Effect of exogenously-applied abscisic acid, putrescine and hydrogen peroxide on drought tolerance of barley

Ernest Skowron¹ · Magdalena Trojak¹

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Abstract

The objective of this study was to identify the effect of abscisic acid (ABA), putrescine (Put) and hydrogen peroxide (H₂O₂) foliar pre-treatment on drought tolerance of barley. Despite water limitation, ABA-sprayed plants preserved increased water content, photosynthetic efficiency of PSII (Φ PSII) and CO₂ assimilation rate (P_n) compared to untreated stressed plants. The ABA-treated plants presented also the lowest rate of lipid peroxidation (MDA), lowered the rate of PSII primary acceptor reduction ($1 - qP$) and increased the yield of regulated energy dissipation (NPQ) with higher accumulation of PGRL1 (PROTON GRADIENT REGULATION LIKE1) protein. These plants preserved a similar level of photochemical efficiency and the rate of electron transport of PSII (ETR_{II}) to the well-watered samples. The significantly less pronounced response was observed in Put-sprayed samples under drought. Additionally, the combined effects of drought and H₂O₂ application increased the $1 - qP$ and quantum yield of non-regulated energy dissipation in PSII (Φ NO) and reduced the accumulation of Rubisco activase (RCA). In conclusion, ABA foliar application allowed to balance water retention and preserve antioxidant capacity resulting in efficient photosynthesis and the restricted risk of oxidative damage under drought. Neither hydrogen peroxide nor putrescine has been able to ameliorate drought stress as effectively as ABA.

Keywords Abscisic acid · Drought stress · Hydrogen peroxide · Oxidative stress · Putrescine

Introduction

Water availability is a crucial factor limiting plant growth in natural arid and semi-arid areas (Evenari et al. 1976) as well as in agricultural regions, constantly expanded to meet the food needs of the growing population (Khush 2001; Mega et al. 2019). Repeatedly occurring episodes of water shortage during the growing season reduce plant growth, flowering, pollination, fertilization and grain development influencing quality and quantity of yield. Drought limits the cell division, reduces the size of the leaf blade, slows down or even inhibits shoot elongation and drastically reduces stomatal conductance, lim-

iting the uptake and transport of water, ions and gas exchange (Farooq et al. 2009). Reduced CO₂ diffusion to the site of carboxylation is the main cause of decreased photosynthesis under drought (Flexas et al. 2009). The water shortage also influences photosynthesis within reduced chlorophyll content and the oxygen-evolving complex (OEC) capacity (Chen et al. 2016). Moreover, thylakoid membranes are distorted as a consequence of the enhanced production of deleterious reactive oxygen species (ROS) (Cruz de Carvalho 2008). Decreased stomatal conductance and disturbed linear electron flow inhibits Calvin cycle activation, resulting in overreduction of stroma NADP⁺ pool and higher leakage of electrons to O₂ via Mehler reaction (Tamburino et al. 2017). This mechanism is responsible for the superoxide radical production, scavenged to hydrogen peroxide via Cu/Zn superoxide dismutase (Cu/Zn SOD). At the same time, when chloroplastic CO₂ concentration is decreased under drought, the photorespiration becomes predominant in H₂O₂ generation, perturbing the redox regulatory state of the plant cells and antioxidant pools (Laxa et al. 2019). Enzyme catalase (CAT), essential for the conversion of H₂O₂ to water and oxygen, exerts most of its

✉ Ernest Skowron
skowron.ernest@gmail.com

Magdalena Trojak
magdalenamariatrojak@gmail.com

¹ Department of Environmental Biology, The Jan Kochanowski University, Uniwersytecka 7, 25-406 Kielce, Poland

activity in the peroxisomes, where it counteracts H_2O_2 generation during photorespiration (Melandri et al. 2020). It has been documented (Alexander et al. 2019) that higher activity of CAT under stress conditions is associated with increased drought tolerance of the plant. Although the accumulation of ROS is generally harmful to cells (Czarnocka and Karpiński 2018), under moderate leaf-water deficits, ROS production is tightly regulated by antioxidant enzymes and non-enzymatic scavengers, thus ROS functions predominantly as the alarm signals that trigger defense response (Cruz de Carvalho 2008). However, prolonged drought stress accompanied by reduction of water content by 35–80% leads to depletion of photosystem II (PSII) core proteins (Giardi et al. 1996) and degradation of Lhcb5, Lhcb6, and PsbQ proteins.

Plants have a variety of physiological and biochemical responses to drought, among them phytohormones such as abscisic acid. ABA plays a role in the plant water status regulation by managing guard cell turgor, plant growth, the expression of dehydration response genes (Zhang et al. 2006), and the accumulation of osmotic compounds such as proline (Pro), polyamines (PAs) and soluble sugars (Sezgin et al. 2018). The hormone activates antioxidant enzymes e.g., SOD and CAT, and attenuates the rate of membrane lipids peroxidation (Li et al. 2011). The earliest plant response to water deficit is also the rapid stomatal closure regulated by a complex network of signaling pathways with ABA and jasmonates. The ABA has traditionally been considered as a major stomatal movement regulator, especially under drought (Daszkowska-Golec and Szarejko 2013). Drought-induced stomatal closure, regulated by the ABA signal, serves as the first line of defense against drought (Li et al. 2020) and controls the rates of water loss and CO_2 intake (Cardoso et al. 2020). Therefore, exogenously applied ABA has been successfully used as an effective regulator of drought-stress response (Brito et al. 2020; Gai et al. 2020). For this reason, we analyzed the basis of ABA-improved drought resistance in barley.

Moreover, as H_2O_2 is the key molecule regulating ABA-induced stomatal closure (Desikan et al. 2004), the influence of its application under drought has been also tested in the present study. The exogenous H_2O_2 application has been successfully employed to induce stomatal closure (Sun et al. 2016) and accumulation of molecules, such as Pro and PAs (Terzi et al. 2014) improving drought tolerance in plants (de Sousa et al. 2016). PAs, including tested putrescine (Put) spermidine and spermine (Mattoo et al. 2010), are small, ubiquitous nitrogenous compounds (Gupta et al. 2012), and regulate the concentration of ABA (Cuevas et al. 2008) and contrariwise - ABA modulates the expression of PAs-biosynthetic genes and their accumulation in response to drought stress (Pottosin and Shabala 2014). Increased PAs concentration is important for the hydraulic conductivity of guard-cell (Alcázar et al. 2010) and has a positive effect on

photosynthetic efficiency due to membrane-stabilizing abilities and antioxidant properties of PAs (Pottosin and Shabala 2014).

Alleviation of drought stress in crops such as analyzed barley is crucial because stress triggers the premature onset of leaf senescence (Janack et al. 2016). The prolonged lifespan of leaves is particularly important for cereals' flag leaf contributing to grain filling (Lee et al. 2017). Barley is an economically important plant that ranks fourth among cereals. It is preferentially used as a feed grain, as raw material for brewing and as food (Sreenivasulu et al. 2008). However, as barley is grown worldwide is often subjected to extreme drought stress that significantly affects production (Guo et al. 2009). It has been previously documented that spring forms of barley, despite their major role in agriculture due to short vegetation period, suffer from soil water limitation as a result of the poorly developed root system, that makes them far more sensitive to drought episodes than winter ones (Pecio and Wach 2015). Thus, for drought-response analyses spring cultivar Carina of barley has been chosen as sensitive to water dehydration.

The objective of the present study was the effect of abscisic acid, putrescine and hydrogen peroxide foliar pre-treatment in barley exposed to the water shortage. Efforts have also been made to know that which one acts more efficiently to improve drought tolerance. For this purpose, we tested the hypothesis that applied regulators alleviate drought stress through (1) higher water retention realized by stomatal closure and increased osmoprotectants accumulation, (2) protection of photosynthetic apparatus with an altered level of proteins engaged in cyclic electron flow (CEF) around photosystem I (PSI) and NPQ activation and (3) increased antioxidant capacity. To this end, we analyzed water retention (RWC) and proline accumulation, the photosynthetic machinery condition (chlorophylls content, PSII photochemical and non-photochemical activity, PsbO protein level), gas exchange (net photosynthetic rate- P_n and stomatal conductance- g_s), Rubisco LSU and Rubisco activase (RCA) contents, as well as the level of PGRL1 (PROTON GRADIENT REGULATION LIKE1) and NdhS protein (subunit S of the chloroplast NADH dehydrogenase-like complex), involved in the alternative routes of electron transport from PSI and the antioxidant defense of plants during drought (SOD, CAT, H_2O_2 , MDA).

Materials and methods

Plant material and growth conditions

The experiments were carried out on the spring barley cultivar (*Hordeum vulgare* L. cv. Carina), selected as a drought-sensitive compared to drought-tolerant winter form Lomerit (Supplementary Material Figs. S1, S2; Table S1). Sterilized

seeds were sown individually in plastic pots (volume $\approx 0.5 \text{ dm}^3$) containing a mixture of soil and sand (2 : 1, v/v, pH 6.0–6.5) and 1 g compound fertilizer (N : P_2O_5 : K_2O = 5 : 2 : 6) added to each pot as base fertilizer. The seedlings were grown in chambers under the uniform white light with a maximum intensity of $130 \mu\text{mol m}^{-2} \text{ s}^{-1}$. The photoperiod was 16/8 h (day/night) and the average temperature was maintained at 26/25 °C with a relative humidity kept at 50–60%. The plants were watered every day with tap water until the drought. Measurement of physiological and biochemical parameters was performed using the third fully expanded leaf.

Experimental systems and treatments used

Drought stress of the 30-day-old plants was imposed by the cessation of watering for seven consecutive days. The plants were divided into five groups and treated as follows: (1) the control plants (CT) continually irrigated with tap water, sprayed with distilled water; (2) drought-stress treatment (D), sprayed with distilled water; (3) the plants subjected to drought stress with 100 μM ABA applied as a foliar spray (D + ABA); (4) the plants subjected to drought stress with 5 mM H_2O_2 applied as a foliar spray (D + H_2O_2); (5) the plants subjected to drought stress with 100 μM Put applied as a foliar spray (D + Put). The optimum concentration of the applied solutions was based on our earlier preliminary experiment. All plants were sprayed twice - on the first and the fourth day of the experiment, each time with 5 ml of water/proper compound per plant. The application scheme proposed was selected to induce and sustain plant acclimation to drought stress, therefore compounds were foliar applied just before and during the water limitation. The time of the second treatment was selected based on the analyzed soil water content when it dropped below 70% of the initial value (data not shown).

Abscissic acid [($\pm$)-ABA] (Sigma-Aldrich, St. Louis, USA) solutions were prepared by dissolving the stock solution of ABA in methanol (40 mg/1 ml) in distilled water. Putrescine (1,4-diaminobutane) (Sigma-Aldrich) solutions were prepared in distilled water. Hydrogen peroxide (Sigma-Aldrich) solutions were prepared by dissolving the 30% stock solution in distilled water. Solutions were immediately foliage-applied with a hand sprayer. Each foliar treatment consisted of an additional 0.01% (v/v) Tween-20 (Bio-Rad, Hercules, USA) as a surfactant and 0.067% (v/v) methanol solution. The compounds were applied at the 14th growth stage of the Zadoks decimal code (Zadoks et al. 1974). Therefore, imposed drought stress mimicked this triggered repeatedly by environmental cues during the seedling early growth stage.

Determination of soil volumetric water contents and leaf relative water content

Changes in the soil moisture during the study were tracked by the Moisture Meter Type HH2 with a ThetaProbe Soil Moisture Sensor-ML2x (Delta-T Devices Ltd., Cambridge, UK), each time placing the probe to a depth of 5 cm. The soil volumetric water contents (SVWC) before the drought was maintained at approximately 26%, corresponding to soil water content at 80% of field capacity (FC). FC of soil was calculated on a weight basis. During drought control plants were irrigated to FC (26% SVWC), and water-deficit plants were not irrigated throughout the experiment. Leaf water status was determined by measuring relative water content (RWC), using 1 cm leaf fragments cut out from the middle of the blade and weighted for fresh weight (FW) determination. Samples were collected from 8:00–10:00 h. To assess turgid weight (TW), fresh leaf fragments were incubated in distilled water for 24 h at 4 °C, weighted and then dried for the next 48 h at 90 °C to determine the dry weight (DW). RWC was calculated as follows (Ishibashi et al. 2011):

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

Analyses were performed in ten replicates for each treatment.

Estimation of proline

The concentration of free proline was determined as described by Giannakoula et al. (2008), with an optimized acid-ninhydrin reaction step proposed by Shabnam et al. (2016). 0.5 g of fresh leaf material was homogenized in 5 ml of 80% (v/v) ethanol, transferred into glass vials and heated at 60 °C for 30 min. The extract was then filtered through Whatman No. 2 filter paper, diluted with 80% (v/v) ethanol up to 10 ml, sealed and mixed thoroughly. Next, 1 ml of the aqueous alcohol extract was transferred into test tubes and 2 ml of reaction mix: 1.25% (w/v) ninhydrin, 60% (v/v) glacial acetic acid, 20% (v/v) ethanol, was added. The tubes were mixed, pierced with the lids and placed in a water bath at 100 °C for 30 min. The reaction mixture was cooled to room temperature (RT) and the absorbance of the proline-ninhydrin condensation product was determined spectrophotometrically (UV VIS Helios Gamma, Thermo Spectronic, Waltham, USA) at 508 nm. For quantification, a calibration curve was obtained using proline standard solutions prepared in 80% (v/v) ethanol added to the reaction mix. Data were calculated as $\mu\text{moles per gram dry weight}$ ($\mu\text{mol g}^{-1} \text{ DW}$) as proposed by Sarker and Oba (2018). The assay was performed in ten replicates for each treatment.

Determination of photosynthetic pigments content and chlorophyll stability index

The collected leaves were ground to a fine powder in liquid nitrogen and chlorophyll *a*, *b* (Chl *a*, *b*) and carotenoids were extracted with dimethyl sulfoxide (DMSO) in a ratio of 1.5 ml DMSO per 10 mg of plant tissue. The samples were incubated for 3 h at 65 °C and the absorbance was determined at 480, 649 and 665 nm, according to the method described by Wellburn (1994). The chlorophyll stability index (CSI) was determined according to Terzi and Kadioglu (2006) and calculated as follows:

$$\text{CSI} = (\text{total Chl } a + b \text{ after treatment} / \text{total Chl } a + b \text{ before treatment}) \times 100$$

Data were calculated as mg per gram dry weight (mg g⁻¹ DW). The assay was performed in ten replicates for each treatment.

Measurement of chlorophyll fluorescence (ChF) induction kinetics

ChF induction kinetics of control and drought-stressed barley leaves was performed using pulse amplitude modulated (PAM) fluorometer (Maxi IMAGING-PAM M-Series, Walz, Effeltrich, Germany). The minimal fluorescence level (*F*₀) with all PSII reaction centers open was measured by the measuring modulated blue light ($\lambda = 450$ nm), which was sufficiently low (0.01 $\mu\text{mol m}^{-2} \text{s}^{-1}$) not to induce any significant variable fluorescence. The maximal fluorescence level (*F*_m) with all PSII reaction centers closed was determined by a 0.8 s saturating pulse at 2700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in 30 min dark-adapted leaves. Then, the leaf was continuously illuminated with blue actinic light (186 $\mu\text{mol m}^{-2} \text{s}^{-1}$). After about 4 min, the steady-state value of fluorescence (*F*_s) was thereafter recorded and a second saturating pulse at 2700 $\mu\text{mol m}^{-2} \text{s}^{-1}$ was imposed to determine maximal fluorescence level in the light-adapted state (*F*_m'). The maximum quantum yield of PSII (*F*_v/*f*_m), actual photochemical efficiency of PSII (ΦPSII), the quantum yield of regulated energy dissipation in PSII (ΦNPQ) and non-regulated energy dissipation in PSII (ΦNO), non-photochemical quenching (NPQ) and the electron transport rate of PSII (*ETR*_{II}) were measured. Chlorophyll fluorescence decrease ratio (*R*_{Fd}) defined as ChF decrease from maximum (*F*_m) to steady-state fluorescence (*F*_s) was estimated in 3–5 min under continuous saturating light (396 $\mu\text{mol m}^{-2} \text{s}^{-1}$) (based on light response curve, data not shown) according to Lichtenthaler et al. (2005) as follows:

$$R_{Fd} = (F_m - F_s) / F_s$$

The excitation pressure on PSII ($1 - qP$) was estimated as has been shown by Dahal et al. (2017). The electron transport rate of PSII (*ETR*_{II}) was calculated based on the measured ΦPSII , the incident photon flux density of blue actinic light (*PAR*_{AL}) and the absorptivity (*Abs.*), defined as the fraction of the incident light absorbed by the leaf sample first illuminated with red (*R*) and then with near-infrared (*NIR*).

$$\text{ETR}_{II} (\mu\text{mol electrons m}^{-2} \text{s}^{-1}) = 0.5 \times \Phi\text{PSII} \times \text{PAR}_{AL} \times \text{Abs.} \\ \text{Abs.} = 1 - R/NIR$$

All measurements were carried out on the third fully expanded leaves and were performed between 8:00–11:00. Analyses of ChF were performed in six replicates for each treatment.

Leaf gas-exchange measurements

The net photosynthetic rate (*P*_n) and stomatal conductance (*g*_s) of leaves were monitored from 8:30–11:30 h using a Li 6400XT Portable Photosynthesis System (LI-COR, Inc., Lincoln, USA) with 2 × 3 cm LED light source 6400-02B chamber. Cuvette conditions were maintained at a growth-like light intensity of 130 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, air relative humidity of 60%, leaf temperature of 25 °C, 400 $\mu\text{mol mol}^{-1}$ of external CO₂ concentration and gas flow rate of 500 ± 2 $\mu\text{mol s}^{-1}$. Gas-exchange measurements on the first (Day 1) and fourth day (Day 4) were determined 4 h after water/solution application. Analyses were performed in ten replicates for each treatment.

Leaf protein extraction and preparation. The analysis of Rubisco large subunit accumulation

Leaf soluble proteins were extracted with Plant Total Protein Extraction Kit (Sigma-Aldrich) according to the manufacturer's instructions, gel-resolved and stained with Coomassie Brilliant Blue (CBB) for quantifying the amount of Rubisco large subunit (LSU) or electrophoretically transferred to nitrocellulose membranes for Western Blot (WB) analyses as proposed by Martin et al. (2005). In brief, 200 mg of the leaf powder was washed with methanol working solution and acetone, protected from proteolysis with protease inhibitor cocktail. Purified tissue pellet has been used for total protein extraction with chaotropic protein reagent. Protein content was estimated according to Bradford (1976) using Coomassie reagent (ThermoFisher, Waltham, USA) and bovine serum albumin as a standard. Samples mixed with 2X Laemmli Sample Buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8) (Bio-Rad) were incubated at 95 °C for 5 min, cooled down to RT, spun down shortly, loaded and separated onto gels. For PGRL1 analysis sample buffer without a reducing

agent has been used and protein samples were incubated at 70 °C for 10 min as proposed by Hertle et al. (2013).

For CBB staining protein content was standardized to 20 µg per lane and loaded onto precast 10% TGX polyacrylamide gels (Bio-Rad). Four replicates of each treatment were analyzed. Gels were stained with Bio-Safe™ Coomassie Stain (Bio-Rad). The quantification of the protein bands of the CBB stained gels (for Rubisco LSU) was made using densitometric analysis (ImageJ v.1.52, National Institutes of Health, Maryland, USA) (Tercé-Laforgue et al. 2004). The relative amount of Rubisco LSU protein was calculated using as a maximum the value measured in CT plants.

Western blot analysis of photosynthetic proteins

Optimized amounts of extracted proteins were loaded onto precast 4–20% gradient TGX polyacrylamide gels (Bio-Rad) and run with a constant voltage of 200 V for 20 min. Separated proteins were transferred to nitrocellulose membranes (0.45 µm or 0.2 µm pore size; Bio-Rad) by semi-dry electroblotting (1.5 mA per cm², 20 min). Air-dried blots were blocked with 5% non-fat dry milk blocking reagent (1 h, RT) (Bio-Rad) and incubated with primary antibodies against RCA (AS10 700; 1:5000), PsbO (AS06 142–33; 1:5000), PGRL1 (AS10 725; 1:1000), NdhS (AS16 4066; 1:1000) and eEF1a (Elongation factor 1-alpha; AS10 934; 1:5000) (Agrisera, Vännäs, Sweden) overnight at 4 °C, washed in Tween-TBS buffer (TTBS; 0.05% Tween 20, 20 mM Tris, 500 mM NaCl) and incubated with a horseradish peroxidase-conjugated secondary antibody (AS09 602; 1:5000–1:10000) for 1 h at RT with agitation. The blots were washed in TTBS and developed for 5–10 min with colorimetric detection using Pierce™ DAB Substrate Kit (3,3'-diaminobenzidine tetrahydrochloride) (Thermo Fisher Scientific, Waltham, USA). The quantification of the protein bands of the WB membranes visualized with DAB was made using densitometric analysis (ImageJ v.1.52) (Tercé-Laforgue et al. 2004). RCA signal was estimated from both α - and β -isoform bands visualized on the membrane, as an overall RCA content (RCA_{total}). PGRL1 signal was estimated either as an overall protein content (PGRL1_{total}) or as the ratio of individual signal for reduced (PGRL1_{red}) and oxidized (PGRL1_{ox}) monomer. To investigate RCA, PsbO and eEF1a 5 µg, and for PGRL1 and NdhS 25 µg of total protein was separated on gels. eEF1a was used as a loading control. The samples were analyzed in threefold. The relative amount of proteins was calculated using as a maximum the value measured in CT plants.

Determination of antioxidant enzyme activities

The activity of SOD (EC 1.15.1.1) was determined using the modified method of Xu et al. (2008). 200 mg of leaf powder was homogenized with 1 ml of an ice-cold 100 mM phosphate

buffer (pH 7.8) containing 1 mM EDTA (pH 8.0) and 5% (w/v) polyvinylpyrrolidone (PVPP). The homogenates were centrifuged at 10,000 × g for 30 min, 4 °C. Then 40 µl of each enzyme extract was mixed with 100 µl of 0.75 mM nitroblue tetrazolium (NBT), 2 µl of 0.5 M EDTA (pH 8.0) and 20 µl of 0.1 mM riboflavin. Because of the photosensitivity of the solution, the procedure was carried out under low light. The samples containing the reaction solution were irradiated under a set of fluorescent light tubes of 40 µmol m⁻² s⁻¹ for 10 min. The absorbance of the irradiated and non-irradiated samples was determined at 560 nm. One unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50% in comparison with tubes lacking the enzyme (Sarker and Oba 2018). The assay was performed in six replicates for each treatment.

The activity of CAT (EC 1.11.1.6) was determined according to the method of Aebi (1984) with some modifications (Lu et al. 2013). In brief, 20 µl of enzyme extract was added to 405 µl of 50 mM potassium phosphate buffer (pH = 7.0) and 250 µl of water. After 5 min pre-incubation at 26 °C 750 µl of 10 mM H₂O₂ was added to start the reaction. The decomposition of H₂O₂ was followed directly by the decrease in absorbance at 240 nm (GENESYS 10S UV-Vis, Thermo Scientific Waltham, USA). Enzyme activity was computed by calculating the amount of H₂O₂ decomposed, every 20 s for 3 min at 26 °C. Calculations used an absorbance coefficient of 43.6 M⁻¹ cm⁻¹ at 240 nm (Pilarska et al. 2016). The assay was performed in six replicates for each treatment.

Determination of endogenous hydrogen peroxide level

The endogenous H₂O₂ content was assessed as has been proposed by Barreto et al. (2017). Approximately 150 mg of collected leaf tissue was frozen immediately in liquid nitrogen and ground into a fine powder. The powder was directly homogenized with 1 ml of a solution containing 250 µl of 0.1% (w/v) trichloroacetic acid (TCA), 500 µl of 1 M KI and 250 µl of 10 mM potassium phosphate buffer (pH 7.0) for 10 min, 4 °C in darkness. The samples were centrifuged at 12,000 × g for 15 min, 4 °C. 200 µl of each supernatant was transferred to a new tube and left to incubate without light access at RT for 20 min. Then samples were diluted 10-fold with distilled water and absorbance was measured at 350 nm. For quantification, a calibration curve was obtained using H₂O₂ standard solutions prepared in 0.1% TCA. Data were calculated as µmoles per gram dry weight (µmol g⁻¹ DW). The assay was performed in six replicates for each treatment.

Measurements of leaf lipid peroxidation

The level of oxidative damage to membranes under drought was estimated indirectly with an assessment of

byproducts of lipid peroxidation reacted with thiobarbituric acid (TBA) and among them malondialdehyde (MDA) content. The assay was in accordance with the procedure of Xu et al. (2008). 100 mg of leaf powder was homogenized in 1 ml of 10% (w/v) TCA and centrifuged at $10,000 \times g$ for 15 min. 1 ml of supernatant was mixed with 1 ml of 0.6% (w/v) TBA, heated at 95 °C for 30 min and then quickly cooled down on the ice to RT. After centrifugation at $10,000 \times g$ for 10 min, the absorbance (A) of the 10-fold diluted supernatants was measured at 532 nm and values corresponding to non-specific absorption at 450 nm and a correction factor for non-specific turbidity at 600 nm. The MDA concentration determined on a fresh weight basis was calculated according to Ying et al. (2015) with the following formula:

$$\text{MDA } (\mu\text{mol g}^{-1}\text{FW}) = 6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$$

Then data were calculated as $\mu\text{moles per gram dry weight}$ ($\mu\text{mol g}^{-1}\text{DW}$). The assay was performed in six replicates for each treatment.

Statistical analysis

The normal distribution of variables was verified with Shapiro-Wilk's test and the equality of variances was evaluated using Levene's test. The one-way ANOVA and post-hoc Tukey's HSD test were employed to analyze differences between groups. The data are presented as the mean with standard deviation ($\pm$ SD). Statistical significance was determined at the $\alpha = 0.05$ level. Statistical analyses were performed using STATISTICA 12.0 statistical software (StatSoft Inc., Oklahoma, USA).

Results

The effect of soil water availability on leaf relative water content

The SVWC in the CT treatment was maintained at approximately 26% throughout the experiment, corresponding to soil water content at 80% of FC, while in all dehydrated plants was markedly decreased to approximately 12–15% SVWC (37–46% of FC) on the last day of water deprivation, suggesting that the water deficiency in the samples was the same (Table 1). The leaf relative water content decreased under drought period (Table 1) more than 70% in the non-sprayed plants (D) compared to CT while in ABA-, H_2O_2 - and Put-treated plants, was reduced by 55, 66 and 53%, respectively.

Accumulation of proline in response to drought

Drought significantly induced accumulation of free proline as we noted nearly 1.7-times higher accumulation of this osmoprotectant in D plants compared to CT (Table 1). Among applied regulators only the Put significantly enhanced further Pro accumulation under drought to the nearly 2.5-times higher level than in CT. Neither the ABA nor H_2O_2 influenced the Pro level in the way similar to Put. Consequently, in the ABA treatment, the Pro level was statistically indistinguishable from D samples or its accumulation was even lower with H_2O_2 treatment.

Photosynthetic pigments accumulation in response to dehydration

In CT plants the Chl *a* + *b* content was kept at $15.44 \text{ mg g}^{-1}\text{DW}$ and fell about 76% in response to drought (Table 1). After the application of ABA and Put Chl *a* + *b* declined by 70 and 63%, respectively compared to the CT. H_2O_2 -treated plants showed about 80% decrease Chl *a* + *b* level as compared with the CT and 18% reduction as compared with the D, respectively. Although the analyzed plants showed different Chl *a* + *b* content, the chlorophyll *a/b* ratio remained unaffected. After foliar application of ABA and Put chlorophyll stability index decreased by 70 and 63%, respectively. The strongest decrease – 80 and 76% was recorded for H_2O_2 and D samples, respectively. Carotenoid content also decreased by 72% in D and by 68, 77 and 59% with ABA, H_2O_2 and Put treatment, respectively.

The effects of drought and growth regulators application on the photosynthetic efficiency of PSII

The maximum quantum efficiency of PSII was practically unaltered under drought, except for H_2O_2 application resulting in slightly decreased F_v/f_m (Fig. 1a). The analyzed distribution of absorbed energy showed, however, that drought decreased ΦPSII (Fig. 1b) by approximately 10% with D and H_2O_2 and by 5% with Put. At the same time, the ABA application allowed to maintain the control-like value of ΦPSII . Despite the chlorophyll-loss and membrane damage the photosystems maintained in intact chloroplast thylakoids were still active. Such findings have been proved with the analyzed electron transport rate of PSII (Fig. 1d). Water-stress inhibited ETR_{II} by 17, 18 and 7% in D, H_2O_2 and Put samples, respectively. For the ABA-sprayed plants, the unaltered ETR_{II} value was noted. Estimated R_{Fd} was markedly decreased under drought (Fig. 1c) to 70, 81, 62 and 72% of CT value in D, ABA, H_2O_2 and Put treatment, respectively. In contrast to ΦPSII or ETR_{II} , R_{Fd} measurement was carried out with higher photon densities saturating the photosynthetic quantum

Table 1 Soil volumetric water contents (SVWC); leaf relative water content (RWC); chlorophyll *a*, *b*: content (Chl *a* + *b*), ratio (Chl *a*/*b*) and stability index (CSI); carotenoids (pool) and proline level of barley leaves under non-limiting soil water condition and drought

Treatments	SVWC (% v/v)	RWC (%)	Chl <i>a</i> + <i>b</i> (mg g ⁻¹ DW)	Chl <i>a</i> / <i>b</i>	CSI (%)	carotenoids (mg g ⁻¹ DW)	proline (μmol g ⁻¹ DW)
CT	26.3 ± 1.22 ^a	85.7 ± 3.59 ^a	15.44 ± 0.72 ^a	3.89 ± 0.02 ^a	97.5 ± 4.73 ^a	2.62 ± 0.18 ^a	9.12 ± 1.98 ^d
D	12.5 ± 2.36 ^b	25.1 ± 1.53 ^c	3.77 ± 0.05 ^d	3.77 ± 0.31 ^a	23.7 ± 0.30 ^d	0.74 ± 0.10 ^d	15.83 ± 1.79 ^b
D + ABA	14.5 ± 2.35 ^b	38.3 ± 1.15 ^b	4.66 ± 0.13 ^c	3.81 ± 0.19 ^a	29.6 ± 0.97 ^c	0.85 ± 0.04 ^c	17.16 ± 2.02 ^b
D + H ₂ O ₂	11.9 ± 3.00 ^b	28.9 ± 1.06 ^c	3.10 ± 0.15 ^e	3.52 ± 0.73 ^a	19.6 ± 1.10 ^e	0.59 ± 0.04 ^e	13.26 ± 1.81 ^c
D + Put	11.7 ± 2.21 ^b	40.4 ± 4.00 ^b	5.67 ± 0.09 ^b	3.33 ± 0.49 ^a	36.0 ± 0.71 ^b	1.08 ± 0.05 ^b	22.23 ± 2.20 ^a

Data are shown as means ± SD (*n* = 10). The parameters were measured on the third fully expanded leaf. Different superscript letters in the same column indicate significant differences according to Tukey's HSD test (α = 0.05). CT states for the control plants with maintained irrigation. The rest of the plants were exposed to 7-days dehydration without (D) or with foliar application of 100 μM abscisic acid (D + ABA), 5 mM hydrogen peroxide (D + H₂O₂) and 100 μM putrescine (D + Put). DW – dry weight. Data present values of the last day of the experiment

conversion of nearly all leaf chloroplasts, thereby exposing the actual effects of drought on CO₂ assimilation.

Drought-induced electron transfer limitation and energy quenching

Drought stress increased 1 – qP significantly as it was 2-, 1.46-, 2.24- and 1.91-fold higher for D, ABA, H₂O₂ and Put treatment, respectively (Fig. 2a). Only the ABA-sprayed plants showed markedly reduced 1 – qP under drought and consequently CT-like values of Fv/fm, ΦPSII, and ETR_{II}. A comparison of NPQ (Fig. 2b) or ΦNPQ (Fig. 2c) with ΦNO

values (Fig. 2d) clearly showed that under drought non-photochemical energy dissipation was realized predominantly in a passive manner. Analyzed distribution of absorbed energy showed that water limitation decreased the effectiveness of safety energy dissipation. D plants presented nearly 12% lower ΦNPQ and 29% higher ΦNO than CT, while ABA- and Put-sprayed plants showed 45 and 6% higher ΦNPQ value than CT, and 65 and 21% increased ΦNPQ compared to D plants, respectively. At the same time, H₂O₂ application downregulated ΦNPQ by approximately 23 and 13% compared to CT and D, respectively. Analyzed ΦNO showed that only ABA application allowed reducing this parameter,

Fig. 1 Effects of exogenous growth regulators application on a) Fv/fm, the maximum quantum yield of PSII, b) ΦPSII, actual photochemical efficiency of PSII, c) R_{fd}, the chlorophyll fluorescence decrease ratio, d) ETR_{II}, the electron transport rate of PSII in leaves of barley cv. Carina plants. The parameters were measured on the third fully expanded leaf on the last day of the experiment. Data are shown as

means ± SD (*n* = 6). Different letters indicate significant differences according to Tukey's HSD test (α = 0.05). CT states for control plants with maintained irrigation. The rest of the plants were exposed to 7-days dehydration without (D) or with foliar application of 100 μM abscisic acid (D + ABA), 5 mM hydrogen peroxide (D + H₂O₂) and 100 μM putrescine (D + Put)

Fig. 2 Effects of exogenous growth regulators application on a) $1 - qP$, excitation pressure on PSII, b) NPQ, non-photochemical quenching, c) Φ_{NPQ} , quantum yield of regulated energy dissipation in PSII, d) Φ_{NO} , quantum yield of non-regulated energy dissipation in PSII in leaves of barley cv. Carina plants. The parameters were measured on the third fully expanded leaf on the last day of the experiment. Data are shown as means

$\pm$ SD ($n = 6$). Different letters indicate significant differences according to Tukey's HSD test ($\alpha = 0.05$). CT states for control plants with maintained irrigation. The rest of the plants were exposed to 7-days dehydration without (D) or with foliar application of 100 μ M abscisic acid (D + ABA), 5 mM hydrogen peroxide (D + H₂O₂) and 100 μ M putrescine (D + Put)

presumably as a result of substantially increased Φ_{NPQ} . Increased energy dissipation by the heat of ABA-sprayed samples was also documented with NPQ, as it reached 56% higher level than in CT. H₂O₂- and Put-sprayed plants preserved CT-like NPQ levels, while a significant decrease of NPQ was observed in D samples.

The effects of drought and growth regulators application on the leaf gas-exchange

Compared with the CT, 7 days of water shortage in D and D + H₂O₂ plants decreased P_n by 64 and 75%, respectively (Fig. 3a). Application of 100 μ M Put did not improve the assimilation rate measured on the last day of drought as well. In contrast, exogenously applied ABA increased P_n by over 50% compared with the D sample. The influence of applied plant growth regulators (PGRs) has been also monitored shortly after application. We noted that all applied PGRs decreased the P_n rate after application by 28, 15 and 19% with ABA, H₂O₂ and Put, respectively. The decreased CO₂ assimilation rates resulted from the reduced stomatal conductance (Fig. 3b), however, the magnitude of the parameters decrease was not correlated as documented for the D + ABA sample. ABA has been the most effective in rapid stomatal closure and decreased g_s by nearly 70% shortly after treatment (Day 1). At the same time, for the D, H₂O₂- and Put-treated plants approximately 5, 13 and 31% lower g_s were noted, compared to CT

(Fig. 3b, Day 1). Throughout the experiment, decreasing RWC of leaves progressively reduced stomatal conductance (Fig. 3b, Day 4), slowing CO₂ assimilation (Fig. 3a, Day 4). Consistently, the g_s level of ABA-sprayed plants on the fourth day was the lowest, but the assimilation rate of the ABA sample remained quite stable. On the last day of drought leaf dehydration markedly reduced stomatal conductance by 67, 57, 76 and 70% for D, ABA, H₂O₂ and Put treatments, respectively (Fig. 3b). It should be noted, however, that for the ABA-samples g_s on Day 7 was slightly higher than measured on Day 1 or Day 4, due to lack of ABA-application. Thus, the decreased g_s on the last day was predominantly a result of progressive guard cell dehydration rather than the activity of previously applied ABA.

The effects of water shortage and growth regulators application on plant proteins accumulation

The abundance of the Rubisco LSU protein was decreased by 48% under drought (Fig. 3c). Exogenous application of ABA alleviated this adverse effect and plants maintained almost 90% of the CT level of the enzyme large subunit. In the Put- and H₂O₂-treated samples accumulation of Rubisco LSU was decreased by 23 and 39%, respectively.

Drought decreased total Rubisco activase (RCA_{total}) accumulation by 15, 6 and 24% with D, Put and H₂O₂-sample, respectively. However, an exception to this were plants-

Fig. 3 Effects of exogenous growth regulators application on **a**) P_n , net photosynthetic rate and **b**) g_s , stomatal conductance measurements on the first and fourth day, determined 4 h after water/solution application or on the last day of the experiment (Day 7), **c**) Rubisco large subunit (LSU) relative amount (%), beneath the LSU protein bands (53 kDa) of 20 μg protein resolved in a 10% TGX polyacrylamide gel and visualized with Coomassie Stain. The relative amounts of Rubisco LSU were normalized to CT. The parameters were measured on the third fully expanded leaf on the first, fourth and seventh (**a**, **b**) or the last (seventh) day of experiment

(**c**). Data are shown as means $\pm$ SD ($n = 10$ for **a**, **b** or $n = 4$ for **c**). Different letters indicate significant differences according to Tukey's HSD test ($\alpha = 0.05$). In the case of Fig. **a** and **b** different letters indicate significant differences among means of the same day. CT states for control plants with maintained irrigation. The rest of the plants were exposed to 7-days dehydration without (D) or with foliar application of 100 μM abscisic acid (D + ABA), 5 mM hydrogen peroxide (D + H₂O₂) and 100 μM putrescine (D + Put). DW – dry weight

sprayed with ABA, presenting approximately 6% higher protein level than the CT group (Figs. 4a, b). We did not analyze the differences in the accumulation of particular RCA isoform separately. It should be noted, however, that the level of β -isoform has been significantly higher than α in all the groups tested (Fig. 4a). The accumulation of PsbO protein of the OEC complex also decreased by an average of 10% in D, Put- and ABA-sprayed samples and by 17% in plants sprayed with H₂O₂ (Figs. 4a, c).

As protein samples for PGRL1 were prepared under non-reducing conditions and resolved with gradient gel, two distinct signals: 24 and 29 kDa for PGRL1 were noted (Fig. 4a), corresponding to oxidized (PGRL1_{ox}) and reduced (PGRL1_{red}) PGRL1 monomers, respectively. No additional signal for PGRL1-PGR5 heterodimers was indicated (Fig. 4a). The ABA-sprayed plants showed 2.5-times higher PGRL1_{total} protein level compared to CT, and nearly 4-times higher than non-sprayed plants exposed to drought (D) (Fig. 4d). A comparison of PGRL1_{total} protein accumulation between D and H₂O₂-sprayed plants,

clearly demonstrated that the negative effect of water-stress on PGRL1_{total} protein level was further aggravated with H₂O₂ application. Moreover, the fraction of PGRL1 monomers in the reduced state increased from approximately 60% in the CT to 70% in D and H₂O₂-sprayed plants. The lowest PGRL1_{red}/PGRL1_{ox} ratio was noted for ABA-sprayed plants (Fig. 4f). At the same time, NdhS protein was significantly increased in D and H₂O₂ plants, as 3- and 2-times higher levels of NDH complex subunit S, compared to CT, were noted in both treatments, respectively (Fig. 4e). This indicates that accumulation of NdhS under drought was only partially suppressed by exogenous H₂O₂ since neither ABA- nor Put-sprayed samples showed any alteration in NdhS content under drought compared to CT.

The activity of SOD and CAT in response to drought

Drought induced over 20% decrease of SOD activity in the case of D sample and 6 and 2% with ABA and Put

application, respectively (Fig. 5c). Besides, in relation to the CT, the H₂O₂ treatment enhanced the SOD activity by

29%. Thus, all applied PGRs mitigated the drought-triggered down-regulation of the SOD activity. Analyzed

Fig. 4 **a)** Western Blot analyses of Rubisco activase (RCA) (α - and β -isoform), PsbO, PGRL1 [red(uced) and ox(idized) monomer] and NdhS protein (eEF1a is the loading control). Immunoblotting was done with barley leaf extracts from the third fully expanded leaf on the last day of the experiment. For RCA, PsbO and eEF1a 5 μ g, and for PGRL1 and NdhS 25 μ g protein per lane were loaded, resolved in 4–20% TGX polyacrylamide gels, transferred to nitrocellulose membrane and visualized with DAB (**a**). Densitometric analysis and quantification were conducted using ImageJ v.1.52 (**b**, **c**, **d**, **e**, **f**). **b)** RCA signal was estimated from both α - and β -isoform bands visualized on the membrane, as an overall RCA content (RCA_{total}). **d)** For PGRL1_{total} quantification both redox state forms were combined. **f)** The ratio of PGRL1_{red}/PGRL1_{ox} indicates the ratio of reduced (29 kDa) to oxidized (24 kDa) form of PGRL1 protein. Different letters indicate significant differences according to Tukey's HSD test ($\alpha = 0.05$). The values of **b**, **c**, **d**, **e** are normalized to CT. Data are shown as means $\pm$ SD ($n = 3$ for **b**, **c**, **d**, **e**, **f**). CT states for control plants with maintained irrigation. The rest of the plants were exposed to 7-days dehydration without (D) or with foliar application of 100 μ M putrescine (D + Put), 100 μ M abscisic acid (D + ABA) and 5 mM hydrogen peroxide (D + H₂O₂)

CAT activity presented an opposite trend to SOD as drought increased enzyme activity by approximately 69, 103, 76 and 119% in D, D + ABA, D + H₂O₂, and D + Put samples, respectively (Fig. 5d). It should be noted, however, that while H₂O₂ application effectively induced SOD activity it was less efficient in mediating CAT activation. No significant difference between D and D + H₂O₂ samples was observed in enzyme activity, despite the increased endogenous level of H₂O₂ in the latter one (Figs. 5b, d).

Fig. 5 Effects of exogenous growth regulators application on **a)** MDA, malondialdehyde accumulation, **b)** H₂O₂, endogenous hydrogen peroxide level, **c)** SOD, superoxide dismutase and **d)** CAT, catalase activity in leaves of barley plants. The parameters were measured on the third fully expanded leaf on the last day of the experiment. Data are shown as means $\pm$ SD ($n = 6$). Different letters indicate significant

The effect of growth regulators on endogenous H₂O₂ accumulation and lipid peroxidation rate under drought

Leaves of the CT plants accumulated approximately 2.28 μ mol of H₂O₂ per gram DW (Fig. 5b) and dehydration increased its accumulation in all the samples tested. Yet, there was a clear correlation between the used PGR and H₂O₂ levels. In the plants exposed to drought and not sprayed nearly a 1.5-fold increase of H₂O₂ accumulation was estimated. At the same time, the application of ABA, exogenous H₂O₂ and Put induced 1.8-fold, 2-fold and 1.6-fold H₂O₂ increase, respectively. Comparison between D and ABA samples allows distinguishing between drought-itself and additional ABA-related induction of H₂O₂ accumulation, presumably resulted from hormone-induced signaling pathways. Moreover, the highest H₂O₂ level was noted for the H₂O₂-treated plants along with unchanged CAT activity compared to the D sample. Thus it was rather the increased SOD activity (Fig. 5c) that enhanced the endogenous H₂O₂ level, than the accumulation of the applied PGR as H₂O₂ is a short-lived molecule and its last application was done three days before analyses. Put samples showed a 10% higher accumulation of H₂O₂ compared to D, presumably related to increased amine catabolism with oxidases.

The drought-induced ROS accumulation enhanced the lipid peroxidation rate estimated by malondialdehyde content

differences according to Tukey's HSD test ($\alpha = 0.05$). CT states for control plants with maintained irrigation. The rest of the plants were exposed to 7-days dehydration without (D) or with foliar application of 100 μ M abscisic acid (D + ABA), 5 mM hydrogen peroxide (D + H₂O₂) and 100 μ M putrescine (D + Put). DW – dry weight

(Fig. 5a). MDA accumulation corresponds to the membrane oxidative damage, thus it increased more than 1.8-fold under drought (D). H_2O_2 and Put alleviated lipid peroxidation as despite drought only 1.34- and 1.47-fold higher MDA content was noted for these treatments compared to CT, respectively. ABA application was the most effective in reducing membrane injuries noticed with over 46% lower MDA accumulation compared to the non-sprayed D plants. It should be noted that the MDA level in ABA-sprayed samples under drought was as low as in the well-watered CT plants.

Discussion

Plants during growth are subjected to periodic water-stress episodes which impose defense response realized to compensate for drought-related alterations (De Souza et al. 2015). Current research revealed that decreasing soil water content significantly reduced leaf RWC (Table 1). Put and ABA application was the most effective in maintaining the water status of barley leaves under drought. However, only the ABA treatment improved the water status via stomatal conductance regulation since the first day of its application (Fig. 3b). The effect of Put was related rather to increased Pro accumulation (Table 1) acting as an osmolyte for osmotic adjustment under stress (Farooq et al. 2017). Ishibashi et al. (2011) reported that the leaf wilting might be also delayed by H_2O_2 spraying but, as shown in Table 1 and Fig. 3b, the H_2O_2 application did not improve the leaf water retention at all.

Although the water shortage caused significant chlorophylls and carotenoids loss and decreased the CSI, exogenous ABA and Put also improved chlorophyll retention (Table 1). Similarly, Li et al. (2016) showed that ABA mitigates the degradation of photosynthetic pigments under drought. The mechanism of delayed chlorophyll degradation in the case of Put resulted presumably from its influence on thylakoid membranes by stabilizing photosystem complexes as has been shown by Li et al. (2015) and Shu et al. (2015). We also observed that drought-caused pigments decline did not influence Chl *a/b* ratio among all analyzed plants, suggesting that the rate of decomposition of the reaction center complexes and the Chl *b*-rich external antennae were similar (Kura-Hotta et al. 1987).

Drought (D) induced a significant decline of $\Phi PSII$ (Fig. 1b), indicating that ETR_{II} was reduced in response to drought stress. In contrast, no significant difference between control and stressed plants was observed in F_v/f_m value (Fig. 1a), indicating that this parameter might not be a good indicator of short-term plant drought stress (Yao et al. 2018). Analyzed D and H_2O_2 samples presented markedly increased $1 - q_P$, whereas ABA and Put treatment allowed to maintain Q_A less reduced (Figs. 1d, 2a). The ABA-based protection of the photosynthetic apparatus was also realized by significant

activation of NPQ (Figs. 2b, c) and markedly restricted membrane injury (Fig. 5a). The plants sprayed with Put showed NPQ and ΦNPQ improvement, however significantly less pronounced than has been reported previously by Ioannidis and Kotzabasis (2007). Thus it might be a result of the lower concentration of applied amine and scarce role of putrescine, compared to spermidine or spermine, as a stimulator of non-photochemical quenching (Ioannidis and Kotzabasis 2007). Put application had beneficial effects on $\Phi PSII$ and ETR_{II} , when compared with D sample, but decreased R_{Fd} and P_n value proved that amine did not improve the actual CO_2 -assimilation rate. Results documented that the highest value of R_{Fd} was maintained by ABA- and the lowest by H_2O_2 -sprayed plants (Fig. 1c). Thus, hydrogen peroxide, despite its role in the ABA signaling cascade was not effective to induce drought acclimation as has been proposed by de Sousa et al. (2016) due to the negative adding effect of the H_2O_2 solution with the stress on photosynthetic efficiency (Figs. 1c, 2a, 3a and 4b). Moreover, measurement of absorbed energy distribution in PSII showed that H_2O_2 treatment enhanced predominantly the fraction of energy passively dissipated in the form of heat and fluorescence (Fig. 2d).

Exposure to drought results in reduced SOD activity (Fig. 5c) and increased CAT activity compared to CT (Fig. 5d). Enhanced activity of CAT did not compensate, however, for increased H_2O_2 production in D sample. At the same time, the H_2O_2 application stimulated the leaf SOD activity. In ABA- and Put-sprayed samples SOD activity remained little or almost unaffected compared to CT, respectively, while CAT activity has been significantly increased. Moreover, the mentioned earlier close correlation of the ABA signaling pathway and H_2O_2 was confirmed by the significantly increased level of H_2O_2 in the ABA-treated plants under drought (Fig. 5b). In our study, the H_2O_2 application was however even more effective to stimulate H_2O_2 accumulation under drought than ABA itself. The previous reports of Terzi et al. (2014) and de Sousa et al. (2016) showed that H_2O_2 application induced antioxidant defense lowering its cellular level. Yet, Lu et al. (2013) indicated that plant reaction is related to its drought tolerance and for sensitive cultivar, such as analyzed Carina, an increased level of H_2O_2 after treatment might be noted. In the case of the Put application, sprayed plants showed a moderate increase of H_2O_2 level. Thus, it might be concluded that although Put application enhanced the activity of amine oxidases, the enzyme-drove H_2O_2 accumulation was restricted due to concomitant antioxidant properties of Put (Pottosin and Shabala 2014) and induced CAT activity (Fig. 5d).

The most abundant final product of ROS-triggered lipid peroxidation is MDA (Pilarska et al. 2017) and its accumulation under drought was related to the SOD activity and endogenous H_2O_2 level as shown within the CT and non-sprayed (D) sample (Figs. 5a–c). However, within plants sprayed with tested PGRs, there was no such obvious tendency for MDA

accumulation level under drought. The increased rate of membrane peroxidation, therefore, seems to be related to more than one oxidative agent and changes in the integrated antioxidant system. We noted that H_2O_2 and Put application lowered MDA accumulation, however only the ABA-treated plants showed significantly restricted peroxidation rate, similar to CT samples (Fig. 5a). Reduced accumulation of MDA content after ABA treatment under drought was similar to those found by Sezgin et al. (2018).

According to Castrillo et al. (2001) severe water stress decreases P_n by both the stomatal closure and direct inhibition of the biochemical reaction. The exogenous ABA induced rapid stomatal closure shortly after treatment (Fig. 3b), similarly to action realized with guard cell-autonomously synthesized ABA (Bauer et al. 2013), thus immediately restricting water loss. The rapid decrease of leaf stomatal conductance after the ABA application has been also documented by Wang et al. (2020). In contrast, Put application was significantly less effective in rapid stomatal closure. Despite the enhanced RWC level, the g_s of the Put-treated plants, at the end of the drought, was as low as noted for the non-sprayed plants (Fig. 3b). The effectiveness of foliar ABA pre-treatment is documented by comparison of P_n and g_s values on Day 1. The latter one has been decreased by ABA application by nearly 70% (Fig. 3b), whilst the CO_2 assimilation rate lowered by 28% compared to CT value (Fig. 3a). These results indicated that under nonsaturating light conditions, applied for gas exchange measurements, the decreased CO_2 influx did not translate directly into damped CO_2 assimilation. Brito et al. (2020) also demonstrated that ABA pre-treatment allowed the delay of the negative drought effects on net photosynthesis. Therefore, upon drought stress, an important physiological function of ABA is to reduce the number of ions in the guard cells to decrease the turgor pressure, which induces stomatal closure enhancing water retention capacity (Dong et al. 2019).

ABA links environmental stress perception with the photosynthetic capacity by repressed expression of nuclear- and chloroplast-encoded genes and subsequently the level of the photosynthetic proteins such as Rubisco (Yamburenko et al. 2013). In contrast to this, in ABA-sprayed plants under drought, only limited downregulation of Rubisco LSU and PsbO protein has been documented (Figs. 3c and 4a–e). Also Put application seemed to protect these proteins against proteolysis, likely as a result of induced Pro accumulation (Table 1) (Li et al. 2015). In the case of H_2O_2 , the previous proteomic analysis showed, that at least 144 protein expression was altered upon exposure to H_2O_2 . Among them, Rubisco and RCA were downregulated while PsbO was up-regulated (Wan and Liu 2008). Comparison of the first two proteins accumulation under drought in ABA- and H_2O_2 -sprayed plants showed, however, that increased endogenous

H_2O_2 accumulation (Fig. 5b) is not a sufficient factor for decreased Rubisco and RCA level (Figs. 3c and 4b). The decreased RCA_{total} and even more the Rubisco LSU level was concurrent with the change in the analyzed CO_2 -assimilation rate on the last day of drought (Fig. 3a). However, the magnitude of P_n decrease on the last day of drought in all the groups tested is a clear indication of additional limitations such as g_s and disturbed electron transfer.

Chen et al. (2016) documented that the level of both OEC complex proteins - PsbO and PsbP were kept almost unchanged in response to water deficit stress, which was also confirmed in our study. The disassembly of OEC extrinsic proteins would induce the donor side limitation of PSII. However, we observed rather opposite results. In D and H_2O_2 -sprayed plants the decreased rate of electron transport from PSII toward PSI (Fig. 1d) and increased excitation pressure of PSII was observed (Fig. 2a), thus the ability of PSII to reduce the Q_A was not altered (Sperdouli and Moustakas 2012). These results suggest that OEC activity has been maintained in all groups under drought.

Drought stress modifies the expression of genes encoding proteins involved in cyclic electron flow around PSI (Lehtimäki et al. 2010). Along with the PGR5/PGRL1 main pathway of CEF-PSI, also the chloroplast NDH-complex might compensate the ATP/NADPH production ratio (Nikkanen et al. 2019). We found that ABA-sprayed plants under drought presented increased accumulation of PGRL1 protein (Figs. 4a, d) and less reduced acceptor side of PSII (Fig. 2a) compared to D sample. Based on these findings it is proposed that in ABA-sprayed plants efficient electron transfer from PSI with ferredoxin (Fd) to PGR5/PGRL1 and then to cytochrome b_6f ($cytb_6f$) through plastoquinone (PQ) was noted (Li et al. 2018). Interestingly, as in the report of Hertle et al. (2013), we were also able to fraction two different PGRL1 monomers - larger (29 kDa) reduced and smaller (24 kDa) oxidized form. The analyzed ratio of $PGRL1_{red}$ to $PGRL1_{ox}$ (Fig. 4f) indicated the water limitation induced higher accumulation of $PGRL1_{red}$ form, meaning that PSI is presumably also reduced (Hertle et al. 2013). However, the Put- and ABA-sprayed plants maintained a lower ratio of $PGRL1_{red}$ to $PGRL1_{ox}$, close to the value noted with CT. Thus, it is reasonable to state that Put- and even more the ABA-treated samples maintained a higher pool of $PGRL1_{ox}$ by efficient electron transfer from $PGRL1_{red}$ to PQ interacting with $cytb_6f$ (Hertle et al. 2013). Consequently, increased transmembrane proton gradient was build up in sprayed plants activating protective energy dissipation by non-photochemical quenching (NPQ) (Figs. 2b, c).

In the case of NDH, we analyzed NdhS protein that forms the Fd-binding site of the chloroplast NDH complex (Shikanai 2016). The NdhS increased accumulation noted in D and, to a lesser extent, in H_2O_2 -treated plants might be related to endogenous H_2O_2 level, compared to CT, as NDH can be

activated *in vivo* by H₂O₂ (Casano et al. 2001). However, in the case of ABA-sprayed plants, enhanced hydrogen peroxide level did not induce the NdhS accumulation. It might be the fact that CEF is already operating with ABA-activated PGR5/PGRL1 complex as both CEF routes present compensatory activity (Suorsa 2015). An explanation of NdhS increased level in D and H₂O₂-sprayed plants might be the fact that the NDH complex is activated prior to the onset of plant senescence, as documented by Zapata et al. (2005). The author showed that expression of *ndh* genes is required for leaf senescence as NDH enhanced the reduction of electron transporters, generation of ROS, higher MDA level, decreased activity of the Calvin cycle and finally induction of leaf programmed cell death (PCD) (Zapata et al. 2005). Indeed, a lot of senescence-associated traits such as chlorophyll-loss, photosynthesis downregulation, electron transporters over-reduction, increased MDA and ROS level, were noted for D and H₂O₂-sprayed plants.

Conclusions

The study demonstrated that foliar application of abscisic acid attenuated the effect of drought in barley through the maintenance of water status and protection of the photosynthetic machinery. ABA pre-treatment was also effective in managing stomatal conductance, preserving CO₂ fixation, SOD and CAT activities, reducing the rate of membrane lipid peroxidation and increased the level of PGRL1 protein. Thus, it seems that the mechanism of ABA action on plant acclimation to drought, besides restriction of water-loss, relies on less reduced quinone pool measured with 1 – qP and maintained electron transport from PSII to PSI, protected by NPQ thermal dissipation. A beneficial, but significantly less pronounced acclimation response to drought was observed in plants with putrescine pre-treatment. The hydrogen peroxide treatment has been shown to exacerbate drought stress in many physiological and biochemical aspects.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.2478/s11756-020-00644-2>.

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Author contributions ES and MT contributed equally. ES and MT conceived and designed research, performed all experimental analyses and carried out data analysis. Both authors read the final manuscript and approved the submission.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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