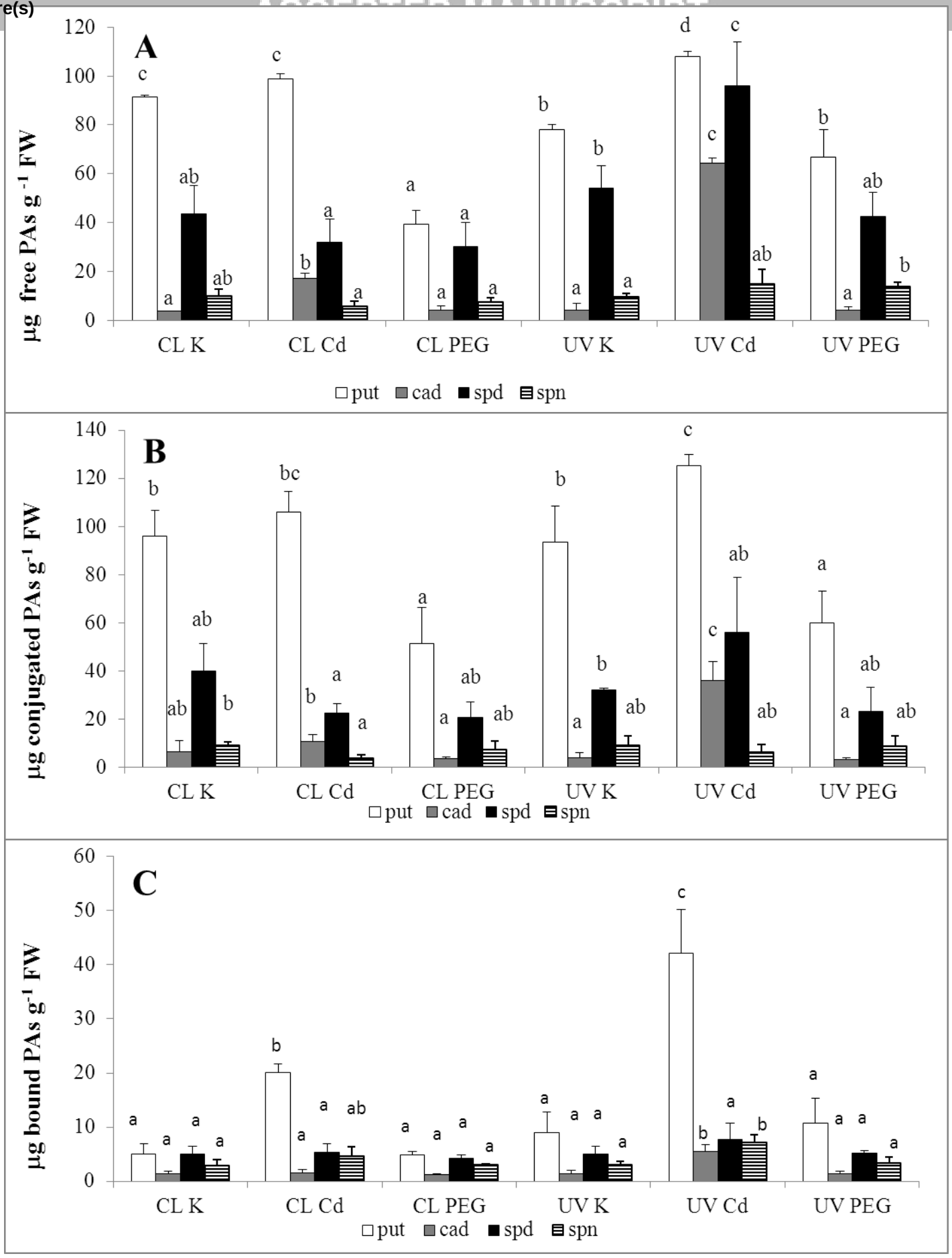
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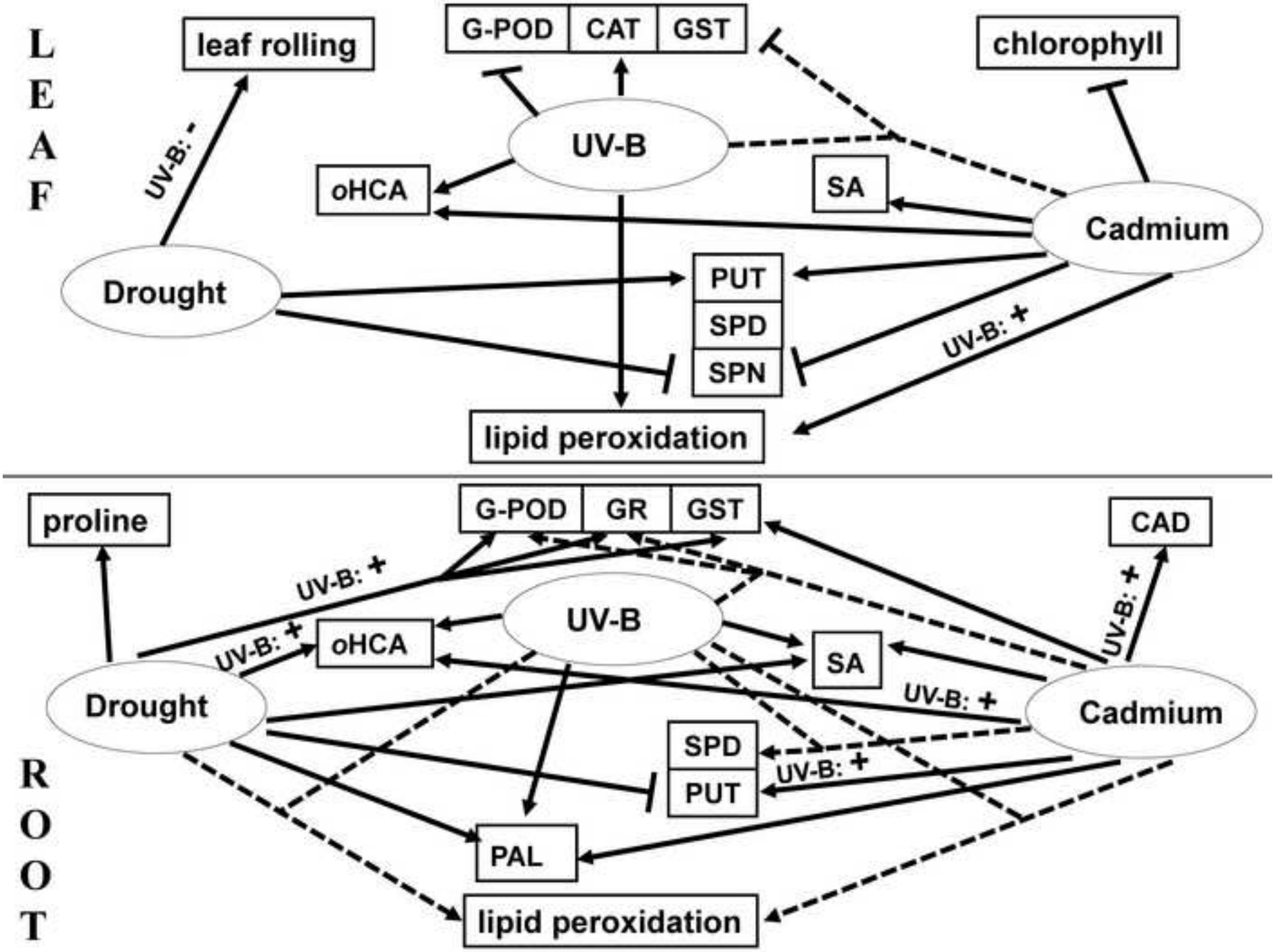
ab
a
a
a
a
a
a
a
a
c
b
a
b
a
a
a
CL K
CL Cd
CL PEG
UV K
UV Cd
UV PEG
put
cad
spd
spn

Figure9


LEAF
leaf rolling
G-POD
CAT
GST
chlorophyll
UV-B: -
UV-B
oHCA
SA
Cadmium
Drought
PUT
SPD
SPN
UV-B: +
lipid peroxidation
ROOT
proline
G-POD
GR
GST
CAD
UV-B: +
UV-B: +
UV-B
oHCA
SA
UV-B: +
Drought
Cadmium
SPD
PUT
UV-B: +
PAL
lipid peroxidation