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Optimising plasma-activated water applications for enhanced growth and antioxidant capacity in maize hybrids: a comparative study of kernel priming, cultivation, and foliar application

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Abstract

Cold atmospheric plasma (CAP) and plasma-activated water (PAW) have emerged as promising tools with potential applications in the agricultural sector. The reactive oxygen and nitrogen species present in CAPs and PAWs have been reported to promote seed germination, enhance plant growth, and improve stress tolerance. The objective of this study is to investigate the effects of PAW on selected maize hybrids, focusing on its application methods, including kernels priming, short cultivation, and foliar application. The application of PAW for kernel priming significantly enhanced growth, with improvements noticed in root and shoot length, leaf area, fresh weight, water uptake, and accelerated lignification. Additionally, an increase in carotenoid and phenolic concentrations was observed in the leaves. When PAW was applied during cultivation, minimal improvements were observed compared to cultivation with tap water. Further, foliar application of PAW was observed to increase carotenoid content in the leaves, enhancing antioxidant capacity. This application also yielded the most notable outcomes in terms of growth parameters and carotenoid concentrations. On the other hand, it did not affect the activity of guaiacol-peroxidase, nor did it influence the concentration of phenolics and chlorophylls. These findings collectively suggest that PAW may be beneficial for enhancing antioxidant capacity in maize, potentially improving resilience under abiotic stress. Further research into the optimization of PAW composition and timing of its application could maximise these benefits, contributing to more sustainable crop production.

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Keywords: maize, transient spark discharge, plasma-activated water, priming, foliar application, peroxidases, phenolics

1. Introduction

In recent years, meeting the demands of agriculture has become increasingly challenging. We are experiencing a growing population, and on the other hand, crop losses due to climate changes and abiotic or biotic stress factors. The search for approaches to traditional agriculture with fewer adverse effects on the environment while efficiently achieving higher yields is therefore of a great interest.

Cold atmospheric plasma (CAP) technology has already proved its potential in agricultural applications. Plasma was first introduced to biomedicine and agriculture as an antibacterial agent due to its physical-chemical properties resulting in reactive nature, i.e. electromagnetic fields, UV radiation, electrons, free radicals, charged particles, or reactive oxygen and nitrogen species (RONS). It can be applied to seeds, seedlings, and plants directly or indirectly (Misra *et al* 2016, Puač *et al* 2018, Attri *et al* 2020).

Effects of direct CAP application on seeds have been extensively studied using different plasma sources, working gases, treatment conditions and types of seeds (Waskow *et al* 2021, Bilea *et al* 2024). CAP sterilizes the seed coat, affects organic polymers in seed, improves its hydrophilicity and increases water uptake. These structural changes together with CAP-triggered signalling pathways in the seeds lead to stimulated germination, improved plant growth and stress tolerance, and changes in metabolism (Bormashenko *et al* 2012, Mitra *et al* 2014, Stolárik *et al* 2015, Los *et al* 2019, Mildaziene *et al* 2021).

Another approach is indirect CAP application via plasma-activated water (PAW). Plasma treatment of liquids induces chemical reactions and alters their physical properties and chemical composition. These changes depend on the type and power of plasma discharge, working gas, treatment time, volume and type of treated liquid (Thirumdas *et al* 2018, Bradu *et al* 2020). RONS generated by the plasma in gas dissolve into water, change its pH, conductivity, oxidation-reduction potential, and its chemical composition. For plasmas generated in air, the main relatively long-lived aqueous RONS in PAW are hydrogen peroxide (H_2O_2), nitrite (NO_2^-), and nitrate (NO_3^-) and PAW often exhibits a strong oxidizing capacity (Lukes *et al* 2012, Machala *et al* 2019). Reactive oxygen species (ROS) trigger various signal and metabolic cascades in plant cells, and reactive nitrogen species (RNS) serve as a source of nitrogen nutrition. Taken together, when PAW is used for seed imbibition or watering, it can promote and accelerate seed germination through induced hormonal changes, enhance seedling development and plant growth, improve photosynthetic pigments content, and alter antioxidant enzymes activities (Zhang *et al* 2017, Kucerova *et al* 2019, Lukacova *et al* 2021, Che *et al* 2024). Due to their antimicrobial nature, plasma and PAW also showed promising effects in plant disease control (Adhikari *et al* 2020, Filatova *et al* 2020).

However, excessive levels of H_2O_2 and NO_2^- may negatively affect physiological processes in plants by triggering oxidative stress (Niu and Liao 2016), a common phenomenon in plants under various stress conditions. Disturbing the RONS homeostasis can lead to the degradation of chlorophylls, reducing photosynthetic activity, and having many other negative effects on the plant developmental processes. In response, plants activate various non-enzymatic (e.g. phenolic compounds, carotenoids) and enzymatic antioxidants (e.g. peroxidases (POX), catalase, superoxide dismutase) or undergo structural modification (e.g. enhanced or stronger lignification) to cope with the adverse conditions caused by increased RONS in the cells (Lukačová *et al* 2013, Mišúthová *at el* 2021, Barzotto *et al* 2023). Interestingly, phenolic compounds, secondary plant metabolites exhibiting wide antioxidant activities (Kumar *et al* 2020), are also precursors for polymeric lignin in the cell walls. On the other hand, tissue-specific lignification and cell wall metabolism are associated with POX (guaiacol peroxidase, G-POX). These antioxidant enzymes do not directly decompose H_2O_2 but oxidize different substrates (e.g. phenolics, monolignols) reducing H_2O_2 to water (Gill and Tuteja 2010).

Contrary to typical stress response, priming, also called an adaptive response, is a technique that involves using a mild amount of a stressor on an organism as a pretreatment to prepare plants for better adaptation to potential stress conditions in the future. It enhances their tolerance to stress by triggering specific signalling pathways, where RONS as signal molecules are involved (Holubova *et al* 2020). Several studies have demonstrated plasma-induced improvements in plant responses to drought stress (Adhikari *et al* 2020), osmotic stress (Gierczik *et al* 2020), and arsenic stress (Lukacova *et al* 2021). The plasma applied directly or indirectly via PAW serves as an efficient priming agent providing plants with a low amount of RONS.

There are numerous studies dealing with the effects of plasma or PAW on maize, an agronomically important crop. They have focused on the direct treatment of kernels with plasma discharge and reported effects such as surface disinfection, germination and seedling growth stimulation, changes in root anatomy and antioxidant enzymes, or higher crop yields (Henselova *et al* 2012, Zahoranova *et al* 2018, Holubova *et al* 2021). On the other hand, the number of research papers on the effects of PAW on maize is limited. PAW has been applied in different ways and at various developmental stages from simple soaking maize kernels in PAW (priming) (Lukacova *et al* 2021, Mogo *et al* 2022), to using PAW for irrigation

seedling and plants (Yemeli *et al* 2020), or combination of both (Dahal *et al* 2024). To our knowledge, only one study focused on PAW's foliar application on maize (Škarpa *et al* 2020).

In the present study, we analysed the effects of PAW generated by transient spark (TS) discharge on three maize hybrids grown in the Slovak field conditions in three separate types of experiments. The effects of PAW were compared across its three application methods: (1) applied solely for the imbibition of kernels, (2) applied during short-term cultivation, and (3) applied foliar during longer-term soil cultivation. First, PAW was tested in maize priming during a 3 day cultivation period in paper rolls. Next, the longer-term effects of PAW priming were examined in 14 day pot experiments. Lastly, one hybrid was selected for further study, where, in addition to the 14 day pot experiment, foliar application of PAW was performed at different developmental stages. The primary objective was to determine the optimal use of PAW and its application method that would effectively prepare plants for enhanced resilience and improved performance under potential stress conditions.

2. Material and methods

2.1. Plant material

Kernels of maize (*Zea mays* L.), hybrids *Bejm, Markiza,* and *Torena,* were purchased from RWA Slovakia Ltd. The kernels were stored in a fridge at 8 °C in the dark. Hybrids used in the present study were selected according to their different growth potential and stress reactions. Hybrid *Bejm* is characterised as sensitive and with weaker growth even in the control conditions. On the other hand, Hybrid *Markiza* and especially *Torena* are well growing hybrids and are more tolerant to various stresses. They are suitable for growing in colder areas and recognized for their resilience to adverse environmental conditions (Lukačová and Lux 2010).

2.2. Transient spark discharge and PAW preparation

TS discharge operated in atmospheric air was applied to tap water (TW) to generate PAW. The TS is a direct current (DC)-driven self-pulsing discharge with a repetitive streamer-to-spark transition with short duration of pulses (10–100 ns), high amplitude of current pulses (10–100 A), and high repetition rate (1–10 kHz). The detailed description of its physical properties has been published in past, namely its electrical characterization (Janda *et al* 2011, 2023), optical characteristics (Janda *et al* 2012) including time-resolved emission spectra, spectral lines broadening, and electron density (Janda *et al* 2014).

Figure 1 depicts the system of TS discharge used for PAW generation. The TS discharge was generated by a DC high voltage (HV) power supply (CX-300 A) in a point-to-plane geometry, where HV needle electrode was placed 1 cm above an inclined (45°) grounded electrode fixed in a polytetrafluoroethylene plate. The size of the plate was 31 × 28 × 18 mm. A V-shaped gutter in the plane was 8 mm wide and 6 mm deep. Grounded metallic electrode of 1 mm width was embedded at the bottom of the gutter. A stream of water circulating through the gutter (without a discharge) was approx. 1 mm deep and 2 mm wide. Water circulation from the test tube, via discharge plasma region and back to the test tube was maintained by a peristaltic pump (Masterflex L/S) with a water flow rate 14 ml·min^{-1}. The water activation time was set to 1 min·ml^{-1}. The test tube was placed in an ice bath to maintain a temperature of the generated PAW constant and avoid undesired evaporation and heating that may decrease or even inhibit the germination and viability of seeds. In our previous work we found that without an ice bath water bulk temperature increased by approximately 10 °C and 0.5 ml of water was lost due to evaporation in case of water activation time of 2 min·ml^{-1} (Kučerová *et al* 2019). The discharge voltage was monitored by a HV probe (Tektronix P6015A) and a current probe (Pearson Electronics 2887) both connected with an oscilloscope (Tektronix TBS 2000). Figure 1 shows a typical voltage and current waveforms of the TS discharge in two different time scales. In this study, the applied voltage was set to 10–13 kV, amplitude of current pulses was approx. 3 A and frequency 2–3 kHz.

2.3. PAW composition

PAW was analysed for its physical and chemical properties. The pH was measured using pH probe (WTW SenTix 60). The chemical composition of PAW was measured spectrophotometrically by UV–VIS spectrometer (Shimadzu UV-1900). The concentration of hydrogen peroxide (H_2O_2) was determined based on its reaction with titanyl ions of titanium oxysulfate ($TiOSO_4$) with an absorption maximum measured at 407 nm (Eisenberg 1943). Sodium azide (NaN_3) was used to prevent H_2O_2 reaction with NO_2^- a and stabilize the sample after the plasma treatment. The concentrations of RNS were measured using commercially available kits in accordance with the protocol provided by the manufacturer. Nitrites (NO_2^-) were measured by Griess assay (Cayman Chemicals) with an absorption maximum at 540 nm. The concentration of nitrates (NO_3^-) was evaluated spectrophotometrically with a Spectroquant nitrate assay kit (Merck Chemicals) with an absorption maximum at 330 nm. The reaction times for both methods were 10 min. Sulfamic acid was used to eliminate cross interaction with nitrites. All measurements were conducted at least in three replicates.

2.4. Experimental procedure

Kernels were imbibed in TW (control) or PAW (priming, PAW cultivation) overnight at room temperature and in dark conditions. After imbibition, three types of experiments were conducted which are described below.

1. **Rolls:** During short 3 day cultivation, kernels of three maize hybrids were wrapped in paper rolls (20–25 kernels per roll) and cultivated in TW (control, priming) or PAW (PAW cultivation) freshly generated every day. Young seedlings were

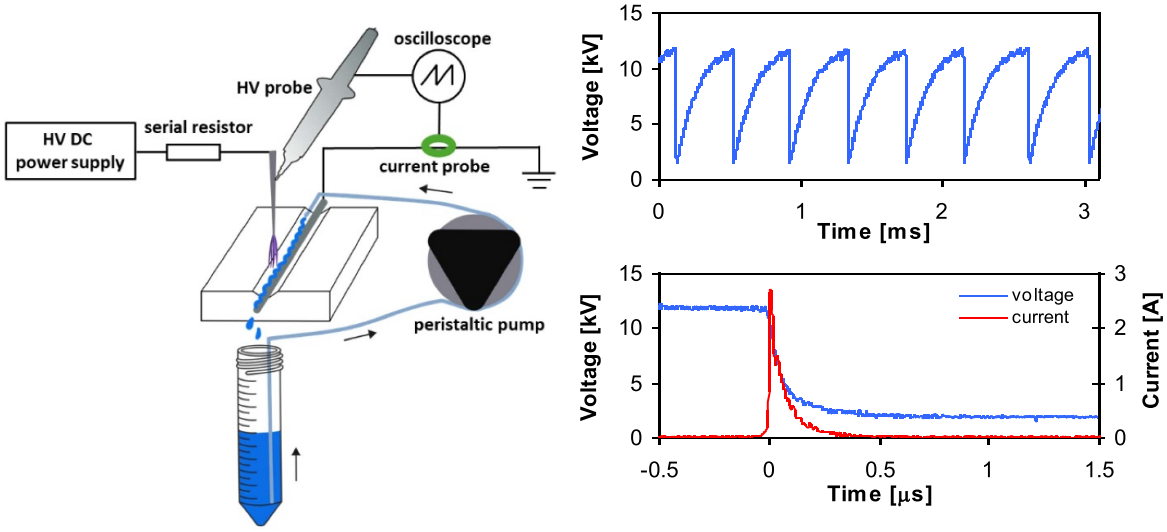

Figure 1. Scheme of the experimental setup for PAW production by TS discharge (left) and voltage and current waveforms in two different time scale (right).

cultivated in dark conditions at 26 °C and 60% relative humidity.

2. **Pot:** 20–25 kernels per pot of control and priming treatments were sown in a 940 g of soil per pot). The soil in pots was watered everyday with 300 ml of TW. After germination (the first 3 d), the experiment was carried out for additional 11 d until the first two leaves were fully developed (total length of experiment was 14 d).
3. **Pot with foliar application:** For the pot experiment with foliar application of PAW, only one hybrid (*Torena*) was chosen. The soil in pots was watered everyday with 300 ml of TW (similar to Pot experiment). In addition, 20 ml of PAW was applied onto the leaves of the control and priming plants on days 3, 6, or 9 after 3 day germination (i.e. on 6th, 9th and 12th day of the 14 day experiment).

After cultivation, below ground and above ground parts of seedlings and plants, respectively, were extracted and analysed as characterised in the table 1.

2.5. Growth parameters

Lengths of the selected organs and fresh weight (FW) were measured. After the FW measurements, samples were wrapped separately and dried at 70 °C for 3 d to constant dry weight (DW). The percentage of DW was then determined as the ratio of dry weight to FW * 100 (%). Leaf area was determined as the ratio of the measured length to the width times the coefficient for maize leaves (0.772) (Květ and Nečas 1966).

2.6. Guaiacol peroxidase (G-POX) activity

To determine activity of G-POX, soluble proteins from maize were extracted. Roots and shoots (approx. 1 g) were gently detached, immediately put into liquid nitrogen, and separately ground. The powder was suspended in 50 mM Na-phosphate protein extraction buffer containing 1 mM EDTA, and 2% PVPP with pH 7.8. The suspension was centrifuged at 12 000 x g and 4 °C for 20 min. The supernatant was used for the measurement of total soluble proteins concentration according to Bradford as a protein reaction with Coomassie Brilliant Blue dye using bovine serum albumin as a standard for calibration. The samples were measured spectrophotometrically at 595 nm (Jenway 6705 UV/Vis) (Bradford 1976).

The specific enzymatic activity of G-POX was evaluated according to (Frič and Fuchs 1970). The method is based on the guaiacol oxidation rate in the formation of brown-red product—tetraguaiacol. The tetraguaiacol production was recorded during the first 60 s of the reaction spectrophotometrically with absorbance at 440 nm (Jenway 6705 UV/Vis). The reaction mixture contained 0.1 M Na-acetic phosphate buffer pH 5.2, the protein sample, 10 mg·ml^{-1} guaiacol, and 1% H_2O_2 which was added to the mixture directly before measuring

$$\text{Specific G} - \text{POX activity} = \frac{\Delta\text{Amin}^{-1} \times 1000}{26.6 \frac{\text{protein content in sample } (\mu g)}{\text{volume of extraction solution (mL)}}}.$$

2.7. Analysis of hydrogen peroxide (H_2O_2) and peroxidase (POX) in situ

Histochemical staining was used to visualise POX activity and H_2O_2 production on the whole seedlings. 4-methoxy-naphtol (4-MN) was used to visualise POX activity. 100 mM Na-acetate buffered solution (pH 5.2) was mixed with 5 mM 4-MN in ethanol. Colourless 4-MN is in the presence of H_2O_2 oxidized by POX into a deep-blue insoluble product (Ferrer *et al* 1990). 3, 3′-diaminobenzidine (DAB) was used as a substrate for a reaction with H_2O_2 and the production of brown-red stain was achieved. DAB (1%) was dissolved in H_2O with pH adjusted to 3.8. For H_2O_2 visualisation, seedlings were incubated in the in dark until the next day.

Table 1. Characteristics of three types of experiments (* indicates an analysed organ).

	1. Roll	2. Pot	3. Pot + foliar application
Duration [days]	3	3 + 11	3 + 11
Cultivation in	Paper rolls	soil	Soil
Hybrids	*Bejm, Markiza, Torena*	*Bejm, Markiza, Torena*	*Torena*
Treatment	**Control** imbibition in TW, cultivation with TW **Priming** imbibition in PAW, cultivation with TW **Cultivation** imbibition in PAW, cultivation with PAW	**Control** imbibition in TW, cultivation with TW **Priming** imbibition in PAW, cultivation with TW	**Control** imbibition in TW, cultivation with TW + foliar application of PAW: day 3, 6, 9 after germination **Priming** imbibition in PAW cultivation with TW + foliar application of PAW: day 3, 6, 9 after germination
Growth parameters	Length, fresh weight, % of the dry weight *primary root and shoot	Leaf area * 1st and 2nd leaf	Leaf area, % of the dry weight * primary main and lateral roots, 1st and 2nd leaf
Analysis of antioxidants	G-POX *primary main root and shoot	G-POX, soluble phenolics *primary main and lateral roots, 1st and 2nd leaf	G-POX, soluble phenolics *primary main and lateral roots, 1st and 2nd leaf
Analysis of peroxidase activity *in situ*	POX, H_2O_2, *primary main root and shoot		
Root anatomy	Lignification *specific tissues		
Analysis of photosynthetic pigments		Chlorophyll *a* and *b*, carotenoids, *1st and 2nd leaf	Chlorophyll *a* and *b*, carotenoids, *1st and 2nd leaf

2.8. Root lignification

To visualise lignin in the free hand cross sections, the primary seminal root of 3 days seedlings was gently washed in the distilled water and cut at the distance of 10% and 50% from the root apex and at the root base to analyse changes in the development of specific root tissue. Autofluorescence of the lignin in the cell walls was observed by fluorescence microscope (Carl Zeiss Axioskop 2 plus), equipped with excitation filter TBP 400 + 495 + 570 nm, chromatic beam splitter TFT 410 + 505 + 585, and emission filter TBP 460 + 530 + 610 nm, documented by a camera system (Olympus DP 72).

2.9. Photosynthetic pigments concentration

The first and second fully developed leaf was selected for the assessment of photosynthetic pigment concentrations. Chlorophylls *a* and chlorophyll *b* (Chl *a* and *b*), and carotenoids were extracted (∼500 mg FW) using a mortar and pestle with 80% acetone (10 ml) and $MgCO_3$ mixed with sea sand to prevent phaeophytin formation. Pigment concentrations were measured spectrophotometrically (Jenway 6400, London, UK) at specific wavelengths (Chl *a*: 663.2 nm, Chl *b*: 646.8 nm, and carotenoids: 470 nm). Calculations of the total chlorophylls (*a* + b) concentration were based on Lichtenthaler's method (Lichtenthaler 1987), with results expressed as milligrams of pigment per gram of FW

$$\text{Chl } a = (12.25 * A663.2 - 2.79 * A646.8) * y$$

$$\text{Chl } b = (21.50 * A646.8 - 5.10 * A663.2) * y$$

$$\text{Chl } a + \text{Chl } b = (7.15 * A663.2) + (18.71 * A646.8)\,/60$$

$$\text{carotenoids} = \frac{(y{*}1000 * A470) - 1.82 * \text{Chl } a - 85.02 * \text{Chl } b}{198}/60$$

$$\text{y} = (\text{volume of acetone used for extraction} * 0.06)\,/\text{FW of material (g)}.$$

2.10. Soluble phenolic concentration

The total soluble phenolic concentration was assessed following the method by Ainsworth and Gillespie (2007) in the first and second leaves and in the roots. The content was quantified using the Folin-Ciocalteau reagent and calculated from a standard curve of gallic acid (0–0.5 $mg{\cdot}ml^{-1}$) and expressed as gallic acid equivalents (GAE) per milligram of FW. Absorbance was measured at a wavelength of 765 nm

$$\text{Total phenolic concentration} = C * V/M$$

where C = concentration of gallic acid established from the calibration curve ($mg{\cdot}ml^{-1}$)

V = volume of the extract solution (ml)

M = weight of the extract (g).

2.11. Statistical analysis

The results were evaluated using Excel (Microsoft Office 365) and statistical software Statgraphics Centurion XVI. One-way ANOVA with *post-hoc* LSD (Least Significant Difference) test was incorporated in all plots to determine the differences between the observed means with the level of significance $P < 0.05$. Also, effects of three independent factors were statistically analysed with three-factor ANOVA (factors hybrid, treatment, and organ) and the results are visualised in the tables. Twenty to thirty plants per one treatment were analysed in every experiment; each experiment was conducted in three independent biological replications.

3. Results and discussion

3.1. PAW composition

The pH of the water remained stable (pH 7.5) and unaffected by the plasma treatment due to its natural hydrocarbon buffering system, unlike of non-buffered liquids (e.g. deionised water) which pH rapidly decreases. The stability of pH is crucial for sustaining plant growth and development, as its changes can induce stress and influence nutrient absorption. The water activation time was set to 1 $ml{\cdot}min^{-1}$ as our previous research indicated the optimal performance in terms of growth parameters (Kučerová *et al* 2019). The concentration of RONS in PAW was 0.66 ± 0.06 mM H_2O_2, 0.88 ± 0.12 mM NO_2^-, and 0.42 ± 0.02 mM NO_3^-. These species are important components of PAW for agricultural purposes, as they can facilitate seed germination, enhance plant growth, or regulate the stress response.

3.2. Rolls experiment

To investigate the early effects of PAW during imbibition and subsequent short cultivation, the chosen maize kernels were imbibed overnight and subsequently cultivated in paper rolls for 3 d in the dark as described previously. We observed inter-hybrid differences in plant responses to the treatment in growth parameters (i.e. length, FW and % of the dry weight) of primary organs, visualisation of POX activity and H_2O_2 accumulation in the whole seedlings and lignification of the root tissue on free hand cross sections.

3.2.1. Growth parameters. Within measured growth parameters, similar trends were observed in the length and FW of both, primary root and shoot (figure 2, table 2), where in controls, hybrid *Bejm* exhibited the shortest length (figure 2) and the lowest FW of the primary root and shoot (figure 3) while *Markiza* showed the greatest root and shoot lengths (figure 2). When controls were compared with priming treatment, significant increase in the length was observed in all hybrids and both organs, while significantly positive effect of priming on FW was observed only in *Bejm* and *Torena* root and *Bejm* shoot (figure 2, table 3). In general, for cultivation in PAW, the only significant improvement in the root and shoot length was seen in *Torena* and in the shoot of *Bejm* (figure 2). Significantly negative effect of cultivation in PAW was observed in *Markiza* root length and FW indicating potential problem of applying higher amounts of PAW during cultivation in terms of primary response of the hybrid. While PAW imbibition stimulated growth, a subsequent cultivation in PAW likely caused stress to the plants resulting in no growth improvement. The stress response in plants is a complex and multifaceted process involving a delicate balance between primary metabolism (e.g. growth, water uptake) and secondary metabolism (e.g. antioxidant defence mechanisms). While hybrid *Markiza* may be more tolerant to stress, the effects of PAW observed in this study were on very young plants. At this early stage, growth reduction or altered primary metabolism might reflect a strategic shift, where the plant diverts energy towards biochemical readiness—such as enhanced antioxidant capacity or other defence mechanisms. These early observations could indicate that the plant is preparing itself for more effective stress mitigation later in development. Therefore, a temporary reduction in growth does not necessarily equate to a negative outcome, but rather a coordinated response aimed at improving long-term stress resilience.

In the previous study, cultivation increased the root and shoot length by 13% comparing with the control, however, different maize hybrid was used in this study (Lukacova *et al* 2021). These findings demonstrate that the impact of PAW on plants is not solely dependent on the specific plant species, but also on the specific hybrid what is a commonly known phenomenon also among the plant stress reactions (Lukačova *et al* 2013).

Contrary to the FW, percentage of the dry weight (% DW) was reduced in all priming treatments, indicating improved water uptake induced by PAW imbibition. Moreover, in the roots of *Bejm* and *Markiza*, the cultivation in PAW resulted in higher biomass accumulation and less water (figure 4).

Several studies have demonstrated enhanced water uptake of seeds after PAW treatment. Dahal *et al* observed decreased water contact angle and improved water uptake of maize and pea seeds induced by PAW imbibition, with aqueous

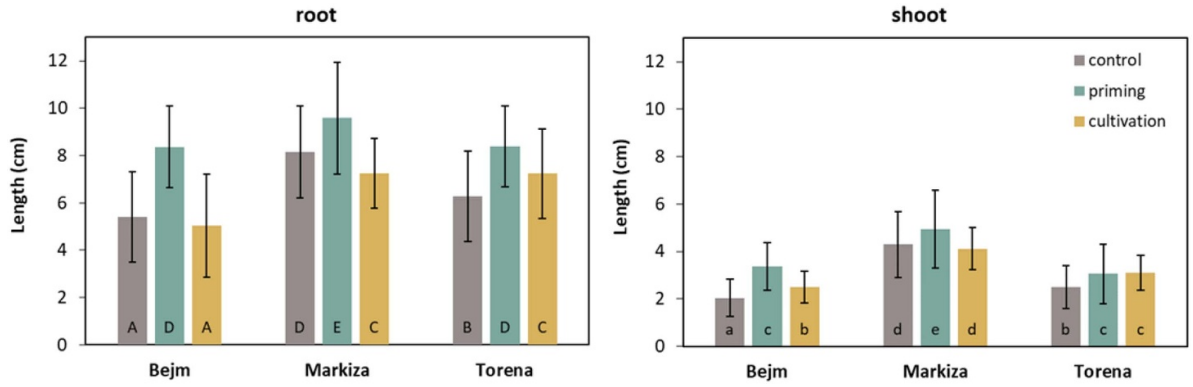

Figure 2. Length of the primary root and shoot of maize seedlings after 3 day cultivation in paper rolls. Letters indicate significant difference between groups based on one-way ANOVA and LSD post-hoc test.

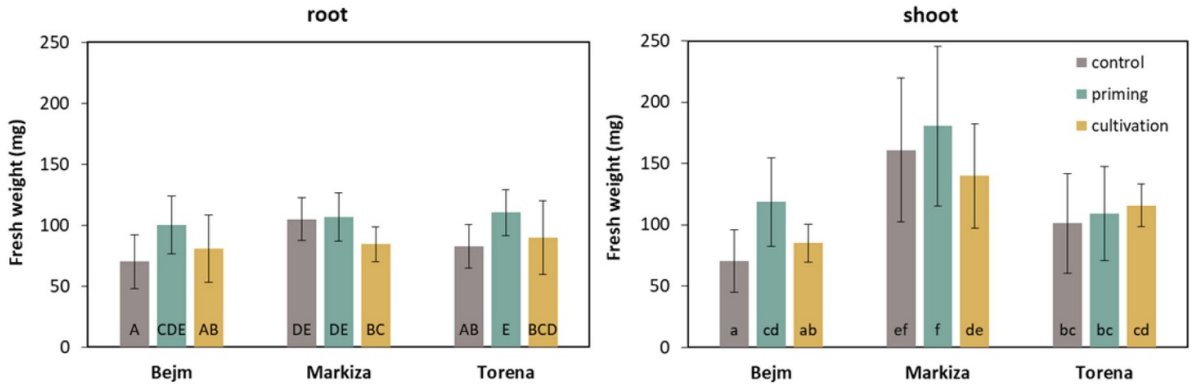

Figure 3. Fresh weight of the primary root and shoot of maize seedlings after 3 day cultivation in paper rolls. Letters indicate significant difference between groups based on one-way ANOVA and LSD post-hoc test.

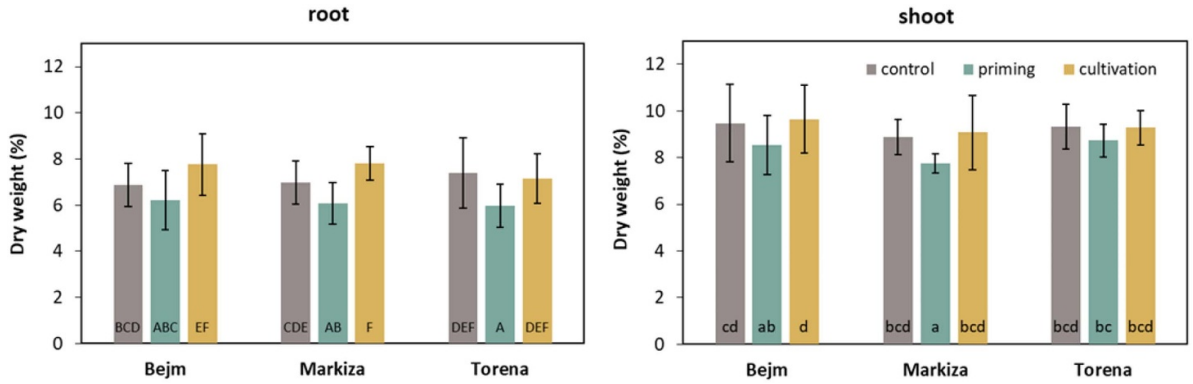

Figure 4. Percentage of the dry weight of the primary root and shoot of maize seedlings after 3 day cultivation in paper rolls. Letters indicate significant difference between groups based on LSD post-hoc test.

RONS playing a key role in these processes (2024). Kučerová *et al* reported improved water uptake of the wheat (*Triticum aestivum L.)* seeds (Kučerová *et al* 2019). In our case improved water uptake contributes to enhanced germination and growth, i.e. length and FW of primary organs.

According to three-factor ANOVA (table 2), the factors treatment and organ have highly significant impact on all three analysed growth parameters. There was no significant difference in the % DW within hybrids, while the treatments and organs had highly significant effect on the % DW.

3.2.2. POX activity. POX are enzymes that participate in multiple processes connected to plant development, and they are responsible for the reduction of H_2O_2. Their production is often triggered in response to various environmental stresses. In our research, PAW insignificantly affect the overall activity of G-POX in roots and shoots after 3 days cultivation (data not shown). This result suggests that RONS in PAW did not induce a stress response in plants in the first three days of their development. Insignificant effect of PAW on G-POX activity in young seedlings was also reported in pea (*Pisum*

Table 2. A three-way analysis of variance (ANOVA) with an impact of the factors 'treatment' (control, priming, cultivation; A), 'organ' (root, shoot; B), 'hybrid' (Bejm, Markiza, Torena; C) or their combination on the observed parameters. 'NS' stands for non-significant impact ($P > 0.05$).

	Treatment (A)	Organ (B)	Hybrid (C)	A × B	A × C	B × C	A × B × C
Length	***	***	***	***	***	**	NS
FW	***	***	***	NS	**	***	NS
% DW	***	***	NS	NS	NS	NS	NS

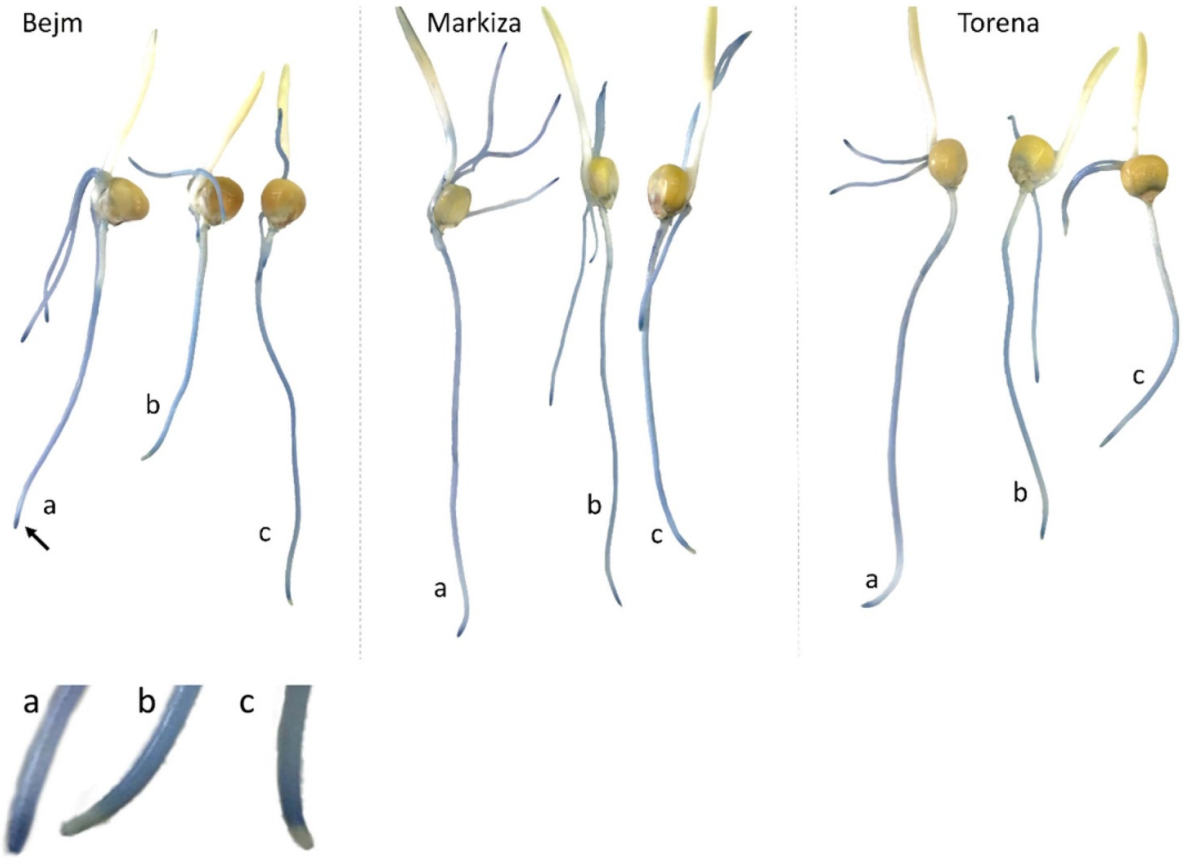

Figure 5. Histochemical visualisation of *in situ* POX activity in 3 day old seedlings. *Bejm, Markiza, Torena*. (a) Control, (b) priming, (c) cultivation. Second picture is a detail on root apexes of *Bejm.*

sativum L.), while significant G-POX activity was reported in barley (*Hordeum vulgare* L.) (Kostoláni *et al* 2021). Changes in the enzymatic activities probably depend on developmental stages and are also plant-species dependent. On the other hand, it is interesting to note that when whole seedlings were stained for POX *in situ* activity visualisation (figure 5), the root apexes (a zone of the highest mitotic activity and cell expansion) of all three controls (indicated as '*a*' in figure 5) exhibited a dark blue staining, indicating a strong POX activity. This is likely related to the normal physiology of the seedlings. However, the seedlings treated with PAW (figures 5(b) and (c)) displayed a lighter or no staining in the apical parts (enlarged picture). This lack of the POX activity in the apical part indicates decrease in the cell expansion capability as the cell wall expansion is joined, behind other factors, with POX. However, mostly an increase in the primary root in PAW priming was noticed, so we propose that this can be an effect of the higher mitotic activity, rather than result of the cell expansion. The consequences of this observation need to be studied more in detail in the future.

On the other hand, PAW treatments (both priming and cultivation) contributed to the accumulation of H_2O_2 in the kernels and along the roots. The highest accumulation was observed in the priming treatment where the kernels were exposed to PAW only during overnight imbibition (figures 6(b) and (c)). The exogenous H_2O_2 in the PAW during the imbibition period likely resulted in the endogenous production of H_2O_2 when the seedlings were no longer exposed to it during the subsequent 3 day cultivation period. Prominent effect of PAW in priming was observed also in length and FW of the primary organs. However, we did not observe any changes in superoxide production along the organs (data not shown). According Kostoláni *et al* 3 day seedlings of pea seeds (*Pisum sativum*) treated with PAW resulted in elevated concentration

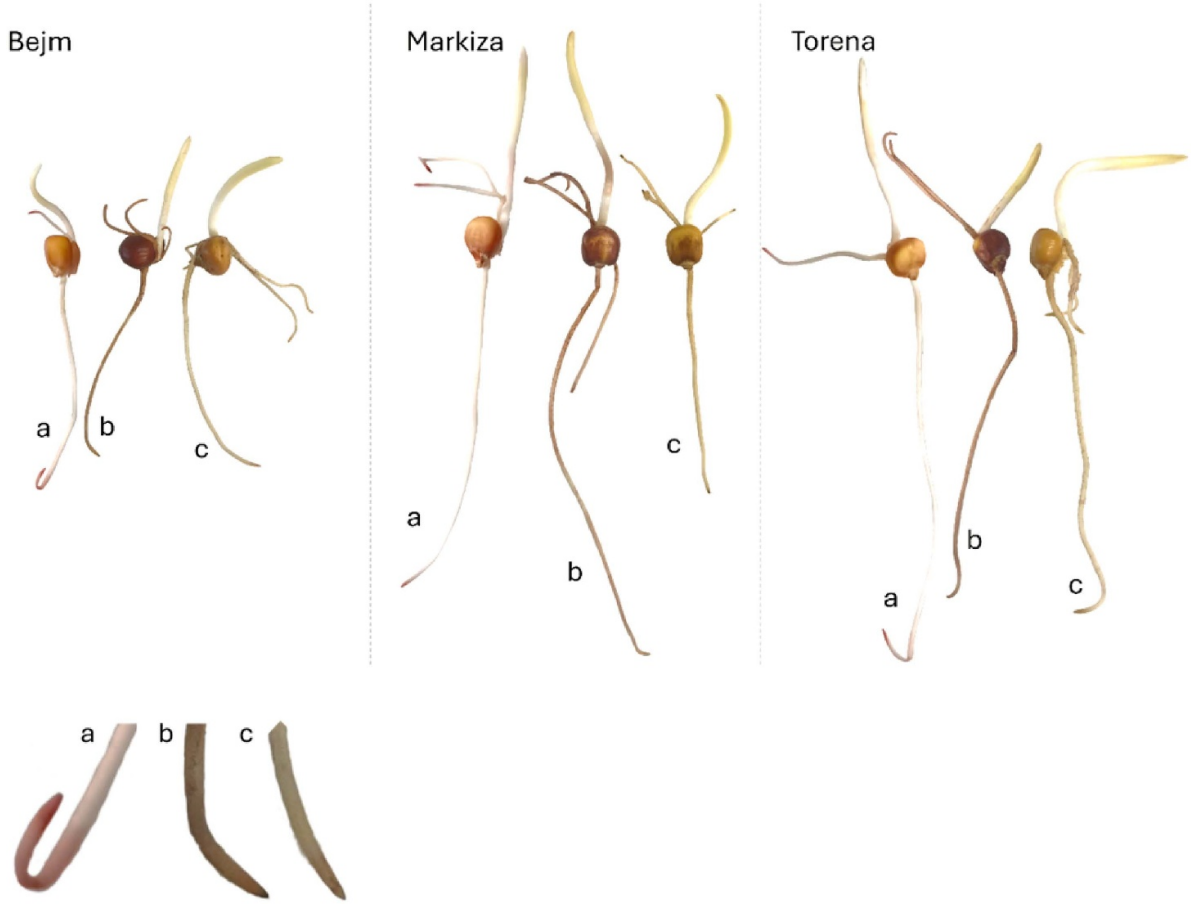

Figure 6. Histochemical visualisation of H_2O_2 in situ in 3 day old seedlings. Bejm, Markiza, Torena. (a) control, (b) priming, (c) cultivation. Second picture is a detail on root apexes of Bejm.

of $\cdot O_2^-$ and H_2O_2 (2021). As mentioned previously, the presence of the elevated H_2O_2 in the root tissues has multiple effect on its development. It serves as a signalling molecule triggering many physiological processes and is also associated with the metabolism of the cell walls (Oh *et al* 2023).

3.2.3. Root lignification. POX in plants are key enzymes that may utilize H_2O_2 to catalyse the formation of cross-links in cell wall components, and lignin formation, helping to fortify the cell wall during stress (Oh *et al* 2023). They catalyse the oxidative polymerisation of lignin precursors, monolignols, and H_2O_2 serves as an electron acceptor (Passardi *et al* 2005). The results demonstrated that the presence of PAW (priming and cultivation) led to accelerated specific root tissue development in terms of their cell walls lignification (figure 7). The lignified secondary cell walls formation was enhanced in the early metaxylems and parenchyma around the late metaxylem vessels in both PAW treatments at the distance of 50% from the root apex. The effect on the metaxylem vessels was obviously stronger in the cultivation (figure 7(c)), especially in *Bejm* and *Markiza* where higher % of the DW were also achieved (figure 4). It was probably associated with more massive lignification on one hand and with the decreased primary growth on the other (figure 2). This observation indicates a positive effect of PAW on root tissue development in the early stages of plant growth. Accelerated lignification enhanced by PAW was also discussed in the previous research (Lukacova *et al* 2021), where improved lignification was observed at the 10% of the root apex, but with no differences at the root base. Root lignification is a crucial process for plants to maintain the integrity and functional water and mineral nutrients transport capacity of the root system. Lignin provides the cell walls also strengthening, structural support and enhancing root defence against pathogens (Bhuiyan *et al* 2009) or abiotic stress factors (Tylová *et al* 2017, Ogorek *et al* 2024) and lignification joined with the apoplasmic barriers development controls the radial flow in the roots.

3.3. Pot experiment

In addition to short 3 day experiments in paper rolls (Chapter 3.2), we conducted an independent set of 14 day pot experiments to compare extended (longer-term) effects of priming the maize kernels without further exposure to PAW. Here we present results in leaf area, photosynthetic pigments concentrations (chlorophyll $a + b$, carotenoids), G-POX activity and soluble phenolics concentration.

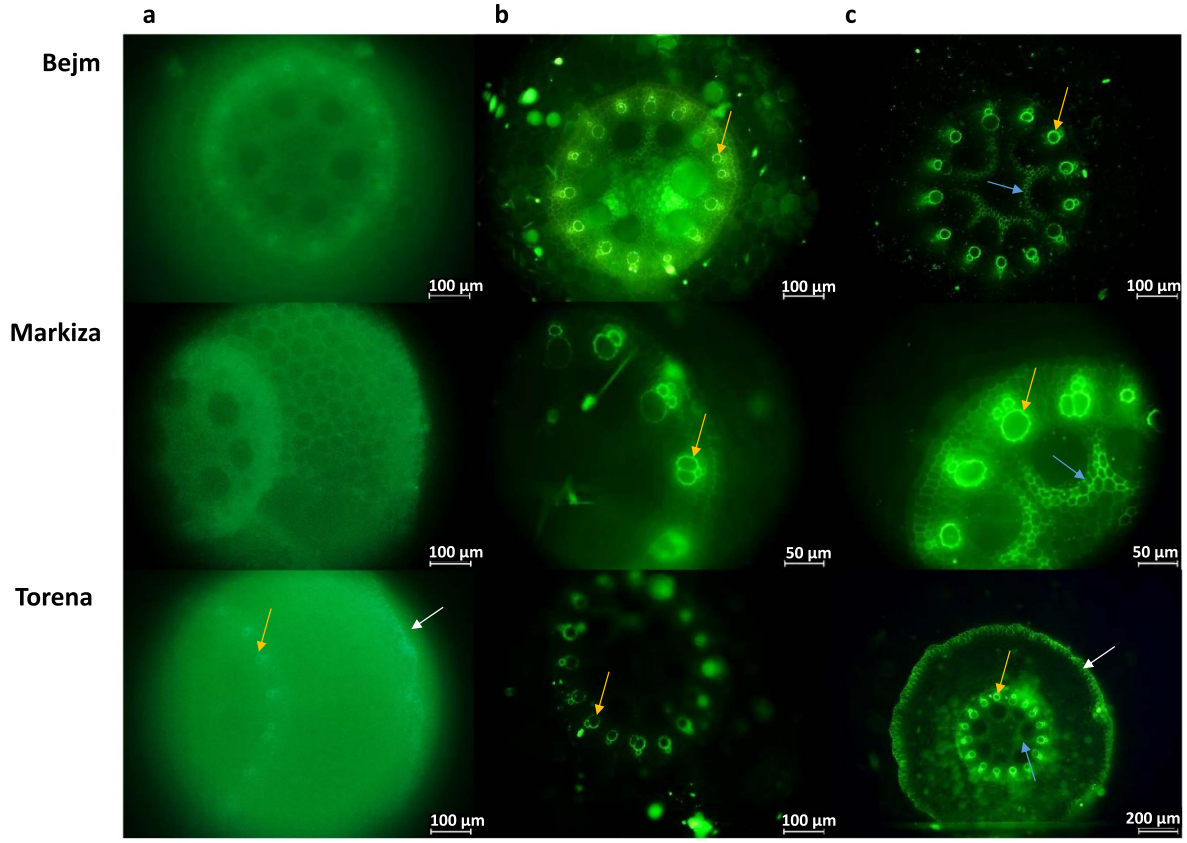

Figure 7. Lignification of the root tissue of three maize hybrids, i.e. Bejm, Markiza, and Torena. Treatments: control (a), priming (b), cultivation (c). The cross sections were taken at 50% from the root apex. The arrows point to exodermis (white), early metaxylem (yellow), and parenchyma around late metaxylem (blue).

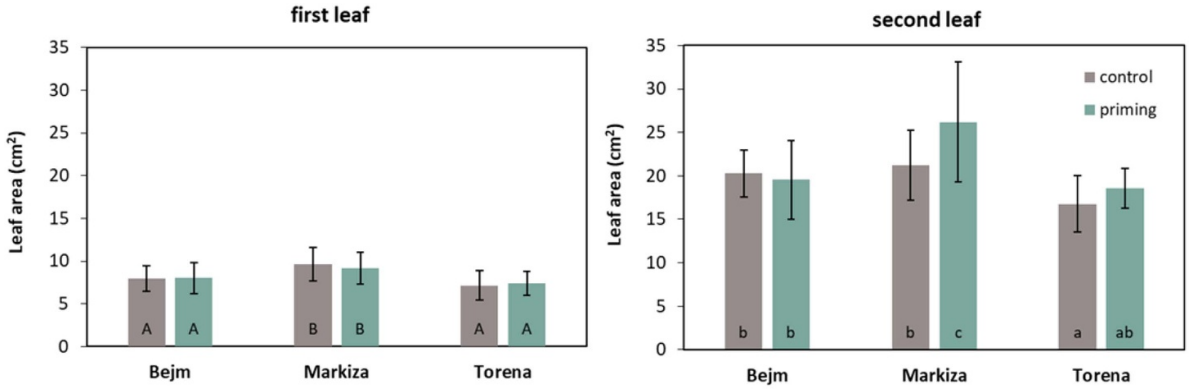

Figure 8. Leaf area of the first and second leaf after 14 day cultivation in pot. Letters indicate significant difference between groups based on LSD post-hoc test.

3.3.1. Growth parameters. In the first leaf, leaf area of controls of *Markiza* was significantly the largest, however with no significant effect of priming. In the second leaf, *Bejm* and *Markiza* controls were significantly greater than *Torena*. Positive effect of the priming was achieved only in *Markiza* (figure 8). Within organs, the priming significantly enhanced the leaf area of the second leaf (figure 8). There are lot of differences between first and second leaf due to their different age. They differ morphologically and mostly biochemically at this stage. Therefore, the response to treatment differs as well. First leaves are typically thicker and shorter undergoing a sequence senescence leading to gradual naturally occurring chlorophyll degradation. Younger leaves are more focused on the photosynthesis; therefore they contain more chlorophylls.

3.3.2. Photosynthetic pigments. Chlorophyll $a + b$ concentration in controls of the first leaf (figure 9) was significantly the lowest in *Bejm*. However, priming caused significant increase of the chlorophylls concentration in *Bejm*, but

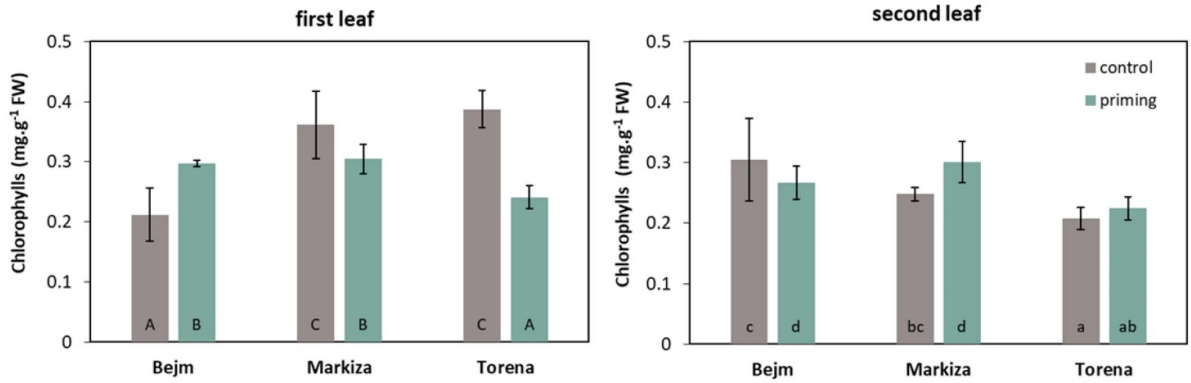

Figure 9. Chlorophyll a + b concentration of the first and second leaf after 14 day cultivation in pot. Letters indicate significant difference between groups based on one-way ANOVA and LSD post-hoc test.

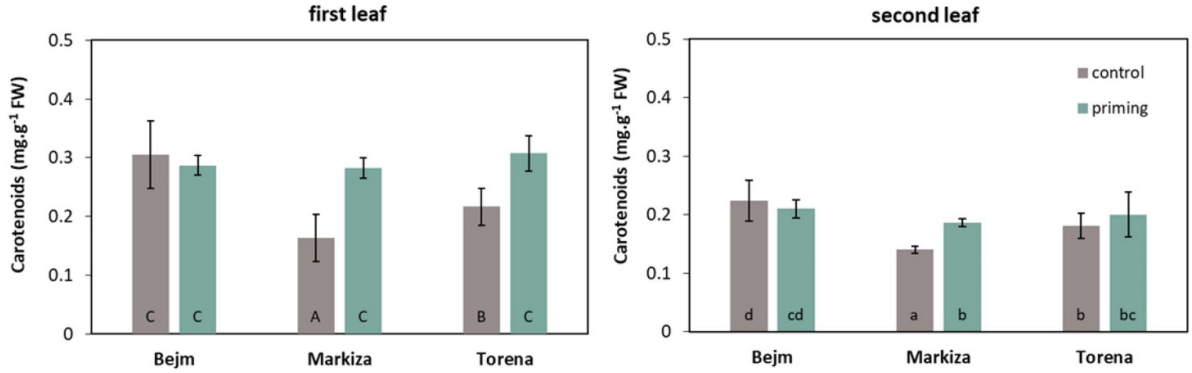

Figure 10. Carotenoids concentration in the first and second leaf after 14 day cultivation in pot. Letters indicate significant difference between groups based on one-way ANOVA and LSD post-hoc test.

a significant decrease in two other hybrids. In the second leaf (figure 9), the lowest concentration of the chlorophylls was achieved in both treatments of *Torena*. Priming had a significantly negative effect on the *Bejm* and positive effect on the *Markiza*, the exact opposite effect as in the first leaf. NO_3^- is an important compound for chlorophyll production (Agnihotri and Seth 2016). Stoleru *et al* (2020) compared effects of two PAWs with different NO_3^- concentrations applied on seeds of *Lactuca sativa* L. and found higher NO_3^- concentration exhibited significant increase of the chlorophyll concentration (Stoleru *et al* 2020). In our study, concentration of NO_3^- in PAW was much higher but production of chlorophylls was not stimulated, indicating the effect of NO_3^- is probably plant-species dependent.

Carotenoids are non-enzymatic antioxidants that play a crucial role in photosynthesis and photoprotection (Swapnil *et al* 2021). Their concentration in the first leaf (figure 10) control was the highest in *Bejm* and the lowest in *Markiza*. Significant increase in priming was observed in *Markiza* and *Torena*. The same was observed in the control of the second leaf (figure 10), however, the only significant increase was shown in *Markiza* when compared with the control. Overall, the carotenoids concentration in the first leaves was significantly enhanced by the priming. The increased accumulation of carotenoids in plants treated with PAW is one of the most significant findings. These antioxidants are essential for enhancing plant responses to abiotic stress. Elevated ROS after PAW treatment can affect metabolic pathways, potentially stimulating the expression of genes involved in carotenoid biosynthesis, including key mevalonate and non-mevalonate pathway enzymes essential for isoprenoid formation (Carvalho *et al* 2011, Misra *et al* 2019).

3.3.3. G-POX. In the plants with PAW-primed kernels, the activity of G-POX was enhanced in the first leaves of all hybrids, and within the second leaves only in *Markiza*. In the roots, where the activity of antioxidant enzymes is generally higher (here by an order of magnitude, G-POX activity was significantly enhanced by priming only in *Torena*. In two other hybrids a decrease was observed (figure 11). POX may not be the primary enzymes responding to PAW-induced changes in roots. Significantly lower activity of antioxidant enzymes (G-POX, catalase, superoxide dismutase) was also observed on wheat (Kučerová *et al* 2019) attributed to the increasing concentration of RNS, mainly nitrates. Other antioxidants or metabolic systems might take precedence, resulting in lower POX activity. Further testing could involve measuring other antioxidant activities (e.g. catalase, G-POX) in both roots and leaves to see if they compensate for the observed POX activity.

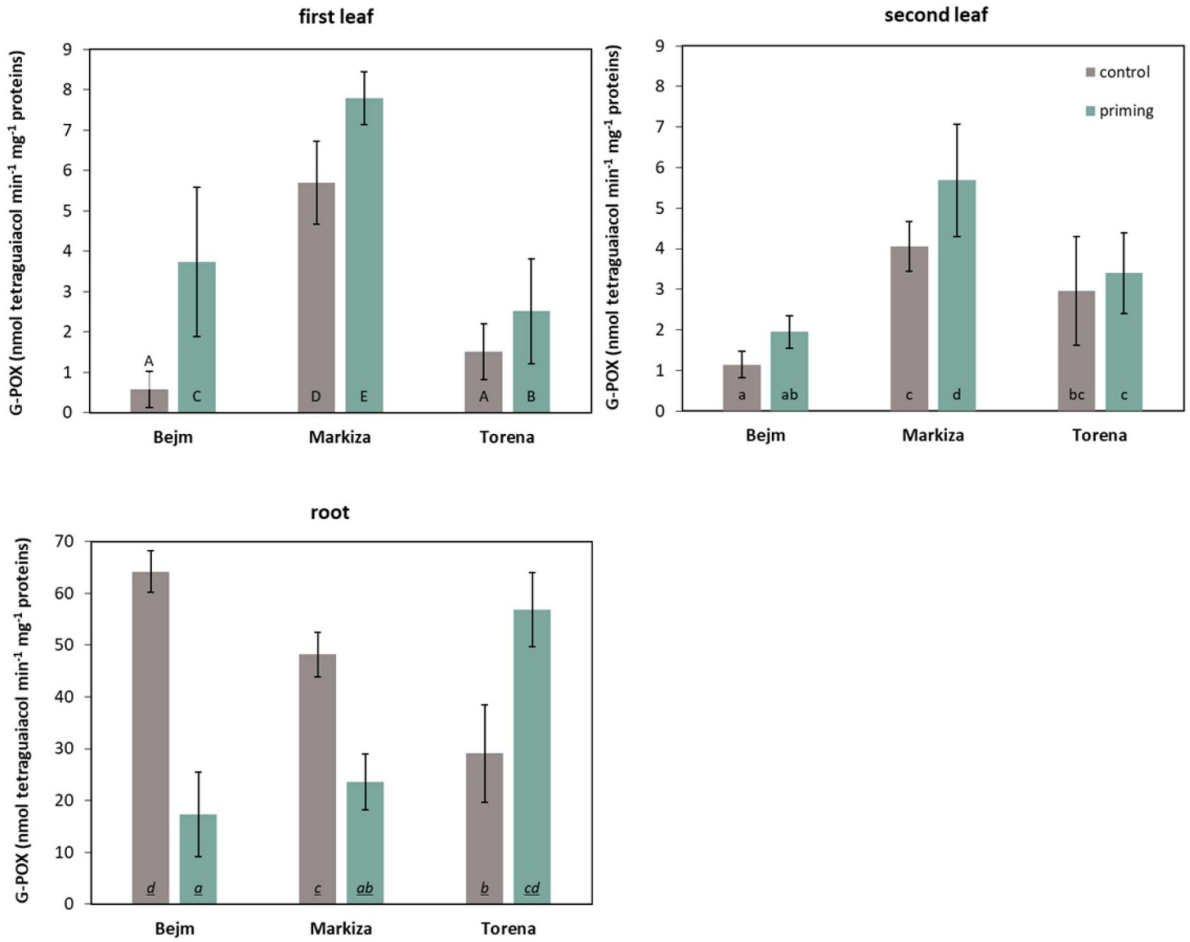

Figure 11. Activity of G-POX in the first, second leaf and root after 14 day cultivation in pot, letters indicate significant difference between groups based on one-way ANOVA and LSD post-hoc test.

3.3.4. Soluble phenolic concentration. Phenolic compounds are other antioxidants with extraordinary significance in the plant growth and development. In our study, significantly highest concentration of soluble phenolics in control was achieved in *Bejm* in all three analysed organs (figure 12). In the first leaf, a negative effect of priming was observed, where concentration of phenolics decreased in all three hybrids in comparison with controls. The opposite effect was observed in the second leaf, where phenolics concentration significantly increased in the priming. In the roots, significant increase was obtained in *Bejm* and *Torena* in the priming.

There are very few studies dealing with and explaining the content of non-enzymatic antioxidants accumulation in the plants after PAW treatment that we could confront our results with. One example is underwater plasma treatment of seeds of water spinach (*Ipomoea aquatica*) significantly improved total phenolic content. Compared with priming of seeds with direct plasma treatment, activities of and soluble phenolics were increased in cayenne (*Capsicum annuum*) (Iranbakhsh *et al* 2018).

The three-factor ANOVA (table 3) showed highly significant effect of treatment, particularly on carotenoid content, G-POX activity, leaf area and phenolics content. The effect on chlorophylls was the least pronounced but still statistically significant. In addition, the hybrids showed highly significant differences in most of the traits, except for G-POX activity. Also, all tested organs achieved statistically significantly different results of all measured parameters.

3.4. Pot with foliar application experiment

For the third set of experiments, a foliar application of PAW during 14 day pot experiment, the hybrid *Torena* was chosen according to the results obtained from two previous experiments, mainly for enhanced carotenoids concentration and G-POX activity. Carotenoids and the activity of antioxidant enzymes (G-POX) are good indicators of how plants internally cope with stress factors or how are they prepared to overcome it in the future. PAW was applied foliar on the day 3, 6, or 9 after germination (the first 3 d after sowing the kernels) either on control plants or plants primed with PAW (described

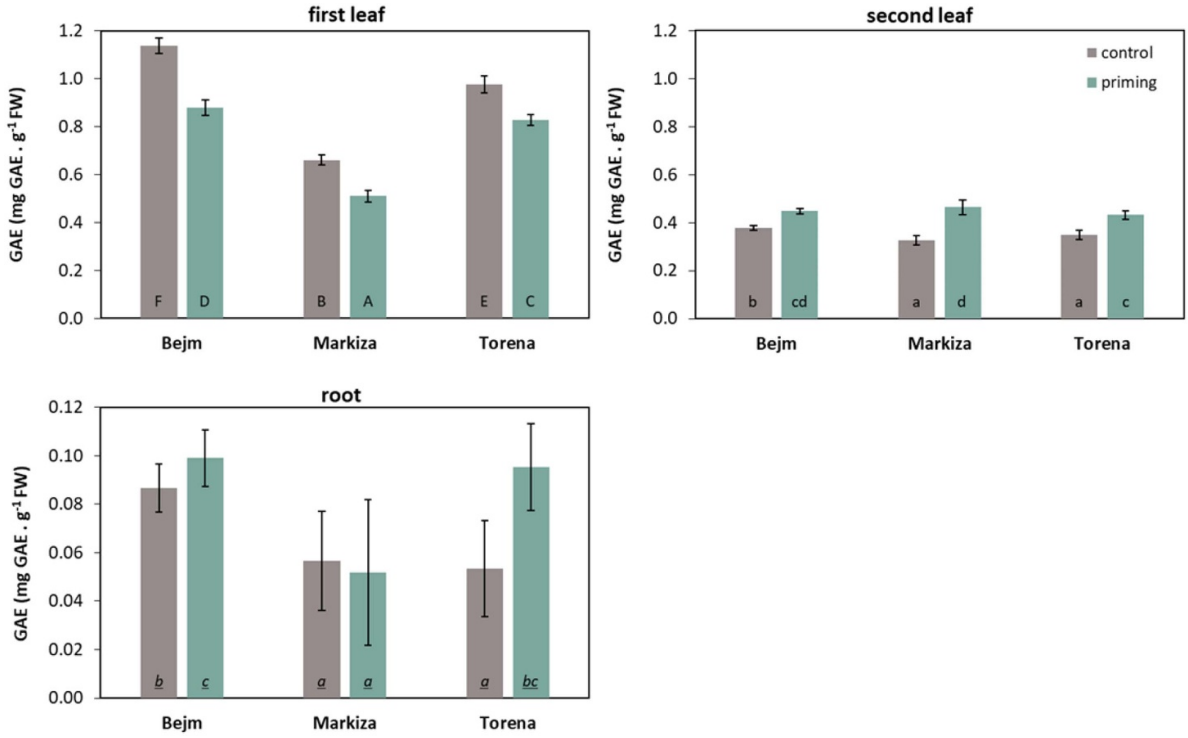

Figure 12. Concentration of soluble phenolics in the first, second leaf, and root after 14 day cultivation in pot. Letters indicate significant difference between groups based on one-way ANOVA and LSD post-hoc test.

Table 3. A three-way analysis of variance (ANOVA) with an impact of the factors 'treatment' (control, priming; A), 'organ' (root, first leaf, second leaf; B), 'hybrid' (Bejm, Markiza, Torena; C) or their combination on the observed parameters. 'NS' stands for non-significant impact ($P > 0.05$).

	Treatment (A)	Organ (B)	Hybrid (C)	A × B	A × C	B × C	A × B × C
Leaf area	**	***	***	**	*	***	**
Chlorophylls	*	***	***	**	***	***	***
Carotenoids	***	***	***	***	***	NS	*
G-POX	***	***	NS	***	***	***	***
Phenolics	**	***	***	**	*	***	**

earlier) to distinguish between the effects of PAW applied at different shoot developmental stages.

3.4.1. Growth parameters. Foliar application of PAW significantly increased leaf area of the first leaf in the priming after 9 d (figure 13). There was a similar effect in the second leaf, but no difference was found between the control and priming; in both cases, the second leaf area was significantly larger.

Effects of PAW on the % DW was significant only in the roots (figure 14). Significantly positive effect of priming with foliar application on the % DW was observed only on day 9. Positive effects of foliar application on day 9 was also observed in the leaf area (figure 13). Škarpa *et al* (2020) reported insignificant effect of periodical foliar application of PAW on dry weight of above ground parts of maize. The repeated application of PAW to leaves of the rice (*Oryza sativa* L.) resulted in a significant enhancement of growth parameters, including dry weight, plant height and stem diameter (Rashid *et al* 2021). According to our observation it is obvious, that the effect of PAW on the plant growth is developmental-stage and amount-dependent.

3.4.2. Photosynthetic pigments. Foliar application of PAW did not have an effect on the total chlorophylls concentration in the leaves (table 4). Although nitrogen uptake is crucial for enhancement of chlorophylls content in the leaves (Nasar *et al* 2022), in our case, nitrogen mostly in the form of $NO3^-$ did not enhance the chlorophyll accumulation. Also, in the study of Škarpa *et al* (2020) the foliar application of PAW on maize resulted in a measurable decrease in chlorophyll content in the leaves compared to the application of distilled water. On contrary, the application of PAW (0.26 mM H_2O_2, 0.17 mM NO_2^-, 0.74 mM NO_3^-) on the leaves of rice (*Oryza sativa* L.) in the field experiment was found to significantly enhance the total chlorophylls and carotenoids content (Rashid *et al* 2021).

In our research, significant changes in carotenoids concentration were achieved only in the second leaf. Significant increase in the concentration was observed in primed plants

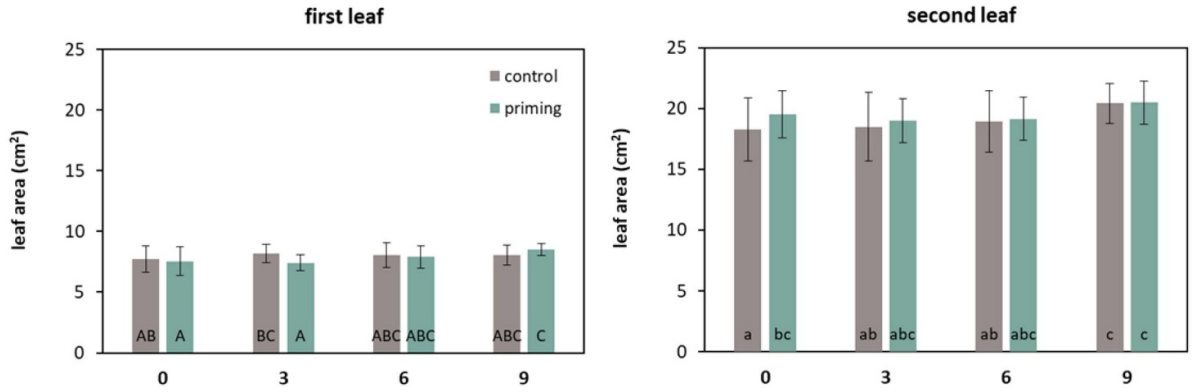

Figure 13. Leaf area of the first and second leaf after 14 day cultivation in pot with additional foliar application of PAW to plants. Letters indicate significant difference based on LSD post-hoc test.

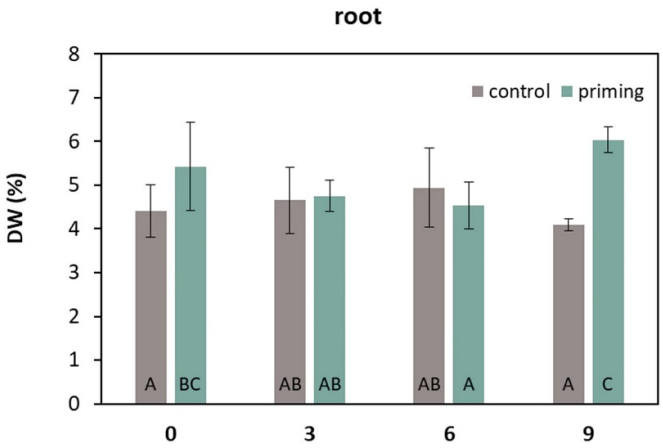

Figure 14. Percentage of the dry weight of the root after 14 day cultivation in pot with additional foliar application of PAW to plants. Letters indicate significant difference based on LSD post-hoc test.

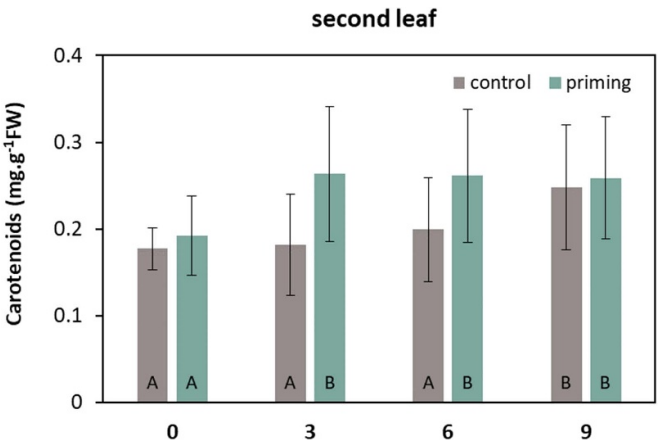

Figure 15. Carotenoids concentration in the second leaf after 14 day cultivation in pot with additional foliar application of PAW to plants. Letters indicate significant difference based on LSD post-hoc test.

after foliar application on day 3 and 6 (figure 15). Rashid *et al* also reported a significant increase in carotenoid content after foliar application. In addition, repeated foliar application of PAW (2–5 times during cultivation) resulted in its higher increase (2021).

3.4.3. Soluble phenolic concentration. Application of PAW insignificantly affected concentration of soluble phenolics in maize leaves (data not shown). On contrary, in the tested roots, the highest concentration of soluble phenolics was in priming with foliar application on day 9 (figure 16). When compared to control and priming, both without foliar application, the only significant increase in phenolics was achieved in the roots in the control and priming 6 and priming 9. To our knowledge, there is no existing research analysing the influence of foliar-applied PAW on the concentration of phenolics, and it has a potential for the future work.

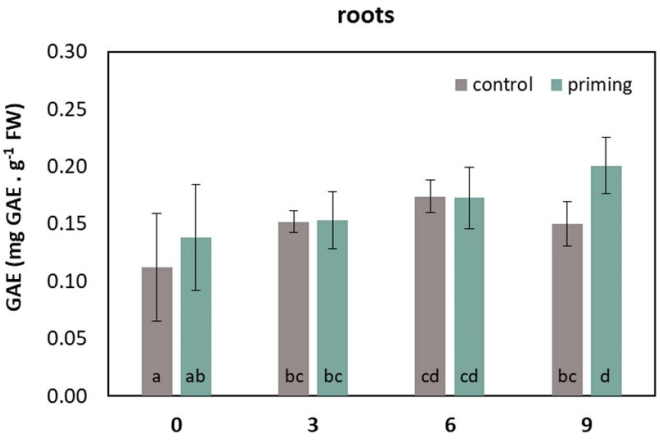

Figure 16. Soluble phenolics in the root after 14 day cultivation in pot with additional foliar application of PAW to plants. Letters indicate significant difference based on LSD post-hoc test.

According to the three-factor ANOVA (table 4), the factor treatment had significant effect only in carotenoids and G-POX. In the latter, the significant effect was measured only in priming and further foliar application of PAW did not affect the activity of G-POX (data not shown). The factor organ had highly significant impact almost on all observed parameters, except for chlorophylls. The factor day, which represents different days of foliar application of PAW had significant impact on the parameters except for %DW and phenolic content. To date, only a limited number of articles have

Table 4. A three-way analysis of variance (ANOVA) with an impact of the factors 'treatment' (control, priming; A), 'organ' (root, first leaf, second leaf; B), 'day' (day of foliar application of PAW, i.e. 0, 3, 6, 9; C) or their combination on the observed parameters. 'NS' stands for non-significant impact ($P > 0.05$).

	Treatment (A)	Organ (B)	Day (C)	A × B	A × C	B × C	A × B × C
Leaf area	NS	***	***	NS	NS	NS	NS
DW	NS	***	NS	*	**	NS	*
Chlorophylls	NS	*	*	NS	*	NS	NS
Carotenoids	*	***	***	*	NS	NS	NS
G-POX	**	***	**	**	***	**	***
Phenolics	NS	***	NS	NS	NS	NS	NS

addressed the application of PAW to leaves and the monitoring of plant responses to this treatment, including the activity of antioxidant enzymes.

4. Conclusion

The effects of PAW generated by TS discharge operated in ambient air on maize (*Zea mays* L.) were investigated in priming, short-term 3 day cultivation in paper rolls and longer-term 14 day cultivation in pot as control or primed plants with or without foliar application of PAW. The response to the PAW treatment was analysed for three different maize hybrids. Priming the maize kernels in PAW contributed to the increase in several monitored parameters, including growth (root and shoot length, FW, and % of the dry weight), accelerated lignification of the root tissue. There were also visible differences in *in situ* staining of the POX activity in the root apexes and subapical zones and roots treated with PAW exhibited visibly more H_2O_2 in the roots than controls. On the contrary, subsequent cultivation in PAW after priming did not result in improvement in most cases, with some effects having even exhibiting a detrimental trend. Only lignification was accelerated, an important result related to root development affected by PAW. In longer-term cultivation in soil, PAW imbibition contributed to higher carotenoid concentration, G-POX activity and total phenolic content in the second leaf in several hybrids. The foliar application of PAW resulted in an enhancement of carotenoid concentration in the leaves, thereby improving the antioxidant capacity. No impact on the concentration of chlorophylls, total soluble phenolics and G-POX activity was observed. It can be concluded that PAW did not induce oxidative stress in the cultivated plants. The foliar application of PAW yielded the most favourable outcomes on day 9. The improvement in antioxidant capacity, mostly in non-enzymatic antioxidants, resulting from PAW treatment could prove beneficial in experiments investigating tolerance to abiotic stresses in the presence of PAW in the future research.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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