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Physiological Response to Different Irradiation Regimes during Barley Seedlings Growth Followed by Drought Stress under Non-Photoinhibitory Light

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Abstract

Differences in physiological response of barley seedlings cultivated under low (LI, ~65 μmol m^{-2} s^{-1}) and elevated irradiation (EI, ~450 μmol m^{-2} s^{-1}) to upcoming drought were evaluated. After ten days of cultivation, drought stress was induced under LI by withholding water and was defined as: mild stress (MS), severe stress (SS), extreme stress (ES) and control (C, well watered). Decreased relative water content (RWC) in both LI and EI grown plants was associated with increased lipid peroxidation (TBARS) and electrolyte leakage (%EL) as well as with decreased total chlorophylls content at SS and ES. Antioxidative response to drought was, generally, indicated by higher accumulation of free proline, increased activities of catalase (CAT), guaiacol peroxidase (GPOD) and ascorbate peroxidase (APX). Even the majority of estimated physiological and biochemical parameters showed no differences between investigated light regimes before drought stress induction, the LI grown plants responded on drought by adjustment of the photosynthetic apparatus to prevent photoinhibitory oxidative damage. Photosynthesis downregulation in EI grown plants under ES was revealed due to reduced values of maximum quantum yield of photosystem II (Fv/Fm) and performance index (PI_{ABS}) showing that acclimatization to EI conditions lowered their tolerance to the following drought stress although it was applied under low irradiation (LI). Therefore, those plants had reduced capability to cope with the challenge of upcoming drought stress showing more pronounced cellular oxidative damage (SS and ES), less efficient and almost dysfunctional photosynthetic apparatus in extreme drought (ES).

Keywords: antioxidative response, drought stress, *Hordeum vulgare*, light regime, lipid peroxidation, photosynthesis

1. Introduction

Drought is one of the most common environmental stresses what strongly affects plant growth and productivity. Therefore, drought impact monitoring is particularly important for crops worldwide as water resources are becoming less available. Understanding the physiological and biochemical processes induced by water deficit is necessary to improve productivity by breeding drought tolerant crops. Also, it was previously revealed that, besides agronomic parameters, investigation of carefully selected parameters from chlorophyll *a* fluorescence measurements could contribute to crop breeding strategies to maximize yield during abiotic stresses (Baker & Rosenqvist, 2004; Narina, Pathak, & Bhardwaj, 2014; Reddy, Chaitanya, & Vivekanandan, 2004). It is well known that drought causes oxidative stress due to an imbalance of production and scavenging the reactive oxygen species (ROS). Though, the regulatory mechanisms of ROS production during water deficit are still not completely understood (Cruz de Carvalho, 2008). Four basic forms of ROS: singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2) are result of partial reduction of atmospheric

O_2. Over-accumulation of ROS during the powerful and long-term stresses can oxidize cell structures and molecules and consequently lead to cell death (Mittler, 2002). During the evolution, plants have developed different mechanisms of adaptation and survival in drought conditions. Enzymatic and non-enzymatic antioxidants function as efficient cooperative systems which are able to eliminate ROS. Besides the main enzymatic and non-enzymatic antioxidants, amino acid proline is also widely used drought stress marker since it plays a significant role in osmotic adjustment and participates in free radical scavenging (Ben Rejeb, Abdelly, & Savouré, 2014). Drought reduces the biochemical capacity for carbon uptake thus decreasing the activity of enzyme Rubisco which, in turn, directly reduces the rate of photosynthesis (Reddy et al., 2004).

The irradiation excess, what is usually coupled with drought, also downregulates the rate of photosynthesis due to induction of photoinhibition (Alves, Magalhães, & Barja, 2002; Murata, Takahaski, Nishiyama, & Allakhverdiev, 2007). Under the conditions of excess irradiation photosynthetic apparatus absorbs more light than can be utilized for CO_2 fixation. This imbalance usually leads to excess ROS production (Foyer & Noctor, 2000). Also, the interaction of shade or daylight with drought in field conditions shows the beneficial impact of shade for leaf-level physiology in drought conditions (Valladares et al., 2008). Björkman and Powles (1984) reported that there were no inhibition of electron-transport activity in shade grown plants even when leaf water potential was low (3.0 MPa) and the degree of inhibition under more severe water stress was much less pronounced than in the plants kept in full daylight. Photoinhibitory effect of the interaction between high light and drought was demonstrated in many publications (Akashi et al., 2008; Šimić, Lepeduš, Jurković, Antunović, & Cesar, 2014), while less is known about the interaction of low light (less than 100 μmol m^{-2} s^{-1}) and drought stress (Dinakar, Djilianov, & Bartels, 2012; Yang, Han, Liu, Lin, & Wang, 2008).

In the present study, barley seedlings cultivated at low (LI, ~65 μmol m^{-2} s^{-1}) and elevated (EI, ~450 μmol m^{-2} s^{-1}) irradiation levels for ten days were further subjected to different drought stress levels for following nine days. Stress levels were marked as C (well watered, control - 0^{th} day), MS (mild stress - 3^{rd} day of drought), SS (severe stress - 6^{th} day of drought) and ES (extreme stress - 9^{th} day of drought). The impact of drought was examined under the low irradiation (LI, ~65 μmol m^{-2} s^{-1}) in order to avoid the cumulative simultaneous effects of two stress factors (increased light level and drought). The aim of the study was to investigate whether the cultivation of barley plants on EI would induce different physiological response to upcoming drought in comparison to cultivation at LI. In our knowledge, this might be the first report of drought stress induced under low light conditions (LI) in barley plants acclimatized at elevated irradiation (EI). We hypothesized that well known adjustment of the photosynthetic apparatus to EI (Alves et al., 2002; Lichtenthaler & Burkart, 1999) might bring about stronger downregulation of the photosynthetic capability and increased oxidative damage in upcoming drought stress.

2. Materials and Methods

2.1 Plant Material and Experiment Design

Seeds of winter barley (*Hordeum vulgare* L. cv. Bravo) were soaked in tap water for 24 h in the dark and sown in plastic containers (53 cm in length, 13 cm in width and 8 cm in height) filled with a mixture of commercial soil substrate and sand in the ratio of 3:1. The seedlings were grown for ten days under controlled conditions of the growth chamber (Vötsch, Germany) with a long day cycle (16 h day, 22±1 ^{o}C; 8 h night, 19 ^{o}C) and 80% relative humidity. During the first ten days of cultivation, plants were grown at two different level of breeding light. One part of plants was grown at low irradiation level (LI, ~65 μmol m^{-2} s^{-1}) what is equivalent to normal morning (30 minutes after dawn) light at the end of the October (45°34′N, 18°42′E) when winter barley usually has one well developed leaf, while the others were grown at elevated irradiation level (EI, ~450 μmol m^{-2} s^{-1}) what is equivalent to midday light of a sunny day. During this period, the seedlings were well-watered daily. Ten-day-old seedlings from both growing conditions were subjected to progressive drought for nine days by water withholding. Drought stress experiment was conducted at low irradiation (LI, ~65 μmol m^{-2} s^{-1}). Other settings of the growth chamber were the same as previously described. The first fully developed leaves of seedlings were sampled to conduct daily analysis during the nine days of drought. In this paper, we showed results obtained from measurements before drought stress induction (0^{th} day, control (C)) and 3^{rd}, 6^{th} and 9^{th} day of drought treatment subsequently marked as mild stress (MS), severe stress (SS) and extreme stress (ES), respectively.

2.2 Leaf Relative Water Content (RWC) Determination

Relative water content (RWC) was estimated as initially described by Weatherley (1950) and adjusted according to Keles and Öncel (2004). Fresh weight (FW) of the 7 mm long leaf pieces was determined immediately after cutting. Turgid weight (TW) was determined after floating leaf segments in distilled water for 3 h at room temperature under dim light (2-3 μmol m^{-2} s^{-1}). Prior to weighing, leaf pieces were quickly dried using soft paper

towels. Dry weight (DW) was determined after 24 h long oven-drying at 105 ^{o}C. Each sample type was measured in ten replicates. RWC was calculated using the equation:

$$RWC\ (\%) = [(FW\text{-}DW)/(TW\text{-}DW)] \times 100 \quad (1)$$

2.3 Electrolyte Leakage (EL) Determination

Cell membrane damage was evaluated by measuring the rate of electrolyte leakage according to Nayyar and Gupta (2006). Ten leaf pieces (7 mm) were washed thrice with de-ionized water to remove external solutions from injured cells and placed in closed glass tubes filled with 20 mL de-ionized water. The initial conductivity of the solution (L_1) was measured after 24 h long incubation at room temperature using the conductivity meter. Then the samples were autoclaved at 121 ^{o}C for 20 min, cooled to room temperature and the final electrical conductivity (L_2) was recorded. Each sample type was measured in ten replicates. The EL was calculated as:

$$EL\ (\%) = (L_1/L_2) \times 100 \quad (2)$$

2.4 Lipid Peroxidation

The concentration of thiobarbituric acid-reacting substances (TBARS), as a measure of lipid peroxidation, was measured as described Verma and Dubey (2003). Leaf samples (~200 mg) were grounded into a fine powder using liquid nitrogen and extracted with 1 mL 0.1% (w/v) trichloroacetic acid (TCA). After centrifugation at 10,000 × g for 10 min, 0.5 mL of the supernatant was added to 1 mL 0.5% (w/v) thiobarbituric acid (TBA) in 20 % TCA. The mixture was heated at 95 °C for 30 min, cooled in an ice-bath and centrifuged again (18,000 × g, 15 min, 4 °C). The absorbance of the supernatant was measured at 532 nm (correction was done by subtracting the absorbance at 600 nm for unspecific turbidity) using spectrophotometer Specord 40 (Analytic Jena, Germany), and the concentration of lipid peroxidation products was expressed as total TBARS in nmol g^{-1} FW (extinction coefficient 155 mM^{-1} cm^{-1}). Each sample type was measured in three replicates.

2.5 Free Proline Determination

Free proline content was extracted and determined according to Bates, Waldren, and Teare (1973), with certain adjustments. Samples of ~200 mg leaf tissue, previously grounded into a fine powder using liquid nitrogen, were mixed with 10 mL of 3% sulphosalicylic acid and immediately centrifuged at 3,500 × g for 15 min. Supernatants (2 mL) were mixed with the equal amount of acetic acid and ninhydrin reagent in a test tube and incubated 1 h at 100 °C. The reaction was terminated by placing tubes in ice bath. Toluene (4 mL) was added after cooling and reaction mixture was well stirred. The absorbance of separated toluene layer was recorded at 520 nm. Proline concentration (nmol $_{PROLINE}$ g^{-1} FW) was determined from the calibration curve using L-Proline (Sigma-Aldrich) as a standard. Determinations were done in triplicate for each sample type.

2.6 Enzyme Extractions and Assays (GPOX, APX, CAT, SOD)

Before enzyme extractions, leaf tissue was grounded to a fine powder using liquid nitrogen. All further steps during the analyzes were performed at 4 °C. Guaiacol peroxidase (GPOX, EC 1.11.1.7) activity was determined according to Siegel and Galston (1967). Samples (~500 mg) were extracted with 100 mM Tris-HCl buffer (pH 8.0) containing polyvinylpyrrolidone (PVP) and centrifuged at 18,000 × g for 10 min. Supernatants were used for enzyme assay. The assay mixture (total volume of 1 mL) contained the reaction mixture (200 mM KH_2PO_4, 200 mM $Na_2HPO_4 \cdot 12H_2O$, 5 mM guaiacol (Sigma-Aldrich), pH 5.8) and 5 - 20 µL of crude protein extract (depending of sample). The activity of GPOX was determined spectrophotometrically by the measuring the absorbance increase at 470 nm due to the formation of tetraguaiacol. For determination of ascorbate peroxidase (APX, EC 1.11.1.11) activity (Nakano & Asada, 1981), samples (~200 mg) were extracted using 100 mM K-phosphate buffer (pH 7.0) containing 5 mM Na-ascorbate, 1 mM EDTA and PVP and centrifuged (10 min, 18,000 × g). The assay mixture contained 100 mM K-phosphate buffer (pH 7.0), 0.1 mM EDTA, 5 mM ascorbic acid, 12 mM H_2O_2 and 40 µL of crude extract in a final assay volume of 1 mL. The decrease in absorbance, due to ascorbate oxidation, was measured at 290 nm. Catalase (CAT, EC. 1.11.1.6) activity was estimated according to Aebi (1984). Samples of ~200 mg were extracted with 100 mM K-phosphate buffer (pH 7.5) containing 1 mM EDTA and centrifuged for 10 min at 18,000 × g. The assay mixture contained 100 mM K-phosphate buffer (pH 7.5), 0.1 M H_2O_2 and 80 µL of crude extract in a final assay volume of 2 mL. The decrease in absorbance due to a reduction in the quantity of H_2O_2, was followed at 240 nm. Superoxide dismutase (SOD, EC 1.15.1.1.) activity was determined according to Giannopolitis and Ries (1977). Samples (~200 mg) were extracted with 100 mM K-phosphate buffer (pH 7.5) containing 1 mM EDTA and PVP, and centrifuged 10 min at 18,000 × g. The assay mixture (total volume of 1 mL) contained 50 mM K-phosphate buffer (pH 7.5), 13 mM methionine, 75 µM nitroblue tetrazolium (Sigma-Aldrich), 0.1 mM EDTA, 2 µM riboflavin and crude extract. The mixture was illuminated (~120 µmol m^{-2} s^{-1}) trough 10 min and the absorbance was read at 560 nm. The same mixture with no

extract added and without illumination was used as blank. One unit (U) of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of nitroblue tetrazolium reduction, compared to the same illuminated sample with no added enzyme.

For all enzyme assays, three extractions per stress type were done and each one was measured in triplicate. The results of GPOX, APX and CAT activity were expressed as change in the absorbance per min per g FW. SOD activity was expressed as U per g FW.

2.7 Measurements of Chlorophyll Fluorescence Parameters and Chlorophyll Content

The chlorophyll *a* fluorescence induction kinetics was measured at room temperature using Plant Efficiency Analyzer (Handy-PEA, Hansatech, UK). The measurements were performed on 25 attached barley seedlings leaves, previously dark adapted for 30 min in order to stimulate opening all chlorophyll reaction centers (RC). The application of saturating red light pulse (peak at 650 nm, 3000 μmol m^{-2} s^{-1}) induced chlorophyll *a* transient. The fast fluorescence kinetics was recorded from 10 μs to 1 s. In conditions when all RC are open it is possible to measure minimal fluorescence intensity (F_0), while maximal fluorescence intensity (F_m) is reached when RC are closed. The fluorescence intensity at F_0, 300 μs, 2 ms and Fm were used to obtain the parameters of JIP-test. Three biophysical parameters describing the photosystem II (PSII) photochemistry were calculated according to Strasser, Tsimilli-Michael, and Srivastava (2004): maximum quantum yield of PSII (Fv/Fm), performance index on absorption basis (PI_{ABS}) and density of the active reaction centers per excited cross-section (RC/CS_0). Detailed calculations of fluorescence parameters are shown in Appendix.

For determination of the total chlorophylls concentration (Chl *a*+*b*) leaves were powdered using liquid nitrogen (~100 mg) and extraction was done using 100% acetone. Total chlorophylls concentration (Chl *a*+*b*) was determined spectrophotometrically according to Lichtenthaler (1987). Pigments concentration was expressed as mg per g DW (fresh samples for DW calculations were oven-dried for 24 h at 105 °C). Total chlorophylls of each stress type were extracted in three replicates.

2.8 Statistical Analysis

The experiment consisted of three independent repetitions. In the first step of statistical analysis, each experiment repetition was analyzed separately by four-factor analysis of variance (ANOVA). Main factors were drought (two treatments – water withholding and control; light (two treatments – low and elevated irradiation); days (ten steps) and measurement replicates: three for lipid peroxidation, free proline, GPOX, APX, SOD and total chlorophyll content; ten for RWC and %EL and twenty-five for chlorophyll *a* fluorescence parameters.

Combined two-factor ANOVA across the experiment repetition was performed using standard errors from the four-factor ANOVAs where main factors were three experiment repetitions and 40 treatments represented by means of the interaction drought × light × day (2 × 2 × 10). Since no significant differences were detected among experimental repetitions, data presented in this study were pooled across the three experiment repetitions using pooled standard error. For all differences among selected means representing stress levels (marked as C for 0^{th}, MS for 3^{rd}, SS for 6^{th} and ES for 9^{th} day) t-test and least significant difference (LSD) at the 0.05 probability level were applied. Different letters represent significant difference between stress levels. All results were statistically analyzed with PLABSTAT (Version 3A) and StatSoft (Version 12).

3. Results

3.1 Relative Water Content Decreased Equally at LI and EI during Dehydration

The relative water content (RWC) of barley seedlings cultivated at low (LI, ~65 μmol m^{-2} s^{-1}) and elevated (EI, ~450 μmol m^{-2} s^{-1}) irradiation levels decreased during the drought stress (Figure 1). Seedlings grown under the both type of cultivation revealed the same dynamics of the RWC reduction during dehydration period. The RWC values at different stress levels in plants cultivated at LI were 95.03% (C), 69.11% (MS), 36.15% (SS) and 21.11 % (ES). The RWC values at the same levels in plants cultivated at EI were 92.93% (C), 69.61% (MS), 34.70% (SS) and 21.78% (ES).

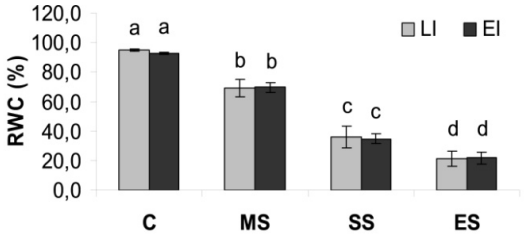

Figure 1. Effects of different drought stress levels (C, MS, SS and ES) conducted at low irradiation (LI, ~65 μmol $m^{-2}s^{-1}$) on the relative water content (RWC) of seedlings grown at (□) low (LI, ~65 μmol m^{-2} s^{-1}) and (■) elevated irradiation (EI, ~450 μmol m^{-2} s^{-1}). The values are the means of 30 replicates ± SE. Different lowercase letters indicate a significant difference at $P < 0.05$ between each of presented values

Notes. C – control; MS – mild stress; SS – severe stress; ES – extreme stress.

3.2 Drought Downregulated Total Chlorophylls Concentration and Photosynthetic Efficiency

The total chlorophylls concentrations (Chl *a+b*) measured at different drought stress levels (C, MS, SS and ES) in barley leaves grown under LI and EI are shown in Figure 2A. The significantly higher concentration of Chl *a+b* was observed in plants cultivated at LI, compared to plants cultivated at EI, during the entire drought period. The total chlorophyll concentration was not significantly affected by MS related to C in both cultivation types. SS and ES caused significant decrease of the chlorophyll content by 1.3-fold and 1.5-fold in plants cultivated at LI, as well as in plants cultivated at EI were it was at SS 1.6-fold and ES 2.9-fold decreased, compared to related control.

In order to evaluate the impact of different drought stress levels on photosynthetic efficiency *in situ* chlorophyll *a* fluorescence was measured. Obtained values of all calculated fluorescence parameters measured before drought stress induction (C) were not significantly different in barley seedlings grown under different types of cultivation (LI and EI) (Figures 2B-D). Maximum quantum yield of PSII (Fv/Fm) was significantly decreased only by ES in plants cultivated at EI (Figure 2C) showing the incompetent PSII as Fv/Fm value was 0.544 ± 0.06 (competent PSII has Fv/Fm > 0.75). Performance index on absorption basis (PI_{ABS}) was slightly affected by SS and ES in seedlings cultivated at LI. In comparison with control, SS and ES caused decrease by 1.3-fold and 1.5-fold (Figure 2D). Higher decrease of PI_{ABS} (compared to control) caused by SS and ES (by 1.8-fold and 6.9-fold, respectively) was observed in seedlings cultivated at EI. Density of active reaction centers per cross-section (RC/CS_0) was not affected by any of drought stress levels in seedlings cultivated at LI (Figure 2B). However, in seedlings cultivated at EI, ES caused significant decrease value of parameter RC/CS_0 by 2.3-fold (compared to control).

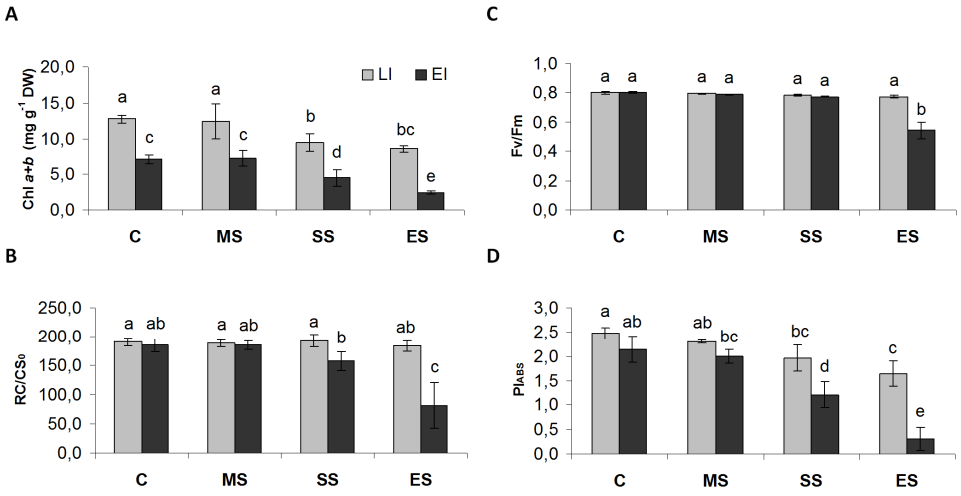

Figure 2. Effects of different drought stress levels (C, MS, SS and ES) conducted at low irradiation (LI, ~65 µmol m^{-2} s^{-1}) on the (A) total chlorophyll concentration (Chl *a*+*b*) and fluorescence parameters: RC/CS_0 (B), Fv/Fm (C) and PI_{ABS} (D) of seedlings grown at (□) low (LI, ~65 µmol m^{-2} s^{-1}) and (■) elevated irradiation (EI, ~450 µmol m^{-2} s^{-1}). The values are the means of 9 replicates ± SE for Chl *a*+*b* and 75 replicates ± SE for fluorescence parameters. Different lowercase letters indicate a significant difference at $P < 0.05$ between each of presented values

Notes. C – control; MS – mild stress; SS – severe stress; ES – extreme stress; RC/CS_0 – density of active reaction centers of photosystem II per excited cross-section; Fv/Fm – maximum quantum yield of photosystem II; PI_{ABS} – performance index on absorption basis.

3.3 Effects of Drought on Electrolyte Leakage, Lipid Peroxidation and Free Proline Content

The values of the electrolyte leakage, lipid peroxidation level and free proline content measured before drought stress induction (C) were not significantly different in barley seedlings grown under the both type of cultivation (LI and EI) (Figure 3).

The lipid peroxidation level, expressed as the concentration of TBARS, was significantly increased by SS and ES in seedlings cultivated at LI, as well as at EI (Figure 3A). Compared to control (C), SS and ES caused an increase by 1.5-fold and 2.5-fold in plants grown at LI, while the increase in plants grown at EI was notably higher, by 2.7-fold at SS and 4.5-fold at ES.

The electrolyte leakage (%EL) in barley seedlings grown under the both type of cultivation (LI and EI) was significantly increased by SS and ES levels of drought, compared to control (C) (Figure 3B). In seedlings cultivated at LI severe stress (SS) caused the increase by 2.8-fold and ES by 5.9-fold, compared to control (C). In seedlings cultivated at EI electrolyte leakage was increased by SS 5.6-fold and ES 14.5-fold, compared to control (C).

The free proline content did not show differences in well watered seedlings (C) as well as in ones subjected to mild stress (MS) of both types of cultivation (Figure 3C). In plants cultivated at LI conditions SS and ES caused a significant increase of proline content by 28.2-fold and by 164.8-fold consequently, compared to control (C). At EI, the increase of proline content was even more pronounced by SS (73.2-fold) and especially by ES (283.7-fold), compared to control (C).

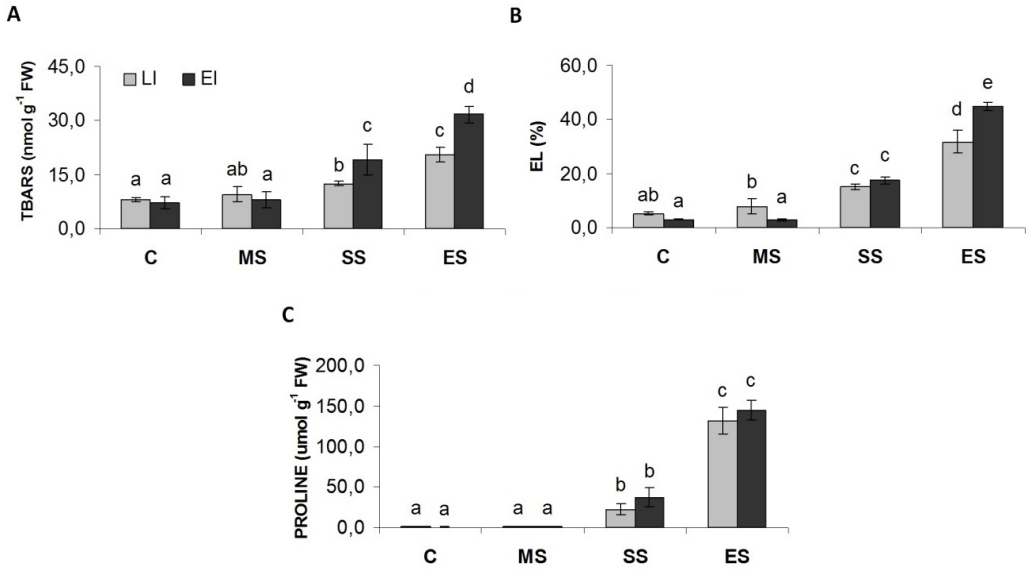

Figure 3. Effects of different drought stress levels (C, MS, SS and ES) conducted at low irradiation (LI, ~65 µmol m^{-2} s^{-1}) on (A) TBARS concentration, (B) electrolyte leakage (% EL) and free proline content (C) of seedlings grown at (□) low (LI, ~65 µmol m^{-2} s^{-1}) and (■) elevated irradiation (EI, ~450 µmol m^{-2} s^{-1}). The values are the means of 30 replicates ± SE for% EL and 9 replicates ± SE for TBARS and proline. Different lowercase letters indicate a significant difference at $P < 0.05$ between each of presented values

Notes. C – control; MS – mild stress; SS – severe stress; ES – extreme stress.

3.4 Drought Differentially Modified Antioxidative Enzymes Activity in LI and EI Grown Seedlings

The measured enzyme activities before drought stress induction (C) were not significantly different in plants grown at EI, in comparison to LI, with the exception of the CAT (Figure 4).

The GPOX activity was not significantly affected by MS while SS and ES caused significant increase at both, LI and EI (Figure 4A). Compared to control, SS caused increase in GPOX activity by 3.9-fold, while the observed increase by ES was 7.9-fold in plants grown at LI. The activity of GPOX in plants grown at EI was increased 4.0-fold by SS and 5.1-fold by ES compared to control (Figure 4A).

No significant differences of the APX activity were observed between the well watered plants (C) and plants subjected to mild stress (MS) for both types of cultivation (Figure 4B). In plants cultivated at LI severe stress (SS) caused significant increase of the APX activity (by 1.8-fold) compared to C and remained unchanged by ES. In plants cultivated at EI severe stress (SS) caused an increase of APX activity by 1.8-fold while ES did it by 2.4-fold, compared to C (Figure 4B).

The CAT activity was not affected by MS in barley cultivated neither at LI nor EI (Figure 4C). Significantly higher activity (1.3-fold compared to C) in plants grown at LI was observed when seedlings were subjected to SS and the activity remained unchanged by ES. In seedlings grown at EI, SS and ES caused significantly increased activity of CAT, compared to control (1.2-fold and 1.6-fold).

The SOD activity (Figure 4D) measured in seedlings cultivated at LI was significantly decreased by SS and ES (1.6-fold), compared to control. In seedlings cultivated at EI the activity of SOD was significantly decreased in MS and SS (1.4-fold and 1.6-fold, respectively), compared to control (C). The extreme stress (ES) treatment regained the SOD activity to the value comparable to well watered plants (C).

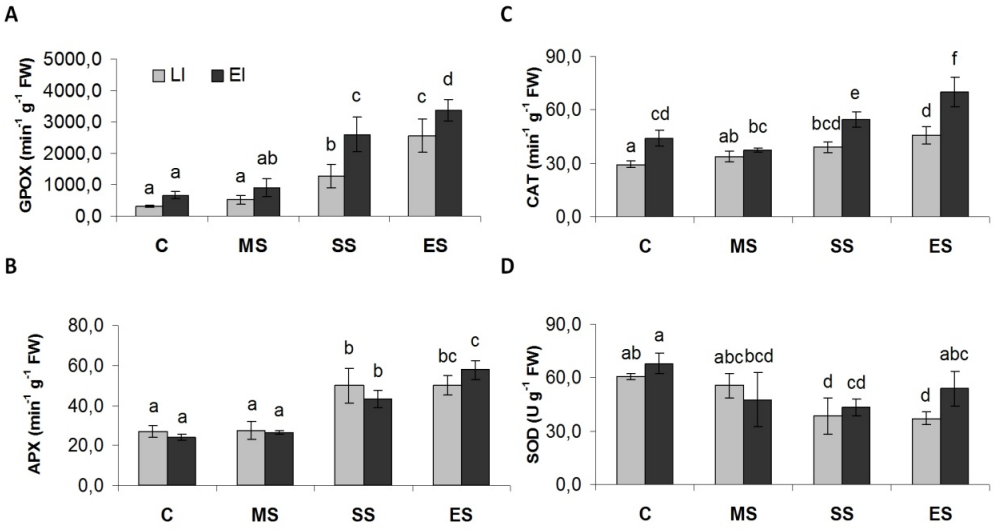

Figure 4. Effects of different drought stress levels (C, MS, SS and ES) conducted at low irradiations (LI, ~65 µmol m^{-2} s^{-1}) on the antioxidative activity of guaiacol peroxidase (GPOX) (A), ascorbate peroxidase (APX) (B), catalase (CAT) (C) and superoxide dismutase (SOD) (D) of seedlings grown at (□) low (LI, ~65 µmol m^{-2} s^{-1}) and (■) elevated irradiation (EI, ~450 µmol m^{-2} s^{-1}). The values are the means of 9 replicates ± SE. Different lowercase letters indicate a significant difference at $P < 0.05$ between each of presented values

Notes. C – control; MS – mild stress; SS – severe stress; ES – extreme stress.

4. Discussion

Physiological and biochemical changes in leaves of young barley seedlings during acclimatization to different drought stress levels (C, MS, SS and ES) at low irradiance, previously cultivated under different light conditions (LI and EI), were the subject of this research. Water status of barley leaves was estimated by measuring the relative water content (RWC) which is generally accepted as a primary indicator of water stress (Uzilday, Turkan, Sekmen, Ozgur, & Karakaya, 2012), and more recently also serving as a trait for selection of drought tolerant cultivars/genotypes of various species (Jäger et al., 2014; Jongdee, Fukai, & Cooper, 2002). Similar categorization of stress levels was found in research of Silvestre, Araújo, Vaz Patto, and Marques da Silva (2014), where RWC of 100-80% was defined as irrigated control group (C), 80-50% as mild stress (MS), 50-30% as severe stress (SS) and 30-20% as extreme stress group (ES). The same pattern of a significant decrease in RWC, affected by water withholding, was revealed in our investigation, in both types of cultivation (Figure 1).

Water loss from plant cells is controlled partly or completely by stomatal closure, the physiological mechanism directly related to decreased CO_2 assimilation. Stomatal limitation downregulates the overall photosynthesis through over-reduction components of photosynthetic electron transport (Yordanov, Velikova, & Tsonev, 2000). This downregulation might consequently lead to enhanced ROS production (Krause & Weis, 1991).

Chlorophyll is the key chromophore involved in photosynthetic light reactions, and PSII is considered being one of the main regulatory components of the electron transport chain in chloroplasts (Foyer & Noctor, 2000). Chlorophyll content depends on the developmental stage of leaves (Lepeduš et al., 2011) as well as the environmental stress factors such as high light and drought (Coelho, Miranda, Melo, & Barbosa, 2015; Yordanov et al., 2000). The elevated irradiation increases the probability of excess ROS formation that induces damage of D1 protein, the key component of PSII reaction center. Light induced damage of D1 is considered to be the primary site of photoinhibition (Foyer & Noctor, 2000). Usually, the decrease in density of active reaction centers of PSII (RC/CS_0) and photosynthetic efficiency (Fv/Fm, PI_{ABS}) were reported upon exposure of leaves to elevated irradiation (Cascio et al., 2010). In their comprehensive study Zivcak, Brestic, Kalaji and Govindjee (2014) discuss the possible protective role of low connectivity between PSII units in shade leaves in keeping the excitation pressure at lower, physiologically more acceptable level under upcoming high light stress. In our study, there was no significant difference in parameters of photosynthetic efficiency before drought treatment (C) (Figures 2B-D). This confirms that cultivation at elevated irradiation (EI) did not cause any photoinhibitory effects. Most of the plants have the ability to modify the density of chloroplasts and the amount of chlorophyll

during acclimatization to different light intensities, what directly affects photosynthetic efficiency (H. Wang, F. Wang, G. Wang, & Majourhat, 2007).

The reduction of chlorophyll content caused by the elevated irradiation was recognized as a protective mechanism to avoid photoinhibition (Lichtenthaler & Burkhart, 1999). Accordingly, significant reduction of total chlorophyll content (Chl *a*+*b*) was observed in seedlings cultivated at EI, compared to LI (Figure 2A). Similar relationship has been described in sun and shade leaves of different species (Sarijeva, Knapp, & Lichtenthaler, 2007; Yang et al., 2008). The total chlorophylls concentration (Chl *a*+*b*) was significantly decreased by SS and remained unchanged during the ES in comparison to C in leaves cultivated at LI (Figure 2A). In plants cultivated at EI (Figure 2A), significant decrease of Chl *a*+*b* was observed during both SS and ES treatments, compared to control. In lot of papers drought stress was shown to be highly related with reduction of chlorophyll content, as a result of slow synthesis or fast breakdown (Li, Guo, Baum, Grando, & Ceccarelli, 2006; Liu et al., 2011).

In this paper, the photosynthetic efficiency of seedlings subjected to different drought stress levels was estimated by measuring chlorophyll *a* fluorescence. Chlorophyll *a* fluorescence was shown to be useful tool for the study of photosynthetic efficiency in plants challenged by drought stress (Lepeduš et al., 2012; Narina et al., 2014; Oukarroum, El Madidi, Schansker, & Strasser, 2007). The maximum quantum yield of PSII (Fv/Fm), that describes the primary photochemistry of PSII and corresponds to the efficiency by which an absorbed photon will be trapped by PSII reaction centers (Maxwell & Johnson, 2000), was not affected in plants cultivated at LI regardless to the severity of drought (Figure 2C). The Fv/Fm values in stressed plants were high (0.78±0.01) as well as in controls (0.79±0.01), confirming a high stability of photochemical efficiency during different drought intensities at LI. For most plant species the maximum quantum yield of PSII (Fv/Fm) is 0.80 to 0.83 in optimal conditions (Schreiber, Bilger, & Neubauer, 1995). Values lower than 0.75 for Fv/Fm are considered to point a dysfunction of photosynthetic apparatus (Bolhár-Nordenkampf et al., 1989). The reports of drought tolerance in different barley cultivars showed only a slight effect of drought on Fv/Fm (Kocheva, Lambrev, Georgiev, Goltsev, & Karabaliev, 2004; Oukarroum et al., 2007), confirming it as not efficient indicator for drought stress. Here, the significant decrease of Fv/Fm was observed in plants cultivated at EI and subjected to ES (Figure 2C) showing less efficient photosynthetic apparatus. Performance index on absorption basis (PI_{ABS}), usually called vitality index, is much more sensitive parameter that can describe stress before visual symptoms (Christen, Schönmann, Jermini, Strasser, & Défago, 2007). This parameter takes into account other important processes besides primary photochemistry such as electron transport beyond primary plastoquinone (Q_A) and heat dissipation of the excess excitation energy (Strasser et al., 2004). PI_{ABS} was decreased strongly at SS and ES in plants cultivated at EI (Figure 2D). Extremely low value (0.31) in plants subjected to ES describes high rate of photosynthetic apparatus dysfunction in leaf seedlings what is in accordance with strongly decreased (RC/CS_0) (Figure 2B). Our results corroborate with these of van Heerden, Swanepoel, and Krüger (2007) who reported drought induced downregulation of PSII due to deactivation of PSII reaction centers in two scrub species.

Drought induced imbalance in chloroplast electron transport would stimulate ROS production (Cruz de Carvalho, 2008) that initiates the oxidative degradation of the membrane lipids (Gill & Tuteja, 2010). To estimate the oxidative damage of membrane lipids, the concentration of TBARS was determined. The absence of oxidative stress during cultivation at EI was confirmed since no difference between EI and LI was found in TBARS levels before the drought treatment (C) (Figure 3A). Although the exposure to drought treatments (SS and ES) induced the increase in TBARS content under the both types of cultivation (LI and EI) (Figure 3A), higher values of TBARS were recorded in EI grown plants. Initiation of lipid peroxidation usually starts by separating the hydrogen atoms of the polyunsaturated fatty acids (PUFA). Since the light was reported to increases PUFA de-saturation (Murphy & Stumpf, 1979) it is likely that EI grown plants would be more predisposed to lipid peroxidation membrane injury during further drought stress (SS and ES) than plants grown at LI. Induced increase of TBARS levels on SS and ES was accompanied by an increase in electrolyte leakage (Figures 3A-B). This was in accordance with previously reported drought stimulated electrolyte leakage from barley leaves due to cell membranes injury by increased lipid peroxidation level (Petrov, Kocheva, Petrova, & Georgiev, 2012). Although the SS caused increased lipid peroxidation in EI grown plants, compared to C as well as to plants grown at LI (Figure 3A), the electrolyte leakage level remained the same as in plants grown at LI (Figure 3B). Since the increased electrolyte leakage in plants grown at EI (compared to LI) was achieved only by ES (Figure 3B) it can be speculated that increased TBARS level at SS was located mainly in chloroplasts but not in cell membranes.

The accumulation of amino acid proline represents the beginning of plant acclimation to drought. It has been reported that the accumulation of compatible metabolites plays an important role during acclimatization in drought conditions, since they participate in osmoregulation and maintain the hydrated state of the proteins

(Hoekstra, Golovina, & Buitink, 2001). Also, it was suggested that the amino acid proline play a role in reducing harmful effects of ROS (Ben Rejeb et al., 2014). In our research, SS caused high accumulation of free proline content while ES caused enormous accumulation (Figure 3C) in seedlings grown under the both cultivation type (LI and EI), compared to C. Although the light was required for drought induced proline accumulation (Szabados & Savoure, 2009) the increased light level (EI) before drought treatment (C) did not affect proline-related response of barley plants exposed to drought later on (SS and ES) (Figure 3C).

Different enzymatic antioxidants were reported to prevent harmful effects of ROS (Gill & Tuteja, 2010). Here we investigated how the activity of main antioxidative enzymes (GPOX, CAT, APX and SOD) in leaves of barley plants grown under different irradiations (LI and EI) were modified by drought stress. The SOD activity showed oscillations through experiment (Figure 4D). In LI and EI grown plants SS caused inhibition of the enzyme activity, in comparison to C. During ES, activity increased at EI grown plants on the value like in C suggesting the enhanced H_2O_2 production upon extreme drought conditions in those plants. Although the SOD is considered as the one of important antioxidative enzymes catalyzing the dismutation of superoxide radical to less harmful H_2O_2 and molecular oxygen (Neill, Desikan, & Hancock, 2002) the conflicting responses of its activity to drought were reported in numerous publications (Cruz de Carvalho, 2008). Activities of GPOX, CAT and APX, generally, increased at SS and ES in both LI and EI grown plants (Figures 4A-C) compared to C. This indicates the occurrence of higher production of ROS, most likely H_2O_2 (Zlatev & Lidon, 2012). However, some differences in dynamics of enzymes activities were noticed during drought stress treatment. Unlike, APX, which activity increased equally in LI and EI grown plants during SS and ES (Figure 4B), GPOX and CAT revealed higher activities in plants grown at EI at the same stress levels (SS, ES) then LI grown plants (Figures 4A-C). The induction of certain GPOX and CAT isoforms by elevated light is well documented (Gechev et al., 2003; Luna et al., 2005). Guaiacol peroxidase (GPOX) participates in the removal of small amounts of H_2O_2, which is then used during the oxidation of various organic substrates. Also, higher activity of GPOX observed in plants grown at EI and affected by SS (compared with ones grown at LI) (Figure 4A), could be an additional source of ·OH (Passardi, Penel, & Dunand, 2004), which may be related to higher lipid peroxidation level (Figure 3A) at the same conditions. APX and CAT activity, as key enzymes responsible for the removal of high amounts of H_2O_2, were investigated in this study. H_2O_2 is small diffusible molecule that can be produced at most cell compartments by different enzymatic or non-enzymatic mediated reactions. H_2O_2 functions as a mobile intracellular signaling molecule which can activate or deactivate certain antioxidant enzymes (Gill & Tuteja, 2010; Neill et al., 2002). In plants cultivated at LI, the exposure to SS caused higher activation of APX (for 86.2%) than of CAT (for 33.5%) in respect to its values measured in C. Similar relation was showed in plants cultivated at EI (Figures 4B-C). These results were not surprising since it is well known that APX has a higher affinity for H_2O_2 in comparison to CAT (Cruz de Carvalho, 2008; Mittler, 2002). Furthermore, H_2O_2 was probably efficiently removed by APX directly at the places of its production in chloroplasts (Baier & Dietz, 2005), and a smaller amount of these molecules diffused to peroxisome, where CAT is positioned.

Combination of enzymatic and non-enzymatic measurements with chlorophyll *a* fluorescence parameters (particularly PI_{ABS}), obtained during early seedling growth stage represents the useful tool for screening drought tolerance in barley cultivars. Further investigations of these multiple parameters in different cultivars might be supportive for plant selection during breeding processes to create drought tolerant crops.

5. Conclusion

In summary, our results suggest that cultivation of barley seedlings under elevated light (EI) induced the adjustment of the photosynthetic apparatus in order to diminish photoinhibitory oxidative damages possible induced by EI. However, this acclimatization lowered the tolerance of EI grown seedlings to the following drought stress although it was applied under low irradiation (LI). Such response was in accordance with our hypothesis, since EI grown plants had reduced capability to cope with the challenge of upcoming drought stress showing more pronounced cellular oxidative damage (SS and ES), less efficient and almost dysfunctional photosynthetic apparatus in extreme drought (ES). So, our future investigation will be focused on exact biochemical and photochemical regulatory mechanisms of the described photosynthetic adjustment to EI and drought.

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Appendix

Appendix 1. Glossary and formulae of JIP-test parameters and expressions

Parameter	Description
	Data extracted from the recorded fluorescence transient
F_0	Minimal Fluorescence Intensity (All PSII Reaction Centers (RCs) Are Open)
F_m	Maximal Fluorescence Intensity (All PSII RCs Are Closed)
F_{300}	Fluorescence Intensity At 300 μs
F_I	Fluorescence Intensity At 2 ms (I Step)
F_J	Fluorescence Intensity At 30 ms (J Step)
F_V	Maximal Variable Fluorescence; $F_V=F_m–F_0$
t_{max}	Time Needed To Reach F_m
V_J	Relative Variable Fluorescence At J Step; $V_J=(F_J–F_0)/(F_m–F_0)$
V_I	Relative Variable Fluorescence At I Step; $V_I=(F_I–F_0)/(F_m–F_0)$
M_0	Initial Slope Of Relative Variable Fluorescence; $M_0=4(F_{300\mu s}–F_0)/(F_m–F_0)$
	Density Of Reaction Centres
RC/CS_0	Density Of Reaction Centres Per Excited Cross Section; $RC/CS_0=F_v/F_m\cdot(V_J/M_0)\cdot F_0$
	Flux Ratio Or Yield
F_v/F_m	Maximum Quantum Yield Of PSII; $F_v/F_m=[1–(F_0/F_m)]=TR_0/ABS$
	Specific Fluxes Per Active Reaction Center
ABS/RC	Absorption Per Active Reaction Centre; $ABS/RC=M_0\cdot(1/V_J)\cdot[1/(F_v/F_m)]$
TR_0/RC	Trapping Per Active Reaction Centre; $TR_0/RC=M_0\cdot(1/V_J)$
ET_0/RC	Electron Transport Per Active Reaction Centre: $ET_0/RC=M_0\cdot(1/V_J)\cdot(1–V_J)$
DI_0/RC	Dissipation Per Active Reaction Centre; $DI_0/RC=(ABS/RC)–(TR_0/RC)$
	Performance Index
PI_{ABS}	Performance Index On Absorption Basis; $PI=(RC/ABS)\cdot(TR_0/DI_0)\cdot[ET_0/(TR_0-ET_0)]$
RC/ABS	Density Of Reaction Centres On Chlorophyll *a* Basis;$RC/ABS=(RC/TR_0)\cdot(TR_0/ABS)=[(F_J–F_0)/4(F_{300\mu s}–F_0)]\cdot(F_v/F_m)$
TR_0/DI_0	Flux Ratio Trapping Per Dissipation; $TR_0/DI_0=F_v/F_0$
$ET_0/(TR_0-ET_0)$	Electron Transport Beyond QA^-; $ET_0/(TR_0–ET_0)=(F_m–F_J)/(F_J–F_0)$

Source: Strasser, R. J., Tsimilli-Michael, M., & Srivastava, A. (2004). Analysis of the chlorophyll a fluorescence transient. In G. C. Papageorgiou & Govindjee (Eds.), *Chlorophyll a fluorescence* (pp. 321-362). Netherlands, Springer.