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Long-Term Effects of Cold Atmospheric Plasma-Treated Water on the Antioxidative System of *Hordeum vulgare*

Frederik Bussmann[1] · Andrea Krüger[1] · Caterina Scholz[1] · Henrike Brust[2] · Christine Stöhr[1]

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Abstract

Facing climate change, the development of innovative agricultural technologies securing food production becomes increasingly important. Plasma-treated water (PTW) might be a promising tool to enhance drought stress tolerance in plants. Knowledge about the effects of PTW on the physiology of plants, especially on their antioxidative system on a long-term scale, is still scarce. In this work, PTW was applied to barley leaves (*Hordeum vulgare* cv. Kosmos) and various constituents of the plants' antioxidative system were analyzed 30 days after treatment. An additional drought stress was performed after foliar PTW application followed by a recovery period to elucidate whether PTW treatment improved stress tolerance. Upon PTW treatment, the Total Antioxidant Capacity (TAC) in leaves and roots was lower in comparison to deionized water treated plants. In contrast, PTW treatment caused a higher content of chlorophyll, quantum yield and total ascorbate content in leaves compared to deionized water treated plants. After additional drought application and subsequent recovery period, an enhancement of values for TAC, contents of malondialdehyde, glutathione as well as activity of ascorbate peroxidase indicated a possible upregulation of antioxidative properties in roots. Hydrogen peroxide and nitric oxide might mediate abiotic stress tolerance and are considered as key components of PTW.

Keywords Antioxidative system · Ascorbate–glutathione cycle · *Hordeum vulgare* · Hydrogen peroxide · Nitric oxide · Plasma-treated water

Abbreviations

AAE	Ascorbic acid equivalents
APX	Ascorbate peroxidase
Asc	Ascorbate
Asc_{ox}	Oxidized ascorbate
Asc_{red}	Reduced ascorbate
Asc_{tot}	Total amount of ascorbate
CAP	Cold atmospheric plasma
DAS	Days after sowing
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DW	Deionized water
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GS_{tot}	Total amount of glutathione
MDA	Malondialdehyde
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
PTW	Plasma-treated water
TAC	Total Antioxidant Capacity
TBARS	Thiobarbituric acid-reactive substances

Handling Author: Nudrat Aisha Akram.

✉ Christine Stöhr
stoehr@uni-greifswald.de

Frederik Bussmann
frederik.bussmann@outlook.com

Andrea Krüger
andrea.krueger@uni-greifswald.de

Caterina Scholz
caterina.scholz@uni-greifswald.de

Henrike Brust
henrike.brust@inp-greifswald.de

[1] Institute of Botany and Landscape Ecology, University of Greifswald, Soldmannstrasse 15, 17487 Greifswald, Germany

[2] Leibniz Institute for Plasma Science and Technology, Felix-Hausdorff-Strasse 2, 17489 Greifswald, Germany

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Introduction

Facing climate change and the continuous population growth, many challenges arise for securing the global demand of crops (Anderson et al. 2020). Climate change in particular risks food security due to the progressive occurrence of extreme weather events. Drought and desertification are two of the most pervasive ecological consequences and exacerbate quality and quantity of crop products (reviewed by Raza et al. 2019). Consequently, extensive strategies encompassing agricultural adaption to climate change are required to deal with climatic future challenges and to ensure food security (Pretty et al. 2010).

Cold atmospheric plasma (CAP) gained considerable attention as promising 'green technology' for future agricultural applications (Puač et al. 2018). Plasma is an ionized gas and referred as the fourth state of matter containing electrons, ions, neutral atoms and molecules, radicals, reactive species, different kinds of electromagnetic radiation (e.g., UV, visible light), and electric fields (Lu et al. 2016; Zhou et al. 2020). A huge variety of methods exists to generate CAP for treatment of biological targets under physiological temperatures, which range from the feed gas to electrical parameters to ignite plasma and configuration of the plasma devices (Šimek and Homola 2021; Zhou et al. 2020). Plasma-treated water (PTW) that is produced by exposing water to plasma, has the advantage that it can be generated in bigger quantities to treat plants roots or shoots and by that, omitting effects of, e.g., UV radiation or electromagnetic fields. The chemistry of PTW is based on complex reactions between plasma-gas–liquid interfaces (Graves et al. 2019; Thirumdas et al. 2018; Zhou et al. 2020). In principle, energy transferred to molecular oxygen- and nitrogen-containing gas leads to the generation of reactive oxygen and nitrogen species (RONS). Gaseous RONS, e.g., ozone (O_3) or nitric oxide (NO), can diffuse to certain extend to the liquid but are relatively unstable due to further reactions. Hydrogen peroxide (H_2O_2), nitrite (NO_2^-), and nitrate (NO_3^-) ions accompanied with decrease in pH are frequently detected in PTW (Hu et al. 2021; Zhou et al. 2020). PTW has multifaceted effects on plants, comprising the activation of plant vitality, inactivation of phytopathogens, enhancing seed germination, and plant growth as well as influencing the antioxidative system (Ito et al. 2018; Zhou et al. 2020; Adhikari et al. 2020). Moreover, recent studies evaluated the effects of PTW to stimulate biotic and abiotic-related stress responses in barley (Gierczik et al. 2020), grapevine (Laurita et al. 2021), maize (Lukacova et al. 2021), periwinkle (Zambon et al. 2020), and tomato (Adhikari et al. 2019).

Plants perceive biotic as well as abiotic changes. If environmental changes extend in strain, outside influences may result in oxidative stress (Kranner et al. 2010; Demidchik 2015). Since plants are aerobic organisms and utilize molecular oxygen (O_2) in several biochemical processes, stress metabolism leads to the enhanced generation of toxic byproducts called reactive oxygen species (ROS) which include singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydroxyl radical ($OH^\bullet$), and H_2O_2 (Choudhury et al. 2017; Mittler 2002). ROS naturally occur upon the partial reduction of O_2 in many parts of the metabolism. The reactivity due to the high oxidizing potential can cause damage to nucleic acids, proteins, carbohydrates, and lipids. ROS triggers cell death by overwhelming the redox homeostasis if oxidative stress is severe (Bartosz 1997). If the damage cannot be reversed, programmed cell death might be initiated (Mittler 2002). In contrast, if kept in transient concentrations, ROS may also function as second messengers (Alscher et al. 1997; Foyer and Noctor 2005). They are responsible for fine-tuning several signal transduction processes involved in defense mechanisms against biotic and abiotic stresses (Dumanović et al. 2021). Besides ROS, it was shown that an imbalanced redox homeostasis also results in the production of reactive nitrogen species (RNS), essentially NO and derivates (Wang et al. 2013). NO is the most studied RNS and participates in many physiological processes of higher plants. Versatile interactions between ROS and RNS are noticeable (Astier et al. 2018). Under stress conditions, an accumulation or de-regulated synthesis of RNS can prevail, leading to nitrosative stress which is possibly involved in oxidative stress (Del Río 2015). Since plants are sessile and possess limited capabilities of stress avoidance, they developed a flexible scavenging system as an adaption to changing environmental conditions, which is universally referred to as the antioxidative system. The ascorbate–glutathione cycle plays an important role in the antioxidative system since it facilitates the efficient detoxification of H_2O_2 (Foyer and Halliwell 1976). It consists of the low molecular mass antioxidants ascorbic acid (Asc), glutathione and the enzymes ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR).

Typically, plants cope with drought stress by activating the antioxidative system as well as producing compatible solutes (also known as osmolytes) to counteract osmotic disequilibria (Fang and Xiong 2015). The upregulation of enzymatic and non-enzymatic antioxidants requires a complex network of signaling pathways which to date is still not completely understood. Next to phytohormones, interlinking molecules, protein kinases, and transcription factors, H_2O_2 and NO play a pivotal role in this signaling network (Qiao et al. 2014; Ilyas et al. 2020; Lau et al. 2021). Many strategies have been designed to improve drought tolerance in plants. An improved stress tolerance based on physiological or metabolic adjustment due

to an earlier exposure to a mild stress is referred to as 'priming' (or 'hardening'). It represents one of the most promising crop protection approaches for production of resilient crops (Li and Liu 2016). The exogenous application of certain compounds which can enhance tolerance to above-ground plant parts is a well-established method both in research and agriculture to improve the performance of crops (Merewitz 2016). Since primed plants show improved stress responses, this phenomenon is part of the concept of a 'stress memory' (Hilker and Schmülling 2019). Associated molecular mechanisms are still unclear (Li et al. 2019), although the same compounds involved in the signaling pathways for the 'natural' development of stress tolerance seem to participate in priming induced stress tolerance. The exogenous application of drought-responsive phytohormones, H_2O_2 and NO-donating chemicals resulted in enhanced drought tolerance (Molassiotis et al. 2016). A common feature for these signaling pathways are complex cross-talks between signaling components (Molassiotis and Fotopoulos 2011).

In this work, we investigated the long-term effects of PTW on the antioxidative system of *Hordeum vulgare* leaves and roots under no stress and drought stress conditions. Furthermore, it is discussed whether the foliar application of PTW, and particularly the PTW containing compounds H_2O_2 and NO, sustainably mediates drought stress tolerance.

Materials and Methods

Production of Plasma-Treated Water

The alternating current (AC)-driven plasma system used in this study consisted of a pin-to-liquid discharge configuration with four metal electrodes placed approx. 3 mm from the water surface (Schmidt et al. 2019). Deionized water (DW) was mixed with 7.5% (v/v) of tap water prior to plasma treatment, as sufficient concentration of ions (≥ 80 µS cm^{-1}) was needed to ignite the plasma between electrodes and water surface. Water mixture of 900 ml was treated for 20 min. The spatial boundary layers between transient spark discharges, water surface, and ambient air led to the formation of reactive and excited nitrogen and oxygen species, which were transported into the water volume by constant stirring during treatment. PTW application to plants and physicochemical analysis were performed 5–10 min after treatment.

The pH of the water was measured using the pH 3210 m (WTW, Weilheim, Germany) and the conductivity with help of the TetraCon 325 electrode on an inoLab Multi Level3 with inoLab Terminal Level3 (WTW, Weilheim, Germany).

Determination of Nitrite and Nitrate Concentration in PTW

Nitrate and nitrite ions were analyzed by ion exchange chromatography using Dionex ICS 6000 system (Thermo Scientific, Dreieich, Germany) equipped with an anion-exchange column (Dionex IonPac AS 18, Thermo Scientific, Dreieich, Germany) and a guard column (Dionex IonPac AG 18, Thermo Scientific, Dreieich, Germany) according to manufactures instructions. Undiluted PTW was injected with 5 µl, ions were separated in 23 mM KOH at 0.25 ml min^{-1} under isocratic conditions, and conductivity signals were recorded. Concentration of ions was calculated based on a calibration curve established by Dionex 7-ion standard solution (Thermo Scientific, Dreieich, Germany). Ions were determined from four independent PTW solutions.

Determination of Hydrogen Peroxide Concentration in PTW

The level of H_2O_2 in PTW was determined with the potassium iodide (KI) method according to Junglee et al. (2014). The assay was performed within 1 ml test volume containing 500 µl 1 M KI in MES-KOH (50 mM, pH 6.0) 50 µl PTW and 450 ml H_2O_{deion}. Absorbance was read at 350 nm after 30 min incubation at room temperature. A standard curve was obtained with H_2O_2 standard solution prepared in H_2O_{deion}.

Determination of Nitric Oxide Release from PTW

Constant and specific measurements of gaseous NO were accomplished via ANALYZER LCD 88 sp (Eco Physics) chemiluminescence-based NO detector equipped with an ozone generator (Stöhr et al. 2001; Stöhr and Stremlau 2006). 500 µl PTW were placed on a petri dish (diameter 5 cm) within the custom-made reactor chamber. The experiment was performed at 30 °C under anoxic conditions, and sample was constantly stirred. N_2 carrier gas transported the emitted NO with a flow rate of 400 ml min^{-1} (mass flow meter GFM ANALYT-MTC) to the analyzer. Data were recorded until NO was no longer detectable.

Plant Material and Cultivation

Seeds of *Hordeum vulgare* cv. Kosmos were pre-germinated in Petri dishes on filter paper soaked with 0.5 mM calcium sulfate for 2 days in dark. Each seedling was placed in a pot (∅ 12 cm) with a homogenous mixture of 2:1 (v:v) coarse-grained and fine-grained quartz sand. Plants were grown under a light/dark rhythm of 14/10 h at 22/18 °C air temperature, respectively. Dependent on the weather conditions, sunlight was supplemented by the light of high-pressure sodium

lamps. The pots were rotated 2 times a week to ensure uniform growth conditions. All plants were watered daily with a defined nutrient solution containing 5 mM nitrate (Stöhr and Ullrich 1997) except for the drought period.

PTW Application and Drought Treatments

Four plant groups were treated as follows:

I. "*DW no stress*" was sprayed with deionized water instead of PTW (DW: 1.7 ml) as a control 18, 19, and 20 days after sowing (DAS).
II. "*PTW no stress*" was sprayed with PTW (1.7 ml) 18, 19, and 20 DAS.
III. "*DW drought*" was sprayed with DW as mentioned in (i) and did experience drought stress by omitting/ceasing the nutrient solution on 33, 34, and 35 DAS followed by a recovery: rewatering on 36 DAS with a double amount of nutrient solution and normal watering thereafter.
IV. "*PTW drought*" did experience both PTW application (ii) and drought stress (iii) followed by a recovery as indicated above.

Photosynthetic measurements were performed 49 DAS, one day before harvesting. Plants were harvested in the morning before rewatering 36 DAS for proline determination only (another batch) and 50 DAS after the recovery phase for biochemical assays. For MultispeQ measurements, 10 biological replicates of each treatment were used, and biochemical assays were performed on 4 biological replicates.

Photosynthetic Measurements

Spectroscopic measurements were done with the hand-held MultispeQ (v2.0) spectrophotometer (Kuhlgert et al. 2016) using the 'Photosynthesis RIDES' protocol linked to the PhotosynQ platform (https://photosynq.org). They were performed in the middle of an intact fully expanded leaf (third leaf from top) to estimate the fraction of light energy captured by Photosystem II (quantum yield or operating efficiency of PSII, Phi2, ΦII).

Soil Humidity

The measurement of soil moisture was conducted with the FOM/mts device with non-standard probes LP/ms (*E*-Test Ltd., Stasin, Poland; on the base of Time Domain Reflectometry technique). FOM/mts provides readout of volumetric water content according to the empirical calibration of Malicki et al. (1996). Under well-watered conditions, plants met a soil moisture of about 10% (v/v), whereas the drought-stressed plants experienced a soil moisture of 2% (v/v) at the lowest point.

Biochemical Assays

Fully expanded leaves and roots were harvested 50 DAS (36 DAS for proline determination), ground with liquid nitrogen and stored at − 80 °C. Frozen tissue powder was treated with individual extracting reagents according to each assay.

Determination of Proline Content and Lipid Peroxidation

Proline content was determined according to the method of Bates et al. (1973). Lipid peroxidation was determined and calculated in terms of thiobarbituric acid-reactive substances (TBARS) using the Malondialdehyde (MDA) assay according to Cavalcanti et al. (2004) with some modifications. 0.2 g frozen tissue were treated with 1 ml ice cold 1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 18,000×*g*, 4 °C and 15 min. For the assay, 250 µl of the supernatant were incubated with 750 µl 0.5% (w/v) thiobarbituric acid in 20% (w/v) TCA. After 1 h incubation at 98 °C, the reaction was stopped on ice and centrifuged at 15,000×*g*, 4 °C, and 5 min. The absorbance was read at 532 and 600 nm. Calculation was done with $A_{532\,nm}$–$A_{600\,nm}$ and the extinction coefficient $\varepsilon = 155\ mmol^{-1}\ cm^{-1}$.

Determination of Chlorophyll Content and Total Antioxidant Capacity

Methanolic extraction was done for chlorophyll content and total antioxidant capacity (TAC). 0.1 g frozen tissue was treated with 1 ml of 99% (v/v) methanol and incubated in an ultrasonic bath (62 °C, 15 min, 100% DEGAS). Extraction was repeated 3 times.

Measurements and calculations of chlorophyll in methanolic extracts were performed according to Lichtenthaler and Buschmann (2001).

TAC was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described by Mahieddine et al. (2018) with some modifications. In this study, methanolic extract (100 µl for leaves, 200 µl for roots) was incubated with 900 µl of 0.1 mM DPPH ethanolic solution for 30 min at room temperature in dark and absorbance was read at 520 nm. A blank for each sample was performed by adding 900 µl ethanol to each extract instead of DPPH solution and subtracted from sample value. Ascorbic acid was used for standard and content was calculated in terms of ascorbic acid equivalents (AAE).

Determination of Ascorbate and Glutathione Content

0.1 g frozen tissue were extracted with 1.8 ml 6% TCA. The colorimetric assay was performed according to Gillespie and Ainsworth (2007), and absorbance was read at 525 nm. Asc_{tot} and Asc_{red}: Asc_{ox} ratios were calculated using ascorbic acid as a standard.

For determination of the glutathione content, a modified protocol of the enzymatic recycling procedure according to Noctor et al. (2016) was performed. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were used as a reference. 2-vinylpyridine was used as a masking reagent for GSH. The assay was performed in a 96-well microtiter plate and absorbance read at 405 nm. GS_{tot} and GSH:GSSG ratios were calculated.

APX Activity

The ascorbate peroxidase (APX) activity was determined according to Noctor et al. (2016) with some modifications. 1 ml of the extracting agent (0.1 M sodium phosphate buffer, 0.1 mM EDTA (pH 7.0), 5% (w/v) polyvinylpolypyrrolidone (PVPP), 1 mM Asc) was added to 0.25 g frozen powder and centrifuged at 25,000×*g*, 4 °C, 15 min. The assay was performed in 96-well microtiter plates and absorbance read at 290 nm. A baseline was recorded with 175 µl test buffer (0.1 M potassium phosphate buffer (KPP, pH 7.0) and 0.1 mM EDTA), 25 µl of 5 mM ascorbic acid, and 25 µl of diluted extract. APX activity started with the addition of 25 µl of 1 mM H_2O_2. Specific APX activity was calculated using the extinction coefficient 2800 $l * mol^{-1} * cm^{-1}$. The protein content was determined according to Bradford (1976).

Statistical Analysis

All the obtained data have mean values ± standard deviation (SD). The data were statistically analyzed by student's *t* test with Excel. Significant differences are denoted according to $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

Results

Plasma Treatment of Water Caused Increased Occurrence of NO_x Species and Hydrogen Peroxide

Plasma treatment of DW for 20 min resulted in accumulation of hydrogen peroxide, nitrite, and nitrate ions in µmolar concentrations (Table 1). The observed decrease in pH in PTW (within several publications denoted as 'plasma activated water,' PAW) in comparison to DW is in line with published data (e.g., Adhikari et al. 2019; Hu et al. 2021; Kang et al. 2019). In addition, significant amounts of NO released from stirred PTW into the gas phase were detected. The detection method of NO applied in this study is based on the reaction of NO with ozone resulting in excited nitrogen dioxide species that emit detectable photons when dropping back to the ground state (Stöhr and Stremlau 2006). The occurrence of NO in plasma-treated liquids has been noticed for other plasma treatment systems as well by measuring NO within PTW via amperometric microsensors (Kang et al. 2019) or by EPR spectroscopy (Tian et al. 2017).

Table 1 Physicochemical properties of deionized water. Deionized water was mixed with 7.5% tap water (DW). Plasma treatment of DW (PTW) was applied for 20 min. ($n = 4$)

parameter	DW	PTW
pH	6.5	3.8
Conductivity (µS cm^{-1})	86 ± 4	155 ± 15
Nitrate ions (mM)	n.d.	0.231 ± 0.082
Nitrite ions (mM)	n.d.	0.568 ± 0.046
Hydrogen peroxide (mM)	n.d.	0.234 ± 0.004
Gaseous nitric oxide (mM)	n.d.	0.331 ± 0.097

n.d. Not detected

Proline Content Increased in Leaves and Roots Directly After Drought Stress

Proline content was measured to estimate the stress status of the plants. Directly after drought stress (36 DAS), in leaves, proline content was 150 times higher in *DW drought* compared to *DW no stress* (1048 compared to 7 µmol proline * g FW^{-1}) and 136 times higher in *PTW drought* compared to *PTW no stress* (1023 compared to 7 µmol proline * g FW^{-1}) (Fig. 1a). Regarding roots, proline content was 5.4 times higher in *DW drought* and 7.6 times higher in *PTW drought* compared to the respective *no stress* group (Fig. 1b). After two weeks of recovery following drought application, only minor differences between proline contents under no stress conditions and drought stress conditions could be observed in leaves and roots independent of the PTW treatment (Fig. 1c, d).

PTW Treatment Resulted in Enhanced Chlorophyll Content and Quantum Yield

Assessing the content of photosynthetic pigments is a suitable indicator for photosynthetic activity (Ghosh et al. 2004) as well as photooxidative stress (Pinto-Marijuan and Munné-Bosch 2014) and considered as an overall requirement for the effective cultivation of plants (Sonobe et al. 2020). Under no stress conditions, significantly higher values of

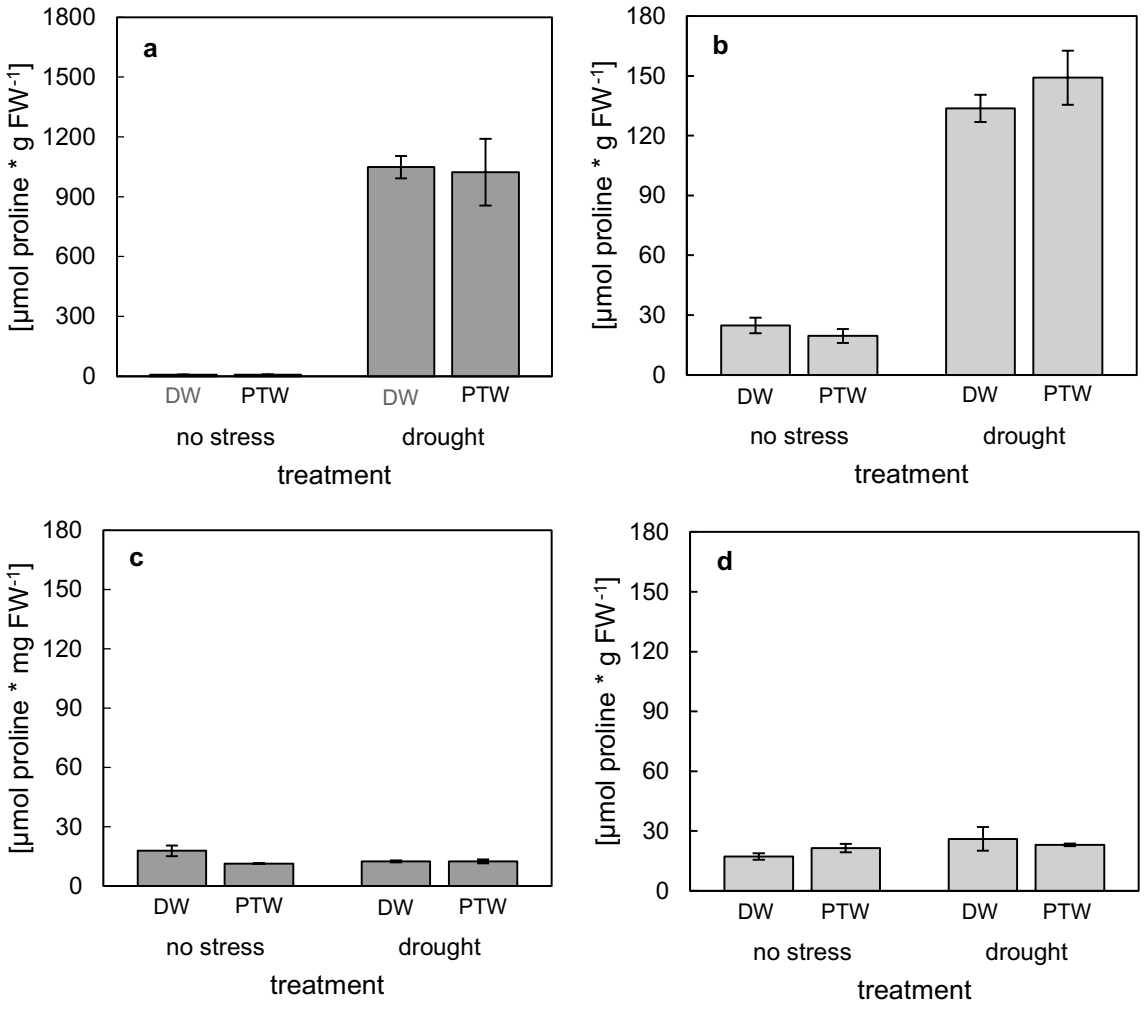

Fig. 1 Proline content determined directly after drought stress treatment in leaves **a** and roots **b** after treatment with deionized water (DW) or plasma-treated water (PTW) under no stress and drought stress conditions. Measurement was performed again 2 weeks after drought stress treatment in leaves **c** and roots **d**. Mean values (±SD) were calculated from four replicates of each treatment. Bars with an asterisk indicate significance differences ($_{*}$: $p<0.05$; $_{**}$: $p<0.01$; $_{***}$: $p<0.001$). Statistical analysis was performed using student's *t*-test

Chl $(a+b)$ content were observable upon PTW treatment relative to DW-treated plants (18%, Fig. 2a). Under drought stress conditions, higher contents of Chl $(a+b)$ in comparison to DW treatment were detected, despite not being significant (10%, Fig. 2a). MultispeQ measurements revealed the same pattern regarding the quantum yield of Photosystem (PS) II: Significantly higher values were obtained upon PTW treatment relative to DW treatment under no stress (11%), whereas under drought conditions, the changes were not significantly higher (7%; Fig. 2b). No morphological differences could be obtained.

Total Antioxidant Capacity Increased Significantly in Leaves and Roots upon PTW Treatment Under Drought Stress Conditions

Plant enzymatic and non-enzymatic antioxidative mechanisms jointly contribute to the TAC. It serves as biochemical

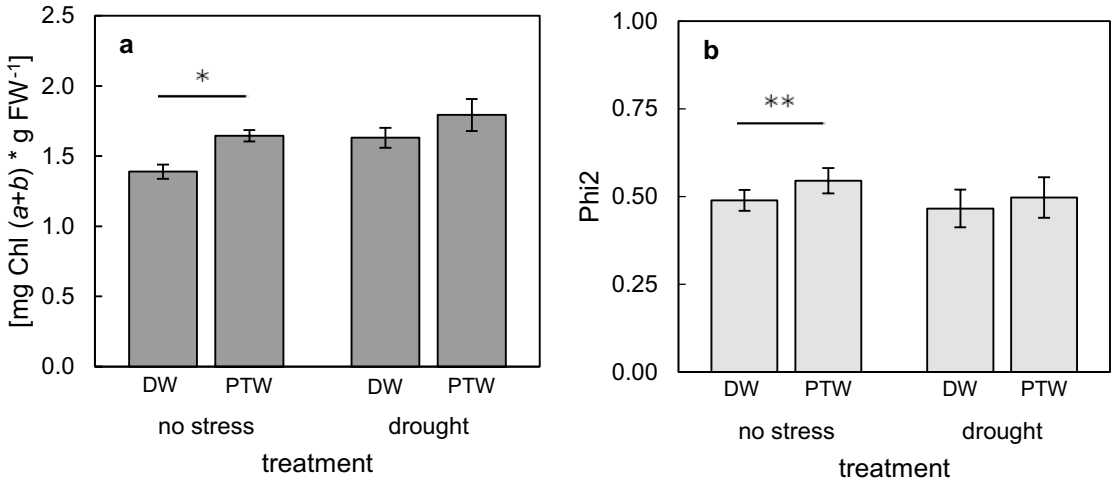

Fig. 2 Chl ($a+b$) content **a** and quantum yield of Photosystem II **b** after treatment with deionized water (DW) or plasma-treated water (PTW) under no stress and drought stress conditions. Mean values (± SD) were calculated from four replicates of each treatment. Bars with an asterisk indicate significance differences ($_{*}$: $p<0.05$; $_{**}$: $p<0.01$; $_{***}$: $p<0.001$). Statistical analysis was performed using student's *t*-test

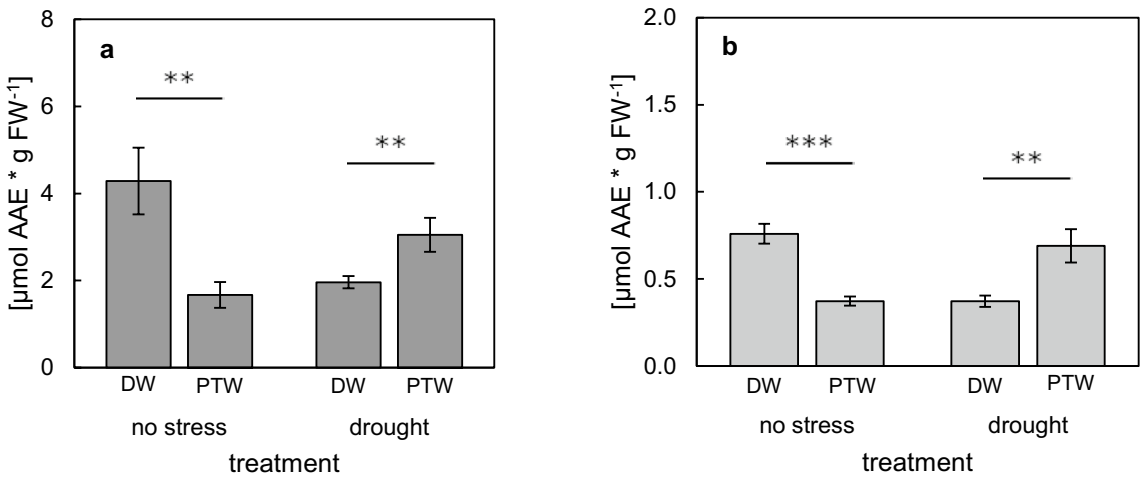

Fig. 3 Total antioxidant capacity (TAC) in leaves **a** and roots **b** after treatment with deionized water (DW) or plasma-treated water (PTW) under no stress and drought stress conditions. Results are expressed in ascorbic acid equivalents (AAE). Mean values (± SD) were calculated from four replicates of each treatment. Bars with an asterisk indicate significance differences ($_{*}$: $p<0.05$; $_{**}$: $p<0.01$; $_{***}$: $p<0.001$). Statistical analysis was performed using student's *t*-test

marker for the plant response to environmental changes since it assesses its redox status (Ghiselli et al. 2000; Gillespie et al. 2007). It reflects the scavenging capacity of reducing agents such as antioxidants towards DPPH (Pyrzynska and Pękal 2013). In both organs, treatment with PTW affected TAC in a similar pattern under no stress and drought stress conditions. Under no stress conditions, treatment resulted in significantly lower values for TAC (61% in leaves, Fig. 3a; 51% in roots, Fig. 3b). Under drought stress conditions, values for TAC were significantly higher upon treatment with PTW (56% in leaves, Fig. 3a; 85% in roots, Fig. 3b).

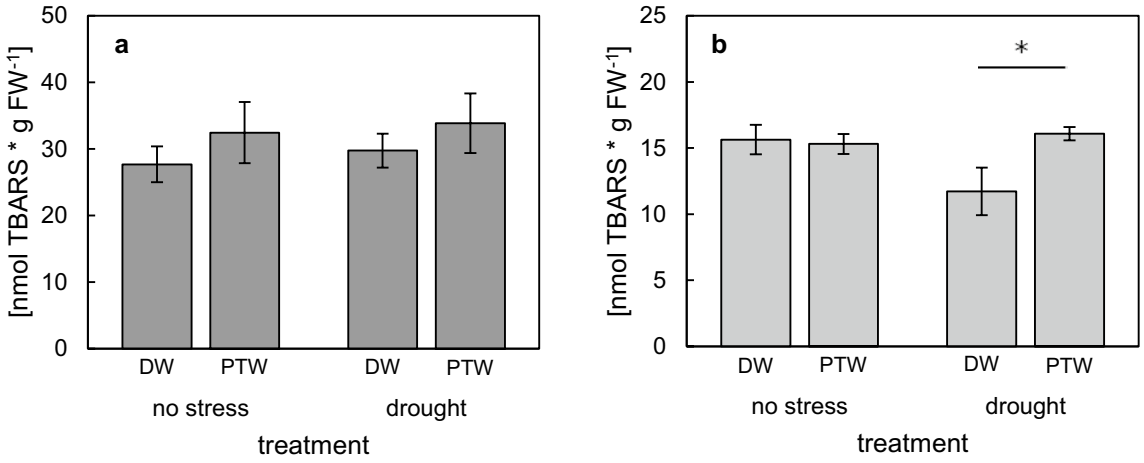

Fig. 4 Lipid peroxidation in terms of TBARS in leaves **a** and root **b** after treatment with deionized water (DW) or plasma-treated water (PTW) under no stress and drought stress conditions. Mean values (± SD) were calculated from four replicates of each treatment. Bars with an asterisk indicate significance differences ($_{*}$: $p < 0.05$; $_{**}$: $p < 0.01$; $_{***}$: $p < 0.001$). Statistical analysis was performed using student's *t*-test

TBARS Content Increased Significantly in Roots upon PTW Treatment Under Drought Stress Conditions

Membrane damage can be a result of oxidation by RONS. Lipid peroxidation estimated as the content of TBARS serves as an indicator for oxidative stress. However, after recovery, TBARS content may also indicate acclimation processes facilitating stress tolerance. In spite of not being significant, a tendentiously higher TBARS content was observed upon PTW treatment under both no stress (17%) and drought conditions (14%) in leaves (Fig. 4a). In roots, no differences in TBARS content were obtained under no stress conditions after PTW application, whereas under drought conditions, significantly higher values were observed in comparison to DW treatment (37%; Fig. 4b).

PTW Treatment Influenced Components of the Ascorbate–Glutathione–Cycle Under no Stress and Drought Stress Conditions

Asc is one of the most important antioxidant metabolites in plants and an essential component of the ascorbate–glutathione–cycle. By controlling the cellular redox state, it contributes to the development of stress tolerance (Latowski et al. 2010). Changes in the Asc_{red}: Asc_{ox} ratio can act as indicators for abiotic stresses as it directly responds to altered turnover rates of antioxidant enzymes (Tausz 2004). The Asc_{tot} content was fivefold higher in leaves compared to the roots (Fig. 5a, b). Treatment with PTW resulted in significantly higher Asc_{tot} contents in leaves under no stress conditions compared to DW-treated plants (32%), while no differences were noticeable in roots. Under drought stress conditions, minor Asc_{tot} contents were observed non-significantly in leaves (23%) and significantly in roots (49%) relative to DW treatment. Based on the difference between Asc_{tot} content and Asc_{red} content, the Asc_{ox} content and further Asc_{red}: Asc_{ox} ratio can be calculated (Fig. 5c, d). The Asc_{ox} content was generally higher in roots than in leaves, although a significant shift towards the Asc_{red} content was notable in roots under drought stress conditions after PTW treatment and subsequent recovery period. Moreover, only minor non-significant changes regarding the Asc_{red}: Asc_{ox} ratio were detected.

Glutathione is the other important metabolite of the ascorbate–glutathione cycle and crucial for the preservation of the cellular redox homeostasis (Latowski et al. 2010; Noctor et al. 1998). The concentration of total glutathione correlates with the adaption to environmental stresses and alterations in the GSH: GSSG ratio may indicate a response to changes in environmental conditions (May et al. 1998). Besides the Asc_{red}: Asc_{ox} ratio, changes in the GSH: GSSG ratio represent a direct consequence of altering turnover rates of enzymes of the ascorbate–glutathione cycle (Tausz 2004). The overall GS_{tot} contents were approximately 10 times higher in leaves compared to the roots (Fig. 6a, b). The difference between GS_{tot} content and GSSG content allows the calculation of GSH content as well as the GSH:GSSG ratio (Fig. 6c, d). Regarding the contents of GS_{tot} and the GSH:GSSG ratio, no long-term effects of treatment with PTW were obtained in leaves, neither under no stress nor under drought stress conditions (Fig. 6a, c). Values of GS_{tot} content were significantly higher in roots upon PTW treatment relative to DW treatment under drought stress conditions (Fig. 6b). With regard to the GSH:GSSG ratio in roots,

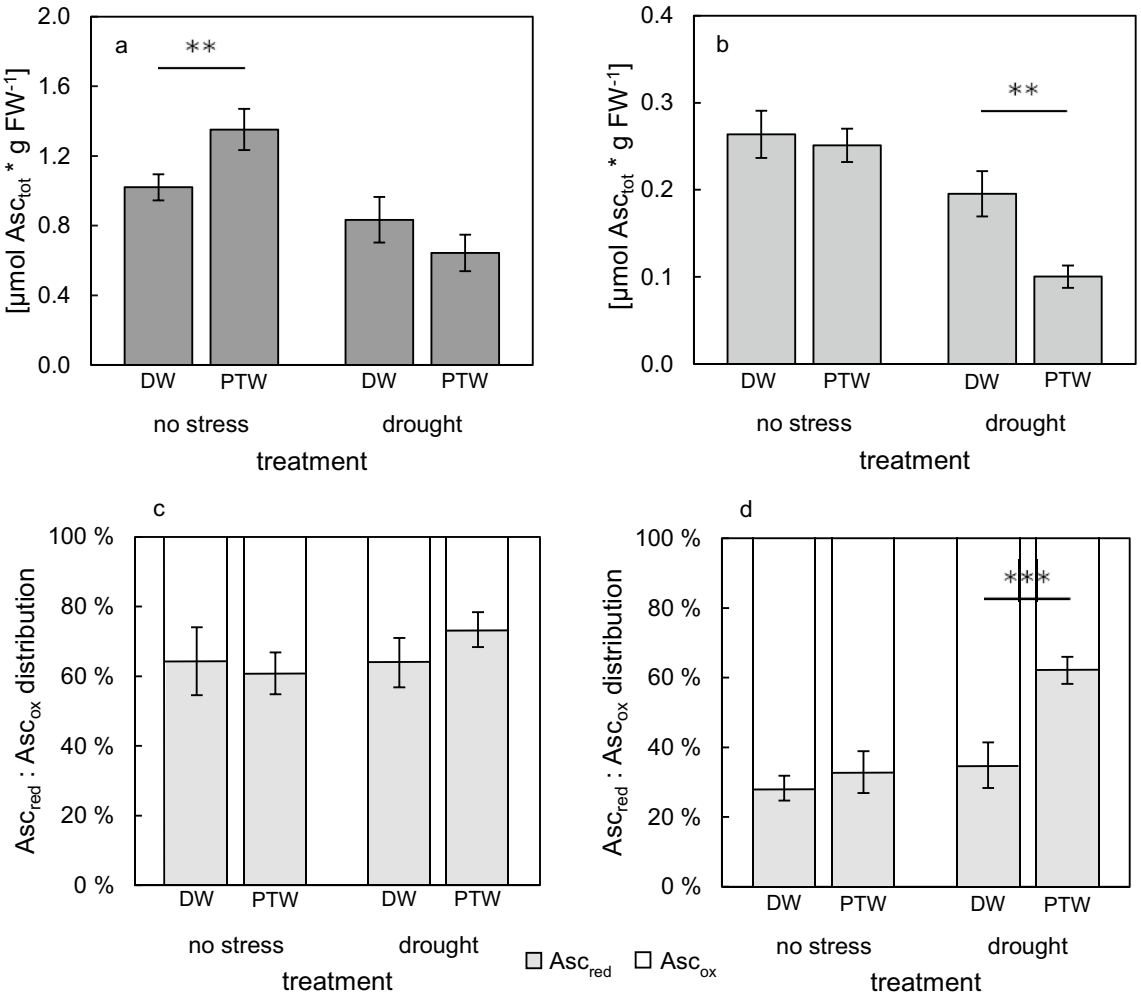

Fig. 5 Total ascorbate content (Asc_{tot}) in leaves **a** and root **b** and distribution **c**, **d** of reduced ascorbate (Asc_{red}; light gray bars) and oxidized ascorbate (Asc_{ox}; white bars) after treatment with deionized water (DW) or plasma-treated water (PTW) under no stress and drought stress conditions. Mean values (± SD) were calculated from four replicates of each treatment. Bars with an asterisk indicate significance differences (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). Statistical analysis was performed using student's *t* test

no differences between DW-treated and PTW-treated plants were obtained (Fig. 6c, d).

In leaves, non-significant alterations regarding the APX activity occurred, as the PTW treatment led to higher values under no stress conditions (33%) and minor values under drought stress conditions (18%) compared to DW treatment (Fig. 7a). In roots, no alterations are noticeable under no stress conditions, whereas under drought stress conditions, values of APX activity were significantly higher upon PTW treatment (45%; Fig. 7b).

Discussion

Numerous physical and chemical reactions between CAP and water lead to the generation of a variety of RONS with different reactivity. Mainly, nitrogen oxides (NO_x) are converted to nitrite and nitrate ions in water while hydroxyls are converted to hydrogen peroxide (Graves et al. 2019). The typical constituents NO_2^-, NO_3^-, and H_2O_2 were also found in the PTW used in this study. Interestingly, PTW contained 331 µM NO that was liberated to the gas phase (Table 1). Only few studies documented the occurrence of NO in PTW (e.g., Kang et al. 2019; Tian et al. 2017). It is known that the radical NO can possess different half-lifetimes from microseconds to hours depending on concentration and chemical environment (Procházková et al. 2015). Kang et al. (2019) could still detect 15–30 µM NO within PTW 16 h after generation. It is proposed that PTW containing H_2O_2, NO_x, and specifically NO might play a prominent role in plant responses to PTW (Kang et al. 2019; Adhikari et al. 2019). It has been reported that PTW irrigation resulted even in enhanced endogenous levels of H_2O_2 and NO_x in tomato seedlings (Adhikari et al. 2019). Both, H_2O_2 and NO, are

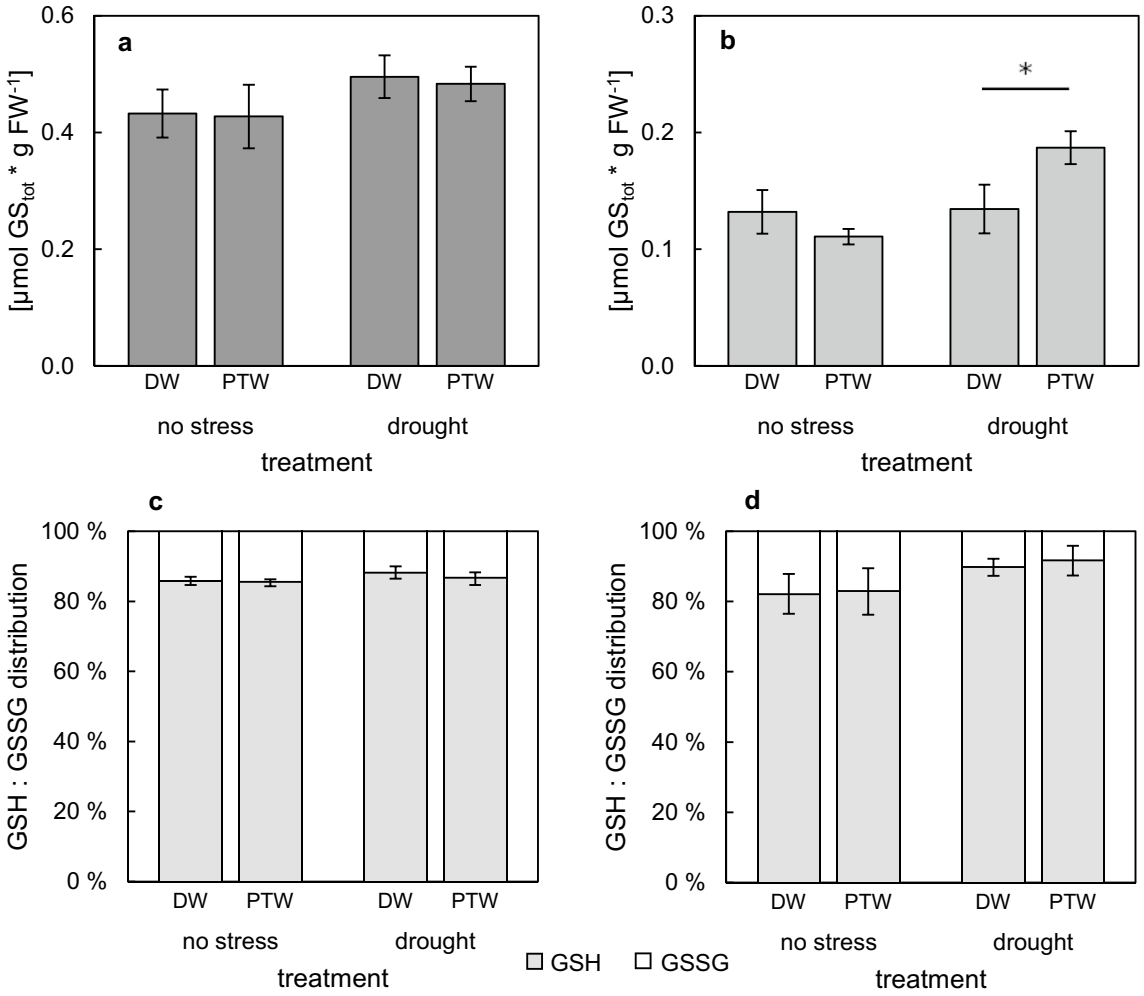

Fig. 6 Total glutathione content (GS_{tot}) in leaves **a** and root **b** and distribution **c**, **d** of reduced glutathione (GSH; light gray bars) and oxidized glutathione (GSSG; white bars) after treatment with deionized water (DW) or plasma-treated water (PTW) under no stress and drought stress conditions. Mean values (±SD) were calculated from four replicates of each treatment. Bars with an asterisk indicate significance differences ($_{*}$: $p < 0.05$; $_{**}$: $p < 0.01$; $_{***}$: $p < 0.001$). Statistical analysis was performed using student's *t*-test

important signaling molecules in plants and responsible for many short-term and long-term reactions to environmental stresses and developmental factors during plants life cycle (Farnese et al. 2016; Neill et al. 2002; Sanz et al. 2015).

In response to environmental stresses, proline accumulates in many plant species including barley (Hanson et al. 1979). Hence, it was described as reasonable indicator of plant reactions to water deficit (Dar et al. 2016). The higher proline content in drought-stressed plants compared to non-stressed plants implied water deficit and corroborates the efficacy of drought stress application (Fig. 1a, b). Two weeks of recovery following drought application the proline content in drought-stressed plants did not differ from non-stressed plants, which indicates that the drought-stressed plants were recovered (Fig. 1c, d) independently of the PTW treatment.

In this study, PTW treatment sustainably resulted in higher chlorophyll content regardless of the application of drought stress, whereby significantly higher values could be observed for non-drought-stressed plants (Fig. 2a). The effect on photosynthetic pigments caused by PTW was reported by other studies as well (Adhikari et al. 2019; Gierczik et al. 2020; Ndiffo Yemeli et al. 2021) and might be a direct effect of H_2O_2 and NO. This conclusion is supported by studies on the treatment of marigold with NO and H_2O_2 (Liao et al. 2012) or on the treatment of *Ficus deltoidea* (Nurnaeimah et al. 2020) and maize (Gondim et al. 2013) with H_2O_2. Additionally, foliar H_2O_2 treatment prior to osmotic stress enhanced chlorophyll content of pistachio (Bagheri et al. 2021), soybean (Guler and Pehlivan 2016), and quinoa (Iqbal et al. 2018). Although the molecular mechanisms have not been investigated yet, it could be shown that H_2O_2 induces osmolyte accumulation under osmotic stress which in turn can lower the destruction of

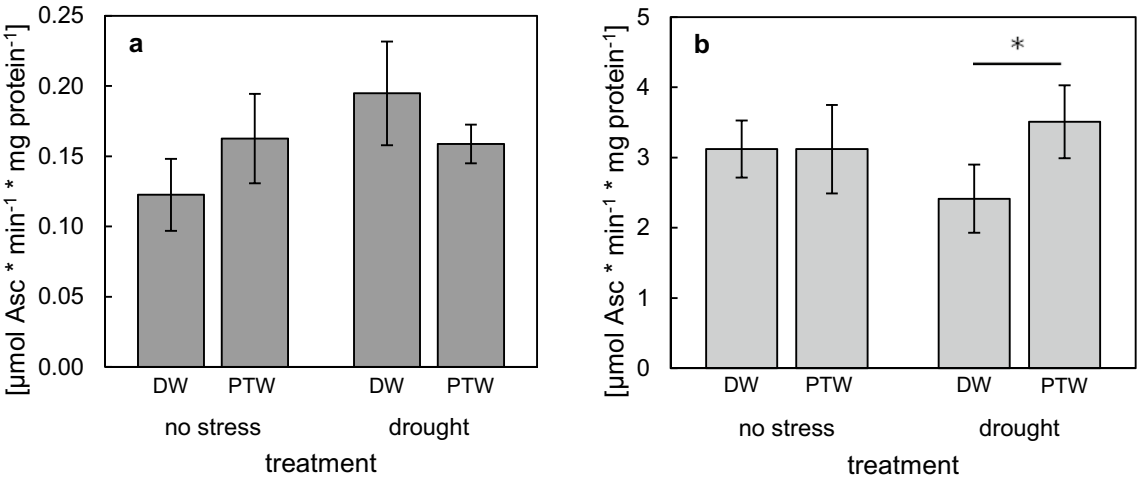

Fig. 7 Ascorbate peroxidase (APX) activity in leaves **a** and root **b** after treatment with deionized water (DW) or plasma-treated water (PTW) under no stress and drought stress conditions. Mean values (± SD) were calculated from four replicates of each treatment. Bars with an asterisk indicate significance differences (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). Statistical analysis was performed using student's *t*-test

chlorophyll by ROS generated in the chloroplast (Farooq et al. 2017).

The quantum yield of PSII followed the pattern of the chlorophyll content: the treatment with PTW sustainably resulted in a higher quantum yield of PSII after no stress and drought stress with significantly higher values for PTW without drought. Škarpa et al. (2020) stated that the quantum yield of the electron transport of the photosystem II was not significantly influenced by foliar PTW application in maize. On the other hand, intensive PTW application on the maize plants leads to damage of the photosystem apparatus (Škarpa et al. 2020). Considering the concentrations of H_2O_2 and NO_x in PTW in this study, they were much lower compared to our treatment. Yemeli et al. (2021) found that watering with PTW increased the concentration of photosynthetic pigments and simultaneously had no or negative impact on net photosynthesis of barley and maize, respectively. They had comparably high concentrations of H_2O_2 (5 times higher) in PTW and watered only with PTW for 4 weeks. Liao et al. (2012) treated marigold explants with NO and H_2O_2. Their data suggest that the application of exogenous NO or H_2O_2 could effectively mitigate the damage of drought stress on leaves by protecting the ultrastructure of mesophyll cells. This was accompanied by a rapid photosynthetic electron transfer rate and higher PS II electron transfer activity under drought conditions. That is in agreement with the presented data, where drought stress resulted in lower values for quantum yield of PS II for DW-treated as well as PTW-treated plants relative to no stress conditions (Fig. 2b). Plants that encountered drought stress after PTW treatment were able to keep the photosynthetic performance up to a higher level as DW-treated plants that did not experience drought.

Asc and GSH have discrete and specific functions in photosynthesis and associated redox signaling (Foyer and Noctor 2009). Following Foyer and Shigeoka (2011), Asc has several important roles in photosynthesis: It is a cofactor for violaxanthin deepoxidase, an enzyme required for nonphotochemical quenching formation, and it participates in the abscisic acid-mediated regulation of stomatal closure. Finally, it can strongly influence the expression of both nuclear and chloroplast genes encoding photosynthetic components (Kiddle et al. 2003). Enhancing the activities of antioxidant enzymes and/or the accumulation of low molecular weight antioxidants by genetic manipulation may increase tolerance to a variety of stresses through more efficient removal of ROS. By improving ROS removal in plant tissue, the photosynthetic processes are desensitized to environmental change (Foyer and Shigeoka 2011).

In this study, the exposure of barley plants to PTW took effect on the plants' antioxidative system. Most of the significant changes occurred in the root after foliar treatment of PTW, drought stress, and subsequent recovery period, revealing that PTW induces systemic signaling.

Values for TAC were significantly higher in leaves and roots upon PTW treatment under drought stress conditions compared to treatment with DW. Elevated values for TAC denote the accumulation of DPPH-reducing agents, which might include antioxidative compounds. In general, higher levels of constitutive or induced antioxidants facilitate tolerance against different environmental stresses including drought stress (Reddy et al. 2004; Miranda et al. 2014). Chutipaijit (2016) stated that TAC indicated by DPPH as reagent may correlate with stress tolerance. In fact, it has been reported that higher levels of DPPH radical scavenging

activity imply abiotic stress tolerance in rice-seedling radicles (Kang and Saltveit 2001) and cucumber-seedling radicles (Kang and Saltveit 2002). In respect of drought, Štajner et al. (2013) and Weidner et al. (2009) used the DPPH assay as a potential parameter for drought stress tolerance. With regard to our study, PTW-treated plants possibly adapted to drought stress more efficiently than DW-treated plants by acquiring the ability to scavenge more RONS as reflected by elevated TAC in leaves and roots. The combined treatment of plants with PTW followed by drought may has resulted in improved detoxification of prooxidants and might has facilitated the induction of drought stress tolerance. It might be possible that the elevated GS_{tot} content in roots under drought stress conditions contributed to elevated values for TAC since the highly reductive thiol group of GSH reacts with DPPH (Viirlaid et al. 2009). In contrast to the elevated values for TAC under drought stress conditions, it must be pointed out that minor values for TAC were observed in leaves and roots under no stress conditions. Considering the recovery phase of 30 days after PTW treatment, it remains questionable whether RONS present in PTW resulted in oxidative stress still visible after that period. With respect to the elevated chlorophyll content caused by treatment with PTW and the low proline content, plants did probably not experience stress at time of harvest. The elevated Asc_{tot} content in leaves of PTW-treated plants compared to DW-treated plants under no stress conditions supports the idea that the antioxidative system was upregulated. Further experiments assessing the TAC with more specific methods are mandatory to evaluate the radical scavenging properties of PTW-treated plants.

The range of lipid peroxidation as determined by the MDA content was significantly higher in roots under drought stress conditions, whereas in leaves, it did not differ significantly between no stress and drought stress conditions upon PTW treatment relative to DW treatment (Fig. 4). Although an elevated MDA content is deemed to be an indicator for oxidative stress, it might also correspond to acclimation processes rather than to damage. Depending on intracellular levels, MDA is described as either toxic or gene activating (Missihoun and Kotchoni 2017) and facilitates expression of abiotic stress genes (Weber et al. 2004). It was suggested that MDA present in low concentrations can implement cell protection under oxidative stress by activating regulatory genes involved in plant defense and development and cellular redox homeostasis. Fine tuning of MDA as a gene activator requires the activity of aldehyde dehydrogenases (ALDHs) (Missihoun and Kotchoni 2017). ALDHs were shown to control MDA levels by catalyzing the oxidation of aldehydes to the corresponding carboxylic acid utilizing $NADP^+$ as the oxidizing agent. For its part, ALDH activity can be induced by H_2O_2 (Zhao et al. 2018). Summarizing, MDA levels are highly balanced in plants and may act as a signal molecule and not as a damager. Cui et al. (2010) suggested that lipid peroxidation and increasing H_2O_2 levels might be involved in the activation of secondary metabolite accumulation. In our study, such an accumulation might count for the elevated TAC in roots under drought stress conditions.

Another indicator of the upregulation of the antioxidative system caused by PTW in roots under drought stress conditions might be the higher percental Asc_{red} pool within the Asc_{tot} content (Fig. 5d). It is hypothesized that the maintenance of a high Asc_{red}: Asc_{ox} ratio might be a key element for the protection against abiotic stress-induced ROS (Fotopoulos et al. 2010). Contrarily, PTW-treated roots under drought stress conditions exhibited higher Asc_{red}:Asc_{ox} ratios while simultaneously featuring decreasing contents of Asc_{tot} compared to DW treatment. The GSH:GSSG ratio remained unaltered, while GS_{tot} content displayed significantly higher values compared to DW treatment (Fig. 6b). Higher total concentrations of glutathione may indicate acclimation processes (Tausz 2004; Cheng et al. 2015). Even though the GSH pool remains unaltered, the higher GS_{tot} content might imply the enhancement of antioxidative traits.

APX activity was significantly higher upon PTW treatment in drought-stressed roots (Fig. 7b). The increase in synthesis or activity of antioxidant enzymes may facilitate drought stress tolerance (Kusvuran et al. 2016; Sallam et al. 2019). Therefore, the PTW induced elevated APX activity in roots under drought stress conditions might indicate enhanced drought stress tolerance. The treatment of rice (Farooq et al. 2009) and turfgrass (Boogar et al. 2014) with NO donors resulted in the drought stress alleviation by upregulating APX activity. This supports the idea that NO might be one of the key components responsible for the reported effects of PTW.

With respect to the overall enhancement of antioxidative traits effected by PTW, the results of this work indicate that the treatment with PTW itself does not show significant evidence of tolerance development before plants meet the stressor (Fig. 8a, c). The application of drought stress was necessary to obtain visible signs of the upregulation of the antioxidative system and systemic signaling caused by components of PTW (Fig. 8b, d).

In conclusion, this study indicates that components of PTW affect the antioxidative system of barley on a long-term scale. These alterations imply that the treatment with PTW

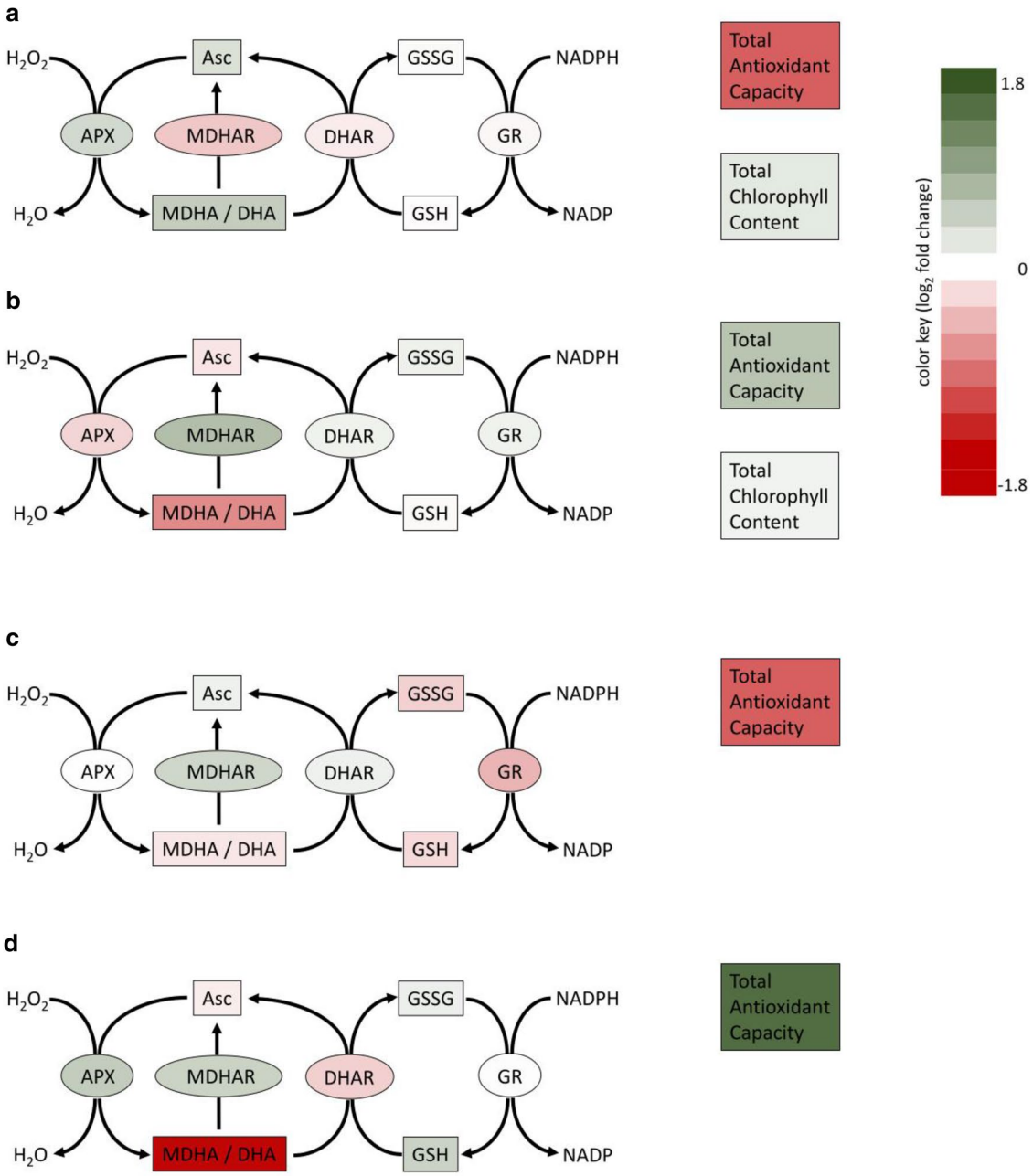

Fig. 8 Ascorbate–glutathione cycle colored with the log_2 fold change after treatment with PTW relative to DW treatment under no stress **a**, **c** and drought stress **b**, **d** conditions in the leaf **a**, **b** and in the root **c**, **d**. Color key of log_2 fold change: green color represents higher values, red color lower values compared to DW treatment. The higher the intensity of the color, the higher the log_2 fold change. *Asc* Ascorbate, *GSSG* Oxidized glutathione, *GSH* Reduced glutathione, *DHA* Dehydroascorbate, *MDHA* Monodehydroascorbate, *APX* Ascorbate peroxidase, *DHAR* Dehydroascorbate reductase, *MDHAR* Monodehydroascorbate reductase, *GR* Glutathione reductase (Color figure online)

might lead to enhanced drought stress tolerance and renders PTW as a putative priming agent.

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Author Contributions FB, CaSch: devising the experiment and statistical analysis; FB, CaSch, AK, HB: data collection; FB: writing; CaSch, AK, HB, ChSt: revising and reviewing the manuscript; ChS: funding acquisition, project design and supervision.

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Data Availability The data supporting the findings of this study are available from the corresponding author, Christine Stöhr, upon request.

Declarations

Competing interest The authors declare no conflict of interest.

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