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PLASMA PROCESSES AND POLYMERS

FULL PAPER

The effect of water activated by nonthermal air plasma on the growth of farm plants: Case of maize and barley

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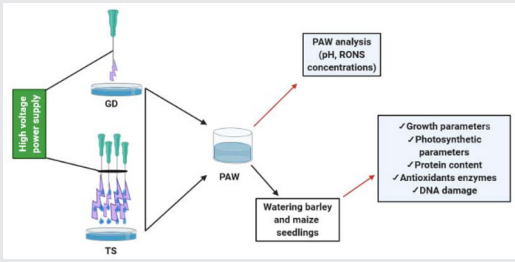
Abstract

The effects of plasma-activated water (PAW) generated by nonthermal air plasmas of transient spark with water electrospray or atmospheric glow discharge were investigated on maize (*Zea mays* L. var Saccharata) and barley (*Hordeum vulgare* L.) seedlings. PAW is characterized by measuring concentrations of reactive oxygen and nitrogen species (H_2O_2, NO_2^-, NO_3^-). After 4 weeks of plants growth, the effects of PAW are analyzed by measuring plant growth and physiological parameters: plant length and fresh weight, photosynthetic pigments concentration and photosynthesis rate, total soluble proteins, antioxidant enzyme activity, and DNA damage. The results suggest that PAW, depending on chemical composition, has the potential to improve the plant growth and influence the physiological parameters, while causing no harmful DNA damage.



KEYWORDS
barley and maize plants, DNA damages, plasma-activated water, reactive nitrogen and oxygen species, total soluble proteins

1 | INTRODUCTION

The constant increase of the world population poses environmental pollution problems and increasing needs in agriculture production. According to FAO (Food and Agriculture Organization of the United Nations),[1] the world's population will reach 9.1 billion in 2050, 34% more than the current number. As a result, it becomes necessary and important to find more efficient and sustainable food production methods and adapt them to the global climate change. With this logic, the recent focus is on the use of new technologies that surrogate chemical products used in agriculture, which are toxic and harmful to humans, animals, water, soil, and the environment in general.[2–4] Amongst them, cold atmospheric plasma generated by electrical discharges is an efficient source of ultraviolet (UV) radiation, radicals, and reactive oxygen and nitrogen species (RONS), often coupled with the effects of electric fields and radiation. Cold atmospheric plasmas provide many new applications: they may cause strong oxidation, antimicrobial effects, and induce other interesting effects in food processing and agriculture,[5–8] without leaving any harmful residues. They have shown immense potential as a simple, safe, and environment-friendly

Abbreviations: CAT, catalase; GD, glow discharge; G-POX, guaiacol peroxidase; HV, high voltage; PAW, plasma-activated water; RONS, reactive oxygen and nitrogen species; SOD, superoxide dismutase; T-1, PAW GD 1 min; T-2, PAW GD 2 min; T-C, tap water control; T-TS, PAW TS; TS, transient spark discharge.

alternative to various chemical processes used in the food processing and agriculture.

Since 2010, the number of published articles related to agricultural applications using cold atmospheric-pressure plasmas has been increasing, thanks to promising results obtained in the enhancement of plant growth, seed sterilization, seed germination improvement effects, and so on.[8–13] The cold plasma technology can be applied in agriculture by two different ways: as a direct treatment of seeds, which is more frequently used and many articles have shown the positive effect of this treatment model in agriculture,[12–21] and as an indirect treatment of seeds/plants with *plasma-activated water (PAW)*.

Regarding the indirect way, which is the purpose of this article, the plasma treatment of seeds/plant is mediated by PAW. Plasma treatment of water is typically generated by the application of cold plasma on the water surface or underneath water using different plasma sources.[22–24] Hybrid plasma–water systems, for example, plasma aerosol,[25,26] or plasma in liquid with gas bubbles,[27] are also efficient. The treatment typically creates an acidified environment (depending on the type of liquid/water used and its buffering capacity), which results in changes of the redox potential, electrical conductivity, and especially in the formation of RONS.[28,29] On the basis of the plasma discharge and the method of its interaction with water, resulting PAW has different chemical composition, especially various concentration of RONS,[30] such as H_2O_2 and NO_3^-, and could serve in agriculture as an alternative to nitrogen-based chemical fertilizers. As shown in several published articles,[31–34] hydrogen peroxide and nitrate (H_2O_2 and NO_3^-) induce seed germination and influence the plant growth enhancement, harvest time, and crop yield. Considering these beneficial effects of H_2O_2 and NO_3^-, which are the typical long-life species in PAW, PAW could be used to increase the germination rate of seeds, enhance the growth of seedlings and plants and their stress tolerance, inactivate plant-related pathogenic organisms, and cure-infected plants. Many other papers have recently shown the beneficial effects of PAW on agriculture.[7,35–39] Gierczik et al.[37] showed that PAW improved the tolerance against combined low temperature and hypoxia stresses during germination, due to its H_2O_2 and NO_3^- content. Zhang et al.[40] showed that by using plasma-activated tap water, they obtained germination rates of 80% instead of 30%. Also, higher stem elongation rates and final stem lengths were obtained using plasma-activated tap water, compared with commercial fertilizer, and they concluded that these improvements strongly depended on the combination of two long-life species: H_2O_2 and NO_3^-. In the same way to show the efficiency of PAW for plants, Kučerová et al.[41] studied the effect of PAW generated by transient spark (TS) discharge at different flow rates on wheat and evaluated germination, plant growth parameters, photosynthetic pigments, soluble protein content, and antioxidant enzymes activity. They concluded that the PAW may effectively improve the germination, the early development of wheat seedlings, but more importantly that the positive stimulation depends on the concentration of RONS in the PAW. In the same way, Fan et al.[42] investigated the effect of PAW on mung bean sprouts by applying the distilled water exposed to nonthermal plasma for 15, 30, 60, and 90 s. They evaluated the PAW effect by measuring parameters, such as the germination rate, growth characteristics, total phenolic and flavonoid contents, and antioxidant enzyme activity, and they deduced that the PAW could stimulate the mung seed germination and growth. In general, the effect depended on the chemical composition of the different PAW.

The purpose of the present work is to investigate the influence of PAW generated by two sources of cold atmospheric air plasma, namely TS with water electrospray and glow discharge (GD) with water cathode on maize (*Zea mays* L. var Saccharata) and barley (*Hordeum vulgare* L.) seedlings. Before applying PAW to irrigate the target plant seedlings, it was characterized to determine the concentration of long-life species (H_2O_2, NO_2^-, NO_3^-) by UV–Vis absorption spectroscopy. We analyzed certain plant growth parameters (plant length and fresh weight) and physiological parameters, such as photosynthetic pigments concentration, photosynthetic rate parameters, total soluble proteins (TSPs) content, antioxidant enzymes activity (superoxide dismutase [SOD], guaiacol peroxidase [G-POX], and catalase [CAT]), and the DNA damage (in barley case), to evaluate how PAW can improve the plant growth.

2 | EXPERIMENTAL METHODS

2.1 | TS with water electrospray and atmospheric-pressure GD plasmas

2.1.1 | TS air discharge with water electrospray

TS discharge with water electrospray has been described in more detail in our previous papers.[6,43,44] Figure 1a shows the experimental setup of TS discharge plasma reactor used in this study. It consists of a high-voltage (HV) DC power supply with the following parameters: $U_{max} = 20$ kV, $I_{max} = 30$ mA, $P_{max} = 600$ W. A positive HV is applied directly through the ballast resistors ($R = 8.8$ MΩ) on the HV electrodes. The HV probe

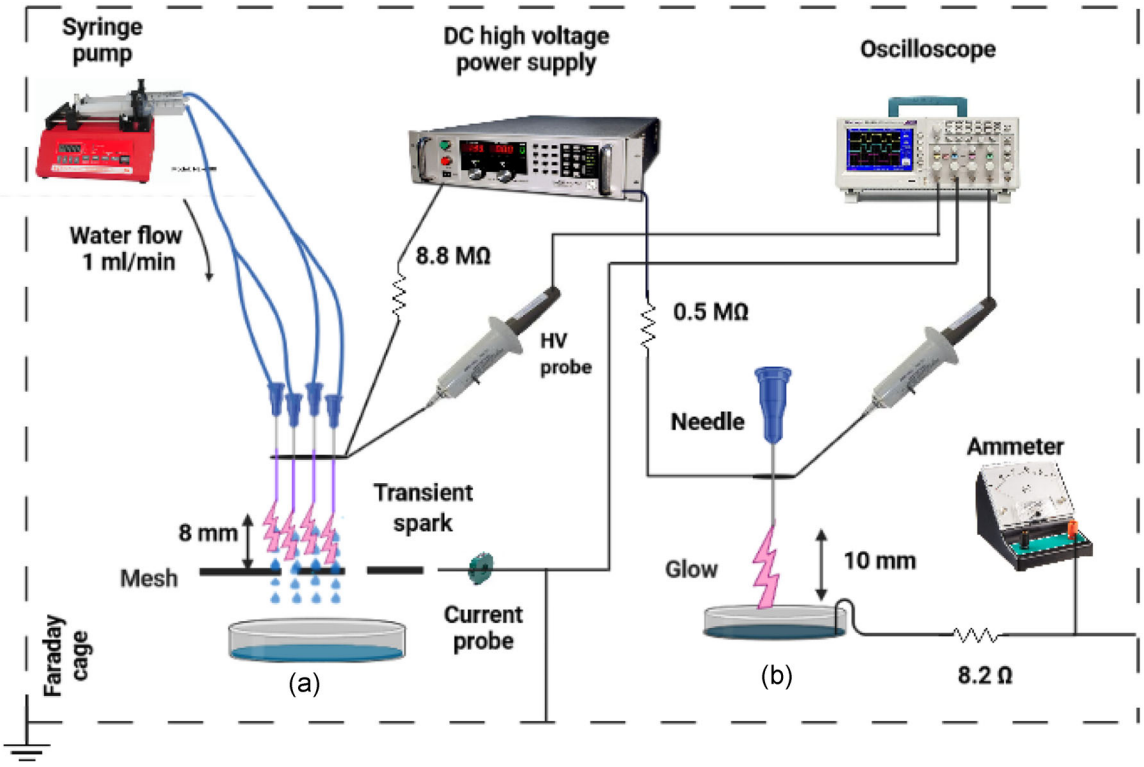


FIGURE 1 The experimental setup of (a) transient spark (TS) discharge with water electrospray and (b) glow discharge (GD) with water cathode

Tektronix P6015A ($R = 100\ \text{M}\Omega$, $C = 3\ \text{pF}$, 1000X attenuation) is used to measure the discharge HV. The discharge current is measured by a Rogowski current monitor Pearson Electronics 2877 (1 V/1 A). The time evolution of electrical parameters of discharge (voltage and current) is recorded and processed by the digitizing oscilloscope *Tektronix TDS 2024* (parameters 200 MHz; 2.5 Gs/s; 4 channels). The syringe pump *NE-300* allows the water flow through the discharge with a constant flow rate. The discharge chamber consists of four parallel electrodes in point-to-plane geometry. The four HV electrodes are represented by hypodermic hollow needles, which are directly joined by the plastic tube to the syringe pump with tap water. The discharge is generated in ambient air at atmospheric pressure between the tip of the needles and the grounded stainless-steel mesh kept at 8-mm distance. The four HV hollow needle electrodes enable us to inject the liquid water through the active zone of TS discharges with the constant flow rate 0.5 ml/min per needle by the syringe pump. The effect of electrostatic spraying of treated aqueous solutions injected directly through the HV needle electrodes occurs when the HV is applied to the needle. The electrosprayed PAW is collected under the metallic mesh in a Petri dish. The experimental device is held in the Faraday cage due to the strong electromagnetic field radiation.

2.1.2 | Atmospheric-pressure GD with water cathode

Figure 1b shows the experimental setup of the GD with water cathode at atmospheric pressure. Such GD has been described in detail in Reference [45]. The setup consists of the same HV DC power supply with the following parameters: $U_{max} = 20\ \text{kV}$, $I_{max} = 30\ \text{mA}$, $P_{max} = 600\ \text{W}$. A positive HV is applied directly through the ballast resistors ($R = 0.5\ \text{M}\Omega$) on a single HV electrode. The same HV probe *Tektronix P6015A* is used to measure the discharge HV. The DC discharge current is measured by the ammeter. The time evolution of electrical parameters of discharge (voltage and current) is recorded and processed by the same oscilloscope *Tektronix TDS 2024* (Figures 2 and 3).

In this study, the typical electrical parameters for both plasma discharges, as shown in Figure 1, are as follows:

- For TS discharge per pulse (Figure 1a): applied generator voltage $U_{app} = 17–18\ \text{kV}$, $I_{max} = 22–26\ \text{A}$, discharge voltage $U_{dis} = 9–11\ \text{kV}$. To keep the constant power dissipated into the discharge, the pulse frequency was controlled at approximately 1 kHz, pulse duration = approximately 30 ns, and P approximately 2.4 W.

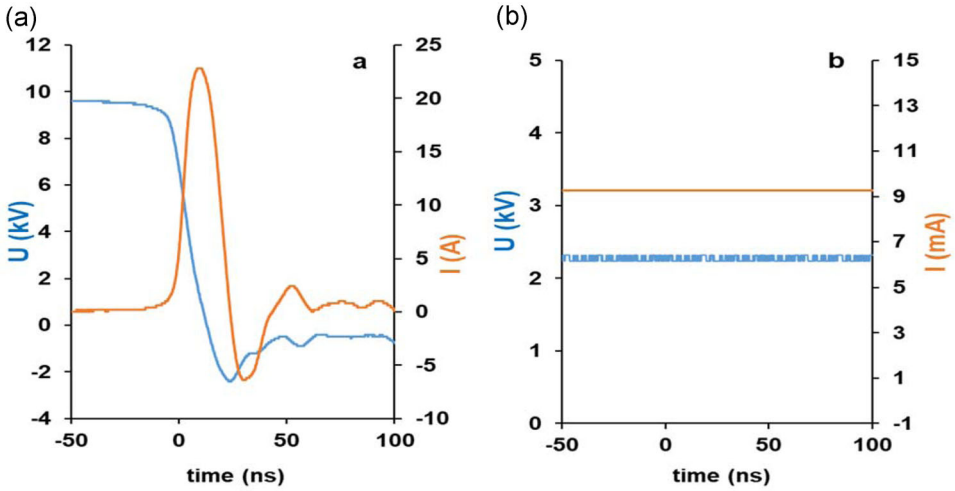


FIGURE 2 Typical discharge voltage and current waveforms of (a) transient spark (TS) with water electrospray and (b) glow discharge (GD) with water cathode

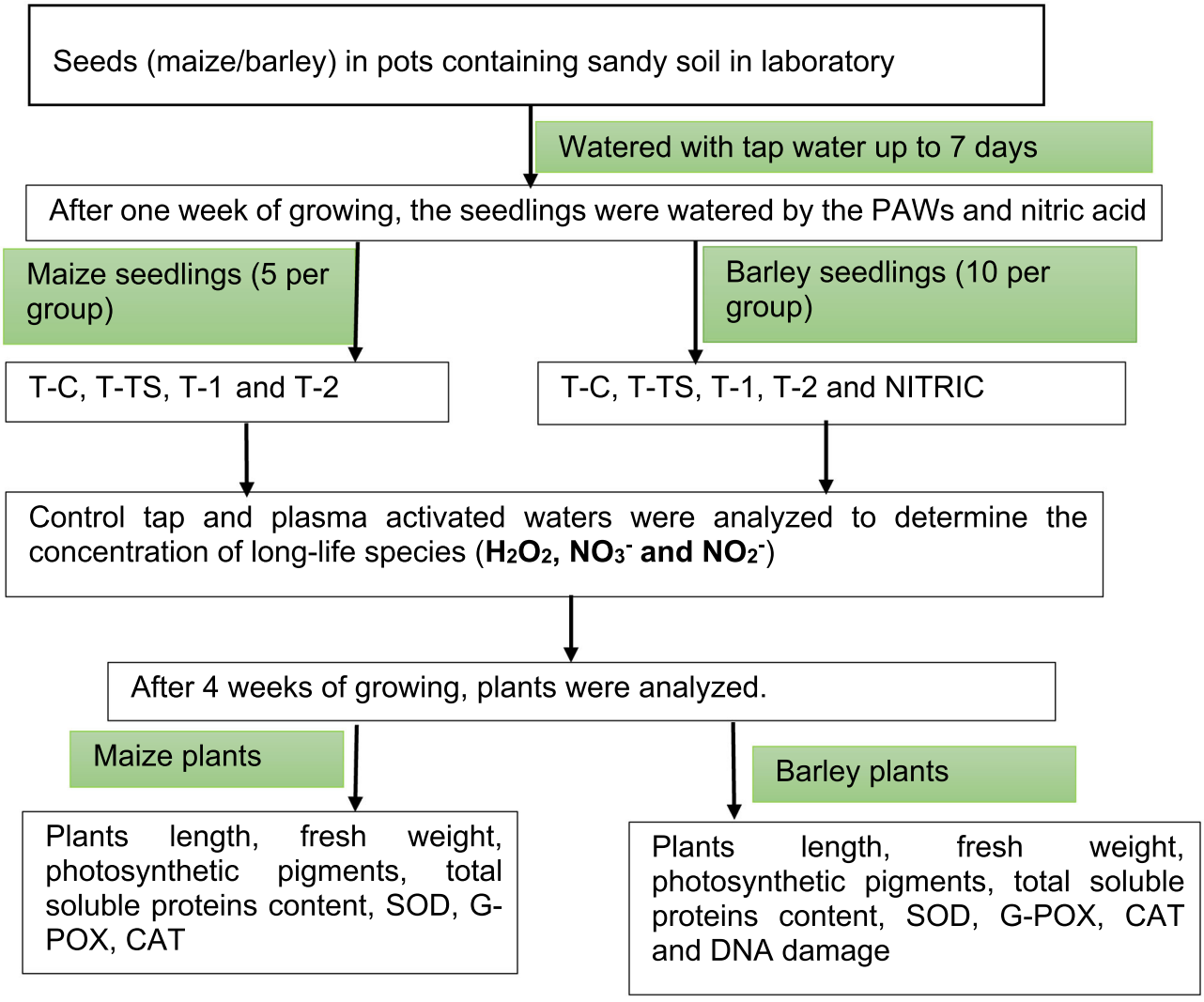


FIGURE 3 A schematic representation of the experiments. CAT, catalase; G-POX, guaiacol peroxidase; PAW, plasma-activated water; SOD, superoxide dismutase

- For GD with water cathode (Figure 1b): $U_{app} = 6–7$ kV, $I_{average} = 9.2$ mA, $U_{dis} = 2.3–2.4$ kV, P approximately 20 W, DC pulseless.

The water activated by both plasma sources is collected and then subjected to chemical analysis before being used for plant growth experiments.

2.2 | PAW analysis

Tap water with characteristic conductivity $\sigma =$ approximately 450 μS/cm and pH approximately 8.0 was used in this study to generate the PAW. Tap water in Bratislava region is "hard," with relatively high pH, due to high concentrations of calcium and bicarbonate ions. The detection of reactive species in PAW (hydrogen peroxide, nitrites/nitrates) was performed by UV–Vis absorption spectroscopy colorimetric methods (Shimadzu UV-1800 Spectrophotometer),[46] as given below.

2.2.1 | Measurement of hydrogen peroxide

Measurement of hydrogen peroxide concentration in PAW is performed by the titanium oxysulfate assay. This colorimetric method is based on the reaction of H_2O_2 with the titanyl (IV) ions in strong acidic conditions, and the yellow-colored product of pertitanic acid is created:

$$Ti^{4+} + H_2O_2 + 2H_2O \rightarrow TiO_2H_2O_2 + 4H^+. \quad (1)$$

The maximum absorption of reaction product at 407 nm is detected by UV–Vis spectrometer *Shimadzu 1800*. The formed yellow-coloured complex is stable for at least 6 h. Due to the presence of nitrites in plasma-treated water, 60-mM solution of sodium azide (NaN_3) is added to the samples with H_2O_2 before mixing with titanium oxysulfate reagent ($TiOSO_4$) to eliminate decomposition of H_2O_2 by nitrites under acidic conditions. Sodium azide immediately reduces nitrites into molecular nitrogen under acidic conditions.

$$3N_3^- + NO_2^- + 4H^+ \rightarrow 5N_2 + 2H_2O. \quad (2)$$

After mixing of the samples with azide, we add the titanium oxysulfate reagent in a sample:$TiOSO_4$ ratio = 2:1.

2.2.2 | Measurement of nitrites/nitrates

The concentration of NO_2^- and NO_3^- in the PAW was determined by colorimetric assay kit of Griess reagents (Cayman Chemicals #780001). This colorimetric method is based on the reaction of nitrites with the Griess reagents (sulfanilamide and *N*-(1-naphtyl) ethylenediamine), which after reaction form a pink-colored azo product. Nitrates are converted to nitrites by nitrate reductase (with the help of coenzyme), and afterward analyzed the same way as nitrites. Both measurements are done at the maximum absorption at 540 nm.

2.3 | Investigation of the effects of PAW on plant growth

2.3.1 | Seedlings

In this study, maize (*Z. mays* L. var Saccharata) and barley (*H. vulgare* L.) seedlings were used as model seeds to investigate the effects of PAW on plant growth.

2.3.2 | Plant cultivation

The plant cultivation was performed in the laboratory, in pots containing sandy soil, to try to recreate the outside field situation for seed germination and seedling growth. Seeds were sown into pots already filled with sandy soil, and these samples were divided into five groups with 5 and 10 plants, respectively, for maize and barley: control (T-C: watered by tap water without any treatment), TS discharge with electrosprayed water flow rate of 0.5 ml/min per needle (T-TS), GD batch water treatment of 1 and 2 min (T-1 and T-2), and 2-mM nitric acid (NITRIC). The nitric acid was used as a positive control as a nitrate ion provider to the plants, and it was used only for barley case. Each plant sample (T-C, T-TS, T-1, T-2, and NITRIC) was watered with only tap water up to first 7 days since seeding, and then either tap water or PAW was supplied to the cultivation pots. The study was carried out for 4 weeks (28 days) in total, and after these 28 days of growth, the maize and barley seedlings were analyzed. Such growth procedure (experimental round) was repeated seven times for barley at different seasons of the year (September 2019–July 2020) and three times for maize (July 2020–September 2020) in the cultivation boxes with controlled light and air temperature and humidity. Two of the seven barley experimental rounds (October 2019 and July 2020) were conducted in ambient

lab room air and natural light conditions by the window with western orientation.

The effect of PAWs and nitric acid (T-C, T-TS, T-1, T-2, and NITRIC) were analyzed by measuring some plant growth parameters (plant height and fresh weight) and physiological parameters, such as photosynthetic pigments concentration and photosynthetic rate, TSPs content, antioxidant enzymes activity (SOD, G-POX, and CAT), and DNA damage.

2.3.3 | Growth parameters

In our experiments we evaluated the PAW and nitric acid effects by measuring the length of the above-ground part of maize and barley plants. We also evaluated the fresh weight of the above-ground parts of both plants.

2.3.4 | Photosynthetic pigments concentration

Photosynthetic pigments (chlorophylls *a* and *b* and carotenoids) were determined in the leaves. An average sample of leaves (0.1 g of fresh weight) in three repetitions per variant was homogenized with sand, $MgCO_3$, and 80% acetone in mortar. After centrifugation (10,000*g*, 10 min, 4°C), the supernatant was filled to certain volume and diluted to the linear absorbance range 0.3–0.7 Au. The concentration of chlorophyll *a*, chlorophyll *b*, and carotenoids (xanthophylls and carotenes, x + c) was evaluated on the basis of the absorbance measured by UV–Vis spectrophotometer (Jenway 6400) at 664, 648, and 470 nm, respectively. The pigment concentrations were calculated according to Lichtenthaler.[47] Three samples from each treatment were taken in each experimental run.

2.3.5 | Photosynthetic rate

After 4 weeks, young, fully developed leaves were used for measurements of the photosynthetic rate (Ciras-2, PP Systems). The central part of the leaf was enclosed in a PLC6 (PP Systems) leaf cuvette connected to a Ciras-2 infrared gas analyzer. Irradiance of 25 mmol m^{-2} s^{-1} PAR was then applied, and photosynthetic light response curves were determined. The light intensity was increased stepwise in seven steps of 4-min irradiation periods until 1830 mmol m^{-2} s^{-1} PAR was reached. Light response curves of photosynthetic rate (P_N) were recorded at a CO_2 concentration of 400 mmol/mol, a temperature of 25 ± 1°C, and a relative air humidity of 65%–70%. As the measured leaf did not cover the entire cuvette area, the photosynthetic rates were recalculated to account for the leaf area enclosed in the leaf cuvette, as measured by calibrated Fluorcam (Fluorcam FC1000LC; Photon Systems Instruments) software. Results shown are the means of five independent measurements.

2.3.6 | Soluble protein content

The extraction and determination of TSPs were done on above-ground part of plants. Samples (1.5 g of fresh weight) were homogenized in a chilled mortar with liquid nitrogen and dissolved in 50-mM Na PB, pH 7.8, containing 1-mM EDTA, PVPP, and protease inhibitor cocktail tablet. The solution was centrifuged at 10,000*g* at 4°C for 20 min, and the supernatant was collected and stored at −70°C for further protein and enzyme analysis. The TSP content was measured using bovine serum albumin as a standard via the specific reaction of Coomassie Brilliant Blue G-250 dye with maximum absorbance at 595 nm.[48]

2.3.7 | Antioxidant enzymes activity

The activity of antioxidant enzymes, namely SOD, G-POX, and CAT, was measured according to standardized assays, with minimum three measurements per sample. We also tested the activity of enzymes that detoxify hydrogen peroxide (POX, E.C.1.11.1.7; CAT, E.C. 1.11.1.6), and superoxide (SOD, E.C.1.15.1.1). The activity of G-POX was established according to Frič and Fuchs,[49] activity of CAT according to Hodges et al.,[50] and the activity of SOD according to Beauchamp and Fridovich.[51] One unit of SOD activity is the amount of proteins required to inhibit 50% initial reduction of NBT under the light. The G-POX activity is expressed in μM of tetraguaiacol min^{-1} mg^{-1} by molar extinction coefficient of tetraguaiacol 26.6. Chemicals were purchased from Sigma-Aldrich Co. The CAT activity was estimated on the basis of the decomposition rate of H_2O_2 in time, which is proportional to the absorbance decrease at 240 nm.[52]

2.3.8 | Comet assay

The alkaline comet assay (single-cell alkaline gel electrophoresis) is a method used for DNA damage measurement in eukaryotic cells. Cells with damaged DNA exhibit increased migration of the chromosomal DNA from the nuclei. The DNA, also called nucleoid, moves from the cathode to the anode during electrophoresis and

the DNA then resembles a comet. By the alkaline comet assay, it is possible to detect different DNA defects, like single-strand breaks, double-strand breaks, cross-links, apyrimidine, and apurine sites.[53,54] Our experiments were performed according to Gichner et al.[55] Briefly, two leaves for each sample were cut by a razor blade, ensuring DNA release in the 150 µl of 0.4 M Tris-HCl buffer solution (pH 7.5) (Sigma-Aldrich Co.), due to the mechanical disruption of the cell and nuclear walls. The slicing and DNA release were realized in the dark on ice. Next, 100 µl of the DNA and buffer suspension were mixed with 100 µl of 1% low melting point agarose (Roth). The final solution was placed on a slide covered by 1% normal melting point agarose (Roth) and then covered by a coverslip. The coverslip was removed after 5 min and the slides were placed in the electrophoretic chamber filled with a cold electrophoretic buffer solution containing 1-mM EDTA (Sigma-Aldrich Co.) and 300-mM NaOH (Centralchem) for 8 min. Then, electrophoresis was conducted at 1.25 V/cm for 15 min at 4°C. Slides were then neutralized three times with 0.4 M Tris-HCl buffer solution (pH 7.5) and stained with fluorescent dye ethidium bromide (0.05 mM, 80 µl for each slide; Serva) for 5 min. The DNA damage was observed using fluorescent microscope OLYMPUS BX 51 with a green excitation filter UMWIG3 under ×400 magnification. Leaves of barley watered by tap water were used as a negative control (NC) and leaves of barley that were treated with 3.5 mM zeocin (InvivoGen) for 60 min were used as a positive control (PC).

2.4 | Statistics

One-way and two-way analysis of variance were performed using Statgraphics Centurion XV.I software for statistical significance at p value less than .05. All the results were expressed as mean $\pm$ SD in the three independent replications. Means were separated using the least significant difference test at a 5% level of significance.

3 | RESULT AND DISCUSSIONS

3.1 | PAW analysis

TS and GD discharges generated in ambient air at atmospheric pressure induced chemical changes in the activated tap water. These chemical changes were investigated by measuring the concentrations of RONS and the pH. We focused on three long-life species often considered as important for seedlings development and plant growth: H_2O_2, NO_2^-, and NO_3^-. Equations 3–16 show the formation of these three species in water.[29,56,57]

OH radicals are mainly formed due to the electron-impact dissociation of H_2O molecules in cold plasmas in humid gases (Equation 3):

$$H_2O + e^- \rightarrow H^o + OH^o + e^-. \quad (3)$$

H_2O_2 is produced in plasma by the recombination of OH radicals (Equation 4):

$$OH^o + OH^o \rightarrow H_2O_2. \quad (4)$$

Nitrogen oxides (NO_x) and nitrogen acids (HNO_2, HNO_3) are formed in gas-phase plasma through Equations (5)–(9)

$$N + O_2 \rightarrow NO + O, \quad (5)$$

$$NO + O_3 \rightarrow O_2 + NO_2, \quad (6)$$

$$NO_2 + O_3 \rightarrow O_2 + NO_3, \quad (7)$$

$$NO + O^o \rightarrow NO_2, \quad (8)$$

$$NO_2 + O^o \rightarrow NO_3. \quad (9)$$

The dissolution of NO_x and HNO_x in water leads to the formation of NO_2^- and NO_3^- and the acidification of water.[5,6,29]

$$2NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+, \quad (10)$$

$$NO + NO_2 + H_2O \rightarrow 2NO_2^- + 2H^+, \quad (11)$$

$$NO + OH^o \rightarrow HNO_2 \rightarrow NO_2^- + H^+, \quad (12)$$

$$NO_2 + OH^o \rightarrow HNO_3 \rightarrow NO_3^- + H^+. \quad (13)$$

The formation of nitrates under acidic conditions may also proceed through the reaction of nitrites with hydrogen peroxide to form peroxynitrite:

$$NO_2^- + H_2O_2 + H^+ \rightarrow O = NOOH + H_2O. \quad (14)$$

Peroxynitrite reactivity and stability are strongly pH-dependent. At pH < 6.8, ONOOH strongly decays into OH and NO_2 radicals (Equation 15), but it dominantly leads to nitrates (Equation 16):

$$O = NOOH \rightarrow OH^o + NO_2^o, \quad (15)$$

$$O = NOOH \rightarrow NO_3^- + H^+. \quad (16)$$

Figure 4 shows the pH and the RONS concentration of water in control (untreated) and the one treated with

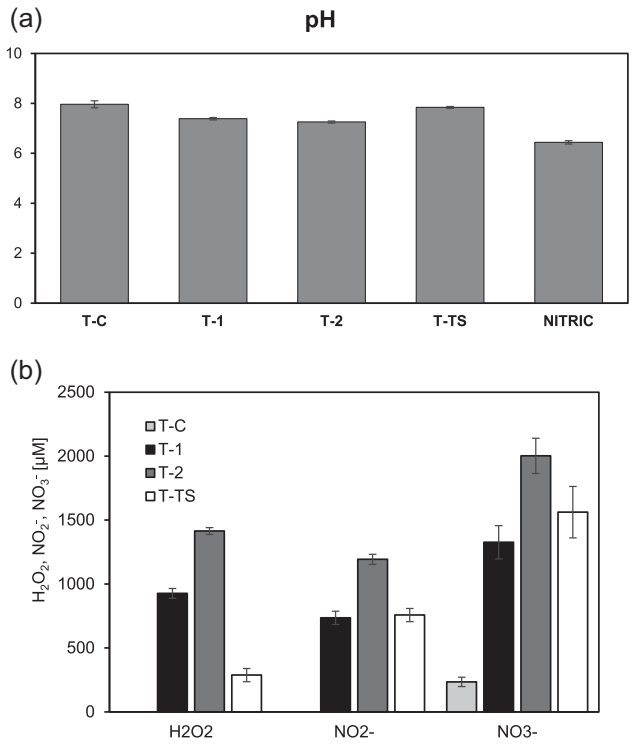


FIGURE 4 (a) pH and (b) concentrations of H_2O_2,NO_2^-,NO_3^- in tap water control (T-C) and plasma-activated water (T-1, T-2, T-TS) generated by glow discharges for 1 and 2 min and transient spark. Values are expressed as a mean ± *SD*, five repetitions

TS and GD. Figure 4a shows a slight decrease of pH between the control and PAWs, from 8.0 to 7.8 for T-TS and from 8 to 7.4 and 7.3 for T-1 and T-2, respectively. The very slight difference of pH before and after the plasma tap water treatment can be explained by relatively strong natural bicarbonate buffer system. This is due to the fact that the bicarbonates (HCO_3^-) and carbonates (CO_3^{2-}) react with the hydrogen ions (H^+) contributed by the acid, preventing them from dropping the pH,[58] as typically occurs in plasma-activated deionized water. It turns out that overall pH variations are negligible, making this parameter as a non-disruptive factor of the plant growth process. The chemical analysis shows an increase of RONS concentration in PAW, see Figure 4b. The H_2O_2, NO_2^-, and NO_3^- concentrations in the PAW were approximately 0.3, 0.6, and 1.6 mM for T-TS, and in GD, the H_2O_2, NO_2^-, and NO_3^- concentrations were approximately 0.9, 0.7, and 1.3 mM, and approximately 1.4, 0.8, 2 mM, respectively, for T-1 and T-2. These results confirmed that the TS with water flow rate of 0.5 ml/min per needle, coupled with electrospray, and GD with water cathode for 1 and 2 min of treatment are efficient sources of RONS production.

3.2 | Effects of PAW on plant growth

The PAW effects were investigated on barley and maize plant growth. As mentioned previously for barley, nitric acid was used to investigate its effect on the growth as a positive control, to compare with two types of air plasma discharges that were used to activate tap water: TS discharge with water electrospray and GD with water cathode at two treatment times.

3.2.1 | Effects of PAW on plant growth parameters

Two growth parameters, the length and the fresh weight of the above-ground part of the plants, were evaluated, and they are depicted by Figures 5 and 6, respectively. As shown in Figure 5a, the average length of barley plants watered with T-1, T-2, T-TS, and NITRIC showed some increase in percentage as compared with control (T-C), with 11%, 4.9%, 7.3%, and 2% of plant length improvement, respectively, for T-1, T-2, T-TS, and NITRIC. In the case of barley plants, T-1 showed an increase in plant length as compared with control. For maize, there was a slight improvement ratio of 1.08, 1.10, and 1.07 times with respect to control, respectively, for T-1, T-2, and T-TS. The difference in the promotion effect could be explained by the difference in concentrations of RONS formed in PAW or NO_3^- in nitric acid, particularly for the case of T-2 and NITRIC for barley, where the plant length is smaller as compared with T-1. The plants watered with T-TS and T-1 showed a higher improvement percentage for barley as compared with the control. These results suggest on the one hand both PAWs lead to improvement of the plant growth, even if the improvement is not very high, and on the other hand, the chemical composition of these PAWs plays a key role. Nitrate absorbed from the soil, besides ammonium, is one of the predominant nitrogen sources necessary for the plant growth. PAW can be an alternative nitrate provider to the plants, as shown in Figure 4b. We observed that the length of plants watered by T-2 is lower as compared to ones watered by T-1; this difference could be due the too high concentration of hydrogen peroxide or nitrate in T-2 PAW.

Approximately 37%, 27%, 29%, and 15% increase of the fresh weight of barley plants was also observed in Figure 6a for T-1, T-2, T-TS, and NITRIC, respectively, compared with control. These barley fresh weight values followed the same trend as the plant length, as showed in Figure 5a. PAW and nitric acid can show a slight improvement in the plant length for barley, but considerably improve the fresh weight of the above-ground part. These results of fresh weight suggest that the PAW

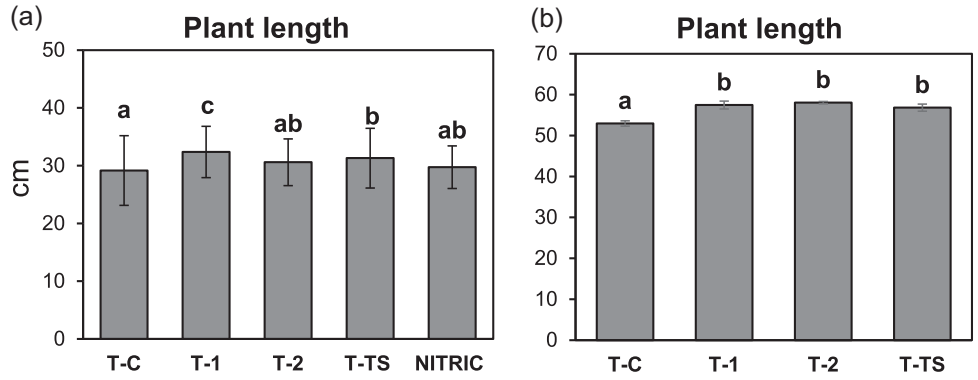


FIGURE 5 The above-ground plant length of (a) barley and (b) maize plants after 4 weeks of growth, watered with tap water (control: T-C), and plasma-activated water (PAW) of glow discharge with water cathode and treatment time 1 and 2 min (T-1 and T-2) and PAW of and transient spark discharge with electrospray (T-TS). Values are expressed as a mean ± *SD* from six repeated experimental rounds for barley and three for maize. Lowercase letters represent statistically significant difference at $p < 0.05$

could be efficient for crops yield improvement. As depicted in Figure 6b, for maize, we observed some improvement of 1.33, 1.31, and 1.30 times, compared with control, respectively, for T-1, T-2, and T-TS. These values confirm that the improvement in plant length can be very slight, but the PAW can improve the fresh weight of the plant and can improve crop yield. The presence of RONS in PAW or nitrate ions in nitric acid used to supply seedlings during the watering could be the reason of these improvements, as we know that the RONS participate in several stimulation pathways along with the plant hormones and also stimulate seed germination.[10,34] Some authors have shown the positive effect of PAW used to irrigate plants; for example, in Reference [41], Kučerová et al. found in experiments with wheat seedling growth that the effect of the PAW on seeds was correlated with the PAW activity and its chemical composition, that is, concentrations of the RONS (H_2O_2, NO_2^-, and NO_3^-). The seeds cultivated in the PAW interact with H_2O_2 mainly in the early growth stages during imbibition and germination, whereas NO_2^- and NO_3^- are metabolized once the seeds start to germinate. Sivachandiran and Khacef[59] observed that PAW treated for 15 and 30 min in the plasma leads to acidic solutions (pH 3) with moderate concentrations of nitrate (NO_3^-) and hydrogen peroxide (H_2O_2). PAW has shown a significant impact on germination as well as plant growth for the three types of seeds (radish, tomato, and pepper).

3.2.2 | Effect of PAW on photosynthetic pigment concentration

Chlorophyll content is an important factor to determine the photosynthesis rate and dry matter production; also, it has a significance in agriculture as an indicator of

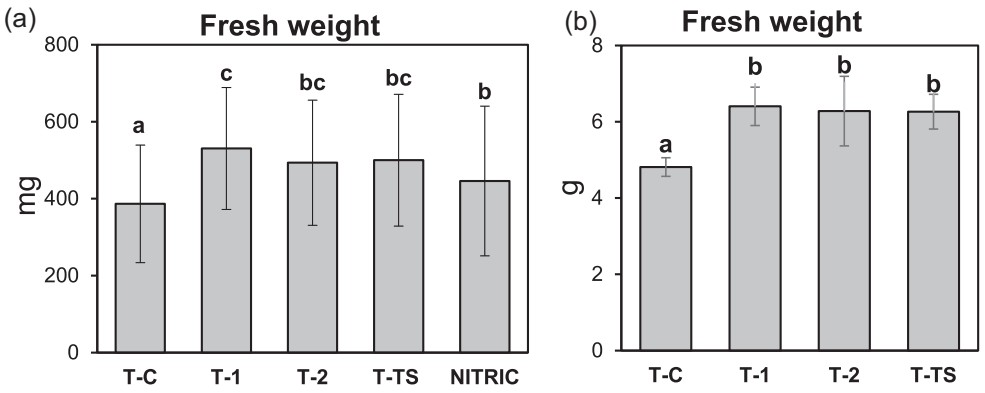


FIGURE 6 Fresh weight of above-ground parts of (a) barley and (b) maize plants after 4 weeks of growth, watered with tap water (control, T-C), and plasma-activated water (PAW) of glow discharge with water cathode and treatment time 1 and 2 min (T-1 and T-2), and PAW of and transient spark discharge with electrospray (T-TS). Values are shown as mean ± *SD* from six repeated experimental rounds for barley and three for maize. Lowercase letters represent statistically significant difference at $p < 0.05$

photosynthetic activity.[60] To evaluate the effect of PAW generated by GD and TS on photosynthetic pigments, chlorophyll *a*, chlorophyll *b*, and carotenoids contents were measured, and the results are presented in Figure 7. As showed, the photosynthetic pigments for plants watered by PAW or nitric acid showed some slight increase of values, probably due to the increase of RONS concentration in water. The barley plants watered with NITRIC showed an increase of chlorophylls *a*, chlorophylls *b*, and carotenoids, 1.21, 1.15, and 1.25 times higher than control, respectively. However, for maize watered with T-1, the increase was 1.11, 1.39, and 1.08 times higher than control, respectively, for chlorophylls *a*, chlorophylls *b*, and carotenoids. Nitrate plays an important role in the photosynthesis process. Without nitrates, the amount of chlorophylls in leaves is reduced, which means leaves turn pale green or yellow.

3.2.3 | Effects of PAW on photosynthetic rate

The effect of PAW generated by GD and TS on the photosynthetic rate P_N was measured, and the results are presented in Figure 8. It can be assumed that PAW had generally no or negative impact on P_N. As shown in Figure 8a, the P_N for barley plants watered by PAW showed a significant decrease of values when compared with control plants. However, maize plants (Figure 8b) showed lower sensitivity to PAW. Only a slight decrease in P_N was observed for variant T-2 under higher light intensities (830–1830 μmol PAR m^{-2} s^{-1}). Differences between barley and maize in sensitivity to plasma treatment were documented, for example, in Švubová et al.[61] Similar to results of this study, Saberi et al.[62] documented disproportionality in photosynthetic pigment concentration to P_N, which could represent different involvement of present pigments in light harvesting complexes.

3.2.4 | Effect of PAW on TSP content

Proteins composed of two-dimensional (2D) or 3D chains of amino acids are basic components of living cells. For healthy growth, plant roots absorb mineral ions including nitrate for producing amino acids, which are then used to form proteins. TSP plays a fundamental role in the growth of the plants, and it is a substantial part of many plant enzymes that indicate the metabolism.[63] Figure 9 shows the results of TSP in the above-ground part of barley (Figure 9a) and maize (Figure 9b) plants watered with control tap water and PAW: T-1, T-2, T-TS, and NITRIC. For barley plants, T-2 and NITRIC showed an increase of 1.23 and 1.18 times, compared with control, and for maize, T-1 and T-2 showed an increase of 1.26 and 1.25 times, as compared with control. The increase of the soluble protein in both barley and maize could be due to nitrates in the watering solution (PAW or nitric acid). Regarding the concentration of the soluble proteins in barley for the plant watered only with nitric acid and knowing that the nitric acid provided only nitrate ions to the plant, we can conclude that the nitrate ion plays an important role in the production of protein in the plant. Also, a rapid observation of both graphs allows us to see a similar trend for both plants watered with PAW, where T-2 shows the highest concentration of soluble protein, which could be associated with the higher concentration of nitrate ions in T-2. These results suggest that PAW improved soluble protein in both plants, under an optimum concentration of nitrate. Sajib et al.[64] and Kučerová et al.[41] also reported the positive effect of using PAW to enhance the TSP in black gram (*Vigna mungo* L.) and wheat (*Triticum aestivum* L.), respectively.

3.2.5 | Effect of PAW on antioxidant enzymes

The expression of antioxidant enzymes enhances as the level of RONS is induced in biotic stress conditions.[65] Figures 10, 11, and 12 show, respectively, the G-POX, SOD, and CAT activities in the above-ground part after 4 weeks of growth of barley and maize plants watered with T-C, T-1, T-2, T-TS, and NITRIC.

In Figure 10a the G-POX activity showed an increase of 2.50, 2.33, 2.34, and 2.28 times in barley plants, respectively, for T-1, T-2, T-TS, and NITRIC, which is probably related to the concentration of H_2O_2. In Figure 10b, for maize plants, we observed a decrease of 1.04, 1.13, and 1.31, compared with control, respectively, for T-1, T-2, and T-TS. Further investigations are needed to better understand these effects.

In Figure 11a,b, showing SOD activity, compared with G-POX activity, we observed a decrease of SOD activity in the above-ground part for plants watered with T-1, T-2, T-TS, and NITRIC with respect to control. The same trend is observed in both barley and maize plants, where T-2 showed the lowest values. These results are similar to Kučerová et al.[41] who also reported the decrease of SOD activity in the above-ground part of wheat plant. Sajib et al.[64] reported the opposite: they showed that the SOD activity increased in black gram root and leaves watered with PAW. PAW is a complex and rich medium of active species, and

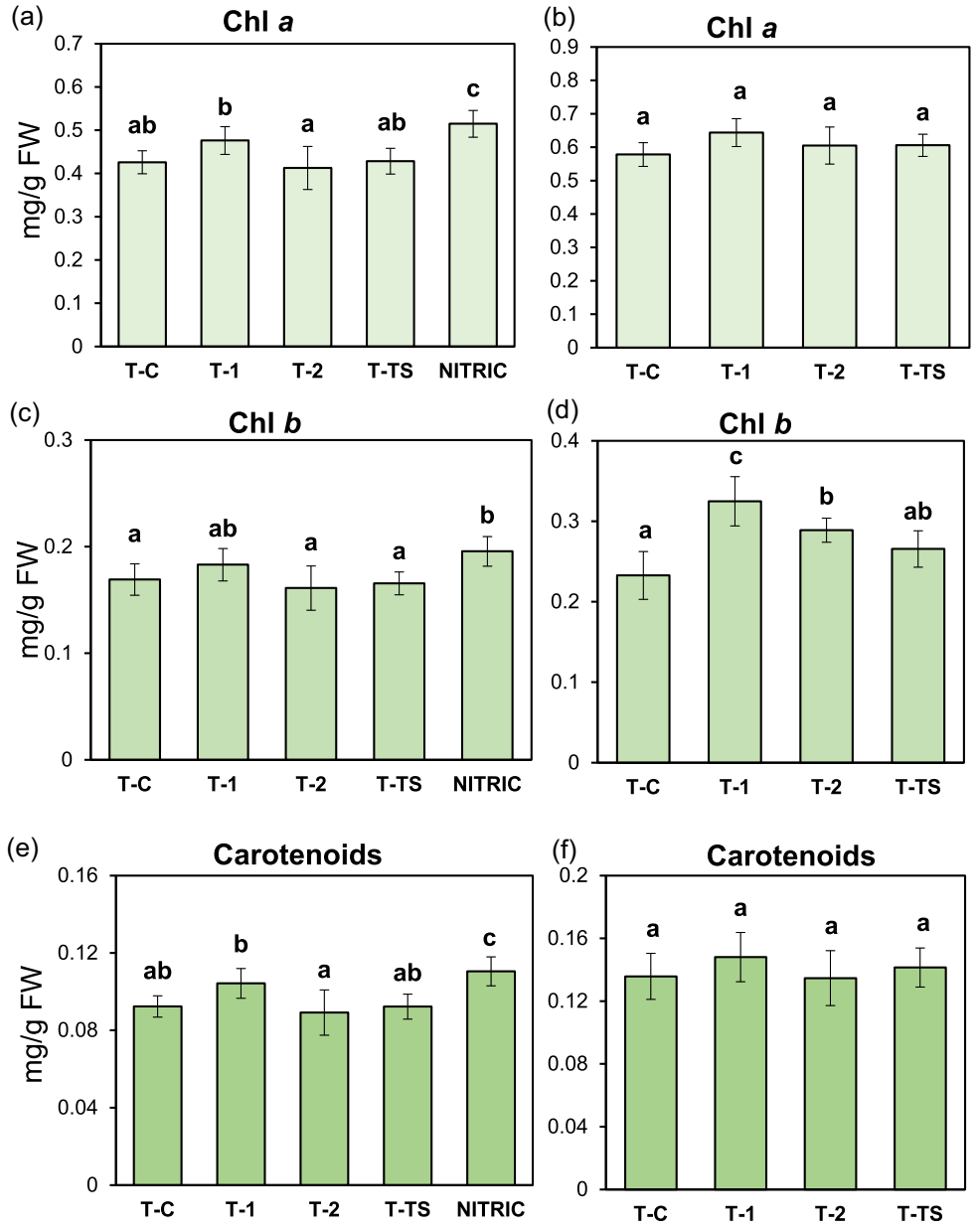


FIGURE 7 Chlorophyll *a*, chlorophyll *b*, and carotenoids (x + c) content in barley (a, c, e) and maize (b, d, f) leaves per fresh weight. Values are shown as mean ± *SD*from two repeated experimental rounds for barley and maize. Lowercase letters represent statistically significant difference at $p < 0.05$

every specific PAW prepared in a different plasma source contains mixtures of RONS of different concentrations, so it is difficult to know exactly which specific mixture species could affect these antioxidant enzymes. The SOD activity reacts to superoxide or oxidative stress in general; hence, the SOD activity decreases despite the fact that PAW contains RONS that are oxidative stressors.

As depicted in Figure 12a,b, we observed an increase of CAT activity in both plants, which is likely related to the scavenging of the oxidative activity of H_2O_2 in PAW.

3.2.6 | Effect of PAWs on DNA damage of barley plants

The potential genotoxic effects of PAW applied on barley plants were determined using the comet assay. As shown in Figure 13, based on results of the comet assay, DNA damage of barley plants treated with PAW was at the level of negative controls (15.19%–16.63%), regardless of the plasma source used for PAW preparation and interval of watering with PAW. The DNA damage in all samples treated with PAW ranged from 18.38% to 21.38%, which represents a statistically insignificant increase as

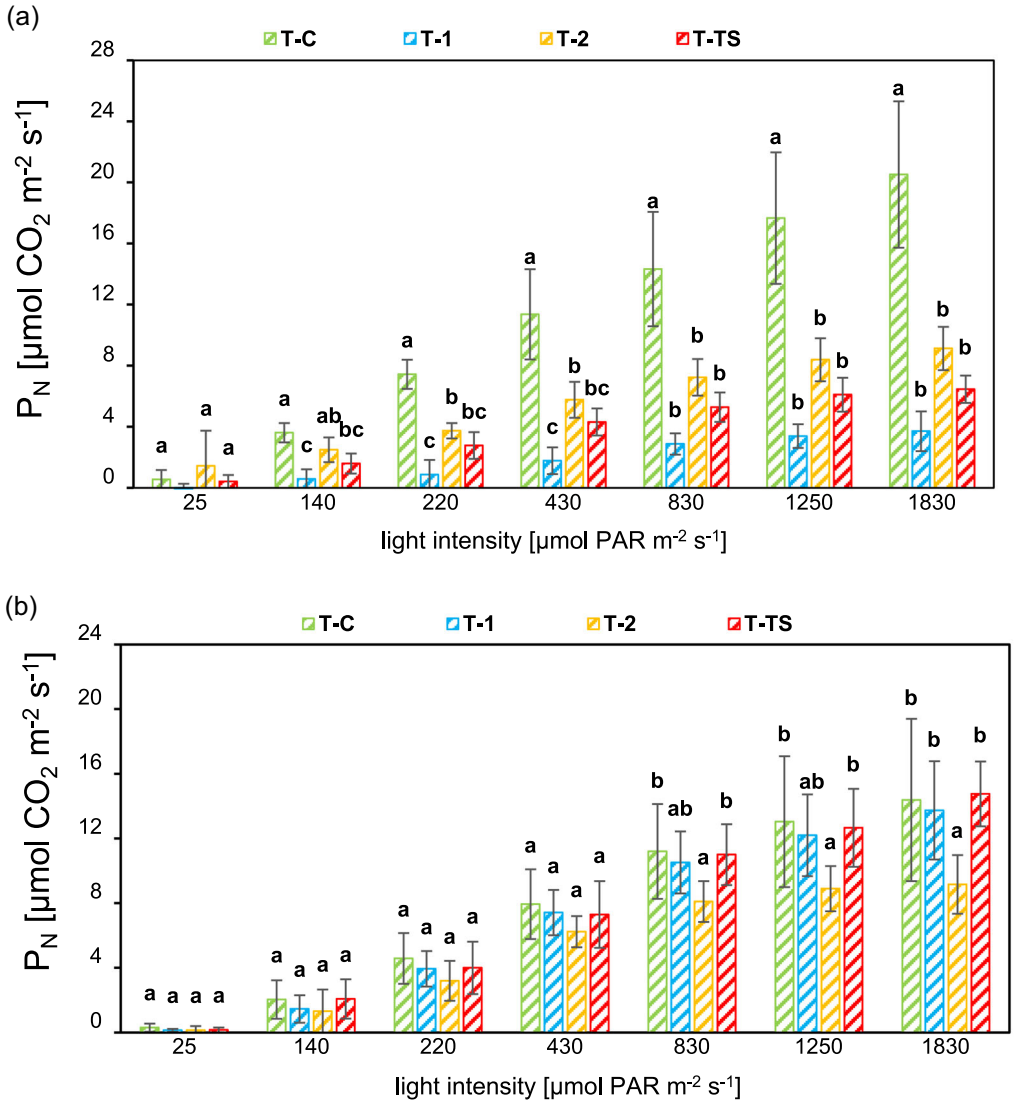


FIGURE 8 The photosynthetic rate of (a) barley and (b) maize. Values are shown as mean ± *SD* from two repeated experimental rounds for barley and maize. Lowercase letters represent statistically significant difference at $p < 0.05$

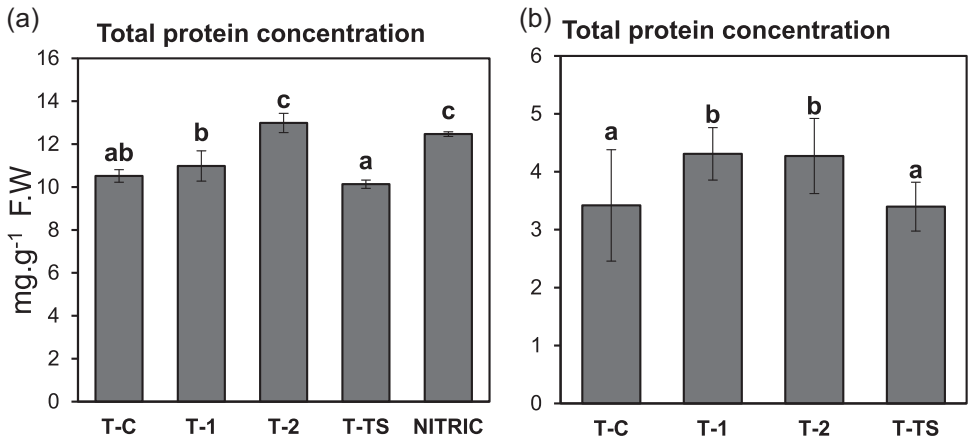


FIGURE 9 The total soluble protein content in the above-ground part of (a) barley and (b) maize plants after 4 weeks of growth and watered with T-C, T-1, T-2, T-TS, and NITRIC. Values are shown as mean ± *SD* from experimental rounds for barley, and for maize, in each round, leaf samples were taken from six plants. Lowercase letters represent statistically significant difference at $p < 0.05$

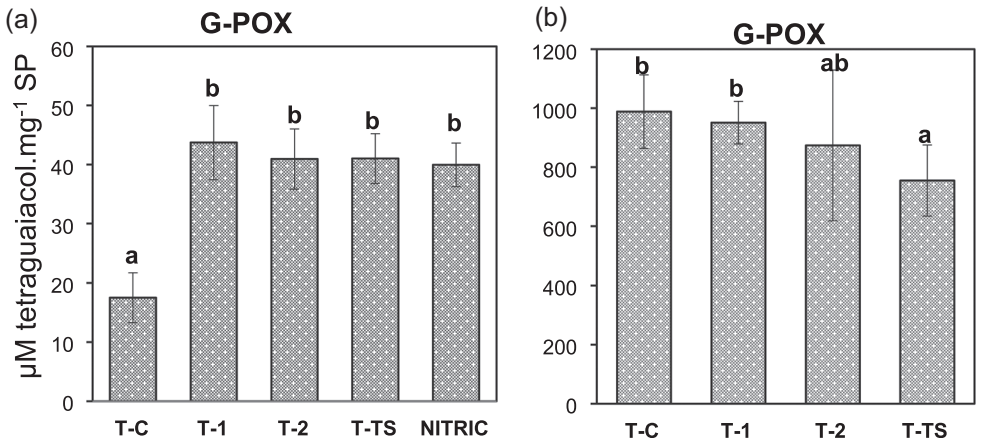


FIGURE 10 Guaiacol peroxidase (G-POX) in the above-ground part of (a) barley and (b) maize plants after 4 weeks of growth. Values are shown as mean ± *SD* from experimental rounds for barley, and for maize, in each round, leaf samples were taken from six plants. Lowercase letters represent statistically significant difference at $p < 0.05$

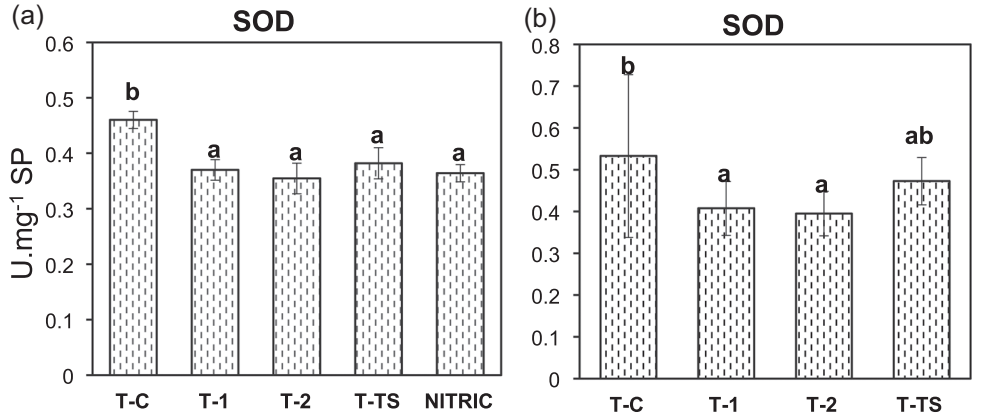


FIGURE 11 Superoxide dismutase (SOD) activity in the above-ground part of (a) barley and (b) maize plants after 4 weeks of growth. Values are shown as mean ± *SD* from experimental rounds for barley, and for maize, in each round, leaf samples were taken from six plants. Lowercase letters represent statistically significant difference at $p < 0.05$

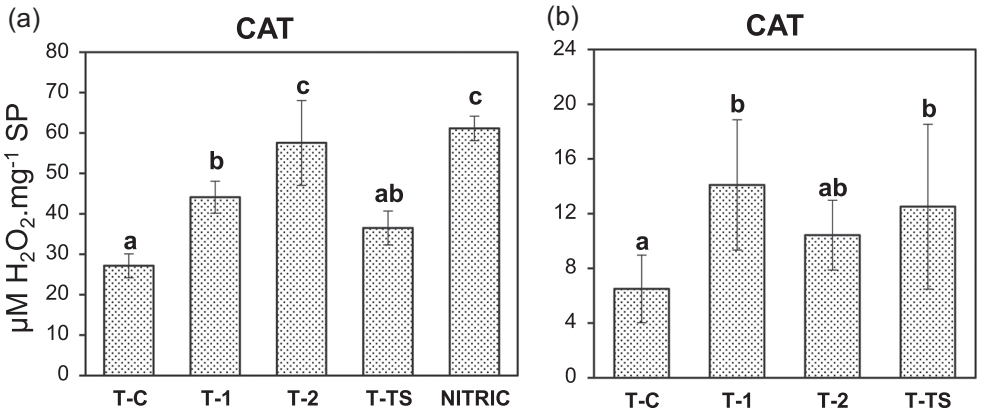


FIGURE 12 Catalase (CAT) activity in the above-ground part of (a) barley and (b) maize plants after 4 weeks of growth. Values are shown as mean ± *SD* from experimental rounds for barley, and for maize, in each round, leaf samples were taken from six plants. Lowercase letters represent statistically significant difference at $p < 0.05$

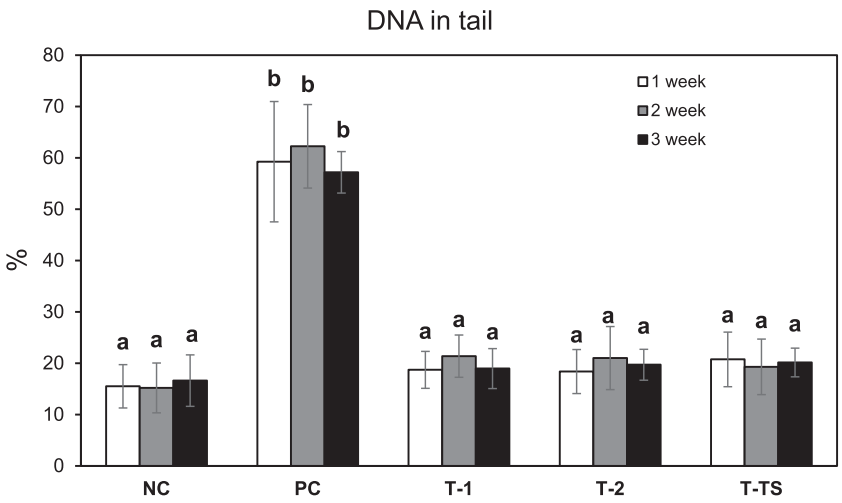


FIGURE 13 A graphical representation of the DNA damage effect of plasma-activated water (T-TS, T-1, and T-2) in leaves of 1-, 2-, and 3-week-old barley plants analyzed by the comet assay. Values are expressed as a mean ± *SD* from two repeated experimental rounds of three samples in each group. NC (T-C): negative control, PC: positive control (3.5-mM zeocin, 60-min incubation). Lowercase letters represent statistically significant difference at $p < 0.05$

compared with the negative controls. The results of the comet assay suggest that PAW prepared by TS and GD did not have any harmful effects on barley DNA. There is no other publication that studied and observed only negligible amount of DNA damage in plant cells after plasma treatment. Švubová et al.[66] evaluated DNA damage in pea seedlings whose seeds were treated with DCSBD plasma generated in ambient air, oxygen, or nitrogen using the comet assay and constant field gel electrophoresis. Their results suggest that the direct exposure of pea seeds to cold atmospheric plasma can cause DNA damage with increasing exposure time, but the rate of single-strand breaks is higher than double-strand breaks. A greater formation of single-strand breaks after plasma treatment was confirmed also on the plasmid DNA.[67–70] Kyzek et al.[71] and Tomeková et al.[72] also observed increased DNA damage in pea seedlings after DCSBD plasma treatment of their seeds. In all these mentioned studies, the effect of direct plasma treatment on DNA was investigated. However, in our experiment, we studied the indirect plasma effect of PAW, which resulted in a lower DNA damage as compared with other studies. Thus, this approach appears to be safe and confirms that PAW used in this study does not have a damaging effect in terms of DNA damage.

4 | CONCLUSION

As reported by many recent studies, cold atmospheric air plasma in contact with water generates RONS, presenting biocidal and germicidal effects, improvement of the seed germination, and induction of plant growth. In this paper, the plasma activation of tap water by the TS discharge with electrospray and GD with water electrode in ambient air at atmospheric pressure induced chemical changes in the water, mostly dominated by production of hydrogen peroxide, nitrites, and nitrates. Maize (*Z. mays* L. var Saccharata) and barley (*H. vulgare* L.) seedlings were used as model farm plants to investigate the effects of PAW on the plant growth during the first 28 days. The following conclusions are obtained:

(1) PAW generated by either TS or GD is a rich source of long-living RONS such as hydrogen peroxide, nitrites, and nitrates.
(2) Watering by PAW slightly enhances the plant growth parameters, plant length and fresh weight, of both maize and barley.
(3) Watering by PAW in some cases gently increases the concentration of photosynthetic pigments and simultaneously has no or negative impact on net photosynthesis.
(4) For both plants, PAW enhances the TSP content, which is an important parameter indicating the growth of the plants and numerous plant enzymes. TSP enhancement correlates with the nitrate concentration in PAW with maximum in the T-2 (GD, 2 min) group.
(5) PAW induced certain changes in the antioxidant enzymes of both plants: decrease of SOD activity in both plants, increase of G-POX activity in barley and a slight decrease in maize, and increase of CAT activity in both plants. Long life-time RONS (H_2O_2, NO_2^-, NO_3^-), especially H_2O_2 in PAW is probably responsible for these effects. Further investigations of these effects are needed.
(6) Plant growth enhancement of barley using these air-plasma based PAWs is not accompanied by any DNA damage.

The concentration of the PAW RONS and their relative ratios, mostly of hydrogen peroxide and nitrate, could be a key for an improvement of the plant growth.

Based on the results obtained in this study we suggest that PAW represents a great potential for applications in agriculture from seedlings to the final stage of the plant growth (harvest).

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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