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FULL PAPER

PLASMA PROCESSES AND POLYMERS

Effects of plasma activated water on wheat: Germination, growth parameters, photosynthetic pigments, soluble protein content, and antioxidant enzymes activity

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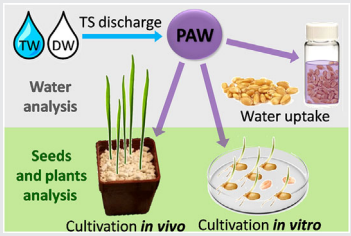
The paper presents the study on the effects of the plasma activated water (PAW) generated by transient spark discharge on the wheat (*Triticum aestivum* L.) cultivated in vitro and in vivo. Water uptake and germination of seeds, growth parameters of seedlings and plants, as well as the content of photosynthetic pigments, soluble proteins and activity of antioxidant enzymes are reported and correlated with the concentrations of reactive oxygen and nitrogen species in the PAW. The PAW improves germination, early development of the seedlings, the content of photosynthetic pigments in the leaves and soluble protein content in the roots, and suppresses the activity of antioxidant enzymes. The results indicate that the PAW may effectively stimulate growth of the wheat seedlings and positively affect their metabolism especially in the soil with low nutrient content.



KEYWORDS
antioxidant enzymes, chlorophylls, germination, plasma activated water, wheat

1 | INTRODUCTION

With the increasing world population and decreasing food sources there is a growing demand for new approaches and technologies in agriculture. Besides basic sources that are necessary for plants growth (as light energy, carbon dioxide CO_2, and water H_2O), they also need nutrition with adequate content of basic macro and micro elements. One of the most important macro nutrient is nitrogen. It represents major cost in plant production as a main compound of commercial fertilizers.[1] Although being necessary for plants growth, nitrogen loss from soils by leaching and runoff to the environment contributes to air and water pollution with serious health hazards. Despite this problem, still almost 50% of food produced today is grown with the help of nitrogen-containing fertilizers.[2] Many efforts have been made to produce plant-available nitrogen and to stimulate seed germination and plant growth by other methods.

The most common method for plant growth enhancement is the use of commercial fertilizers or organic compounds as compost, manure, or sludge. Organic fertilizers, beside their numerous advantages, may contain remains of pathogens, or antibiotics.[3] More natural way is to use nitrogen fixing (growth promoting) bacteria as biofertilizer, although some bacterial strains are detrimental and they are not symbiotic with all plant species.[4] Besides chemical approaches various physical methods have been also tested, namely the use of electromagnetic field, laser or microwave treatment, gamma

irradiation, and ultrasound.[5] The advantage is that in comparison to the chemical methods they leave low or no residues. These methods are mostly applied on dry seeds and only very seldom to water.[6,7]

Another promising novel physical method is a cold (non-thermal, non-equilibrium) plasma (CP). The CP is a plasma that produces highly reactive environment of high energy electrons, charged particles, and various reactive species, although being generated and maintained at room temperature. It can be generated by various kinds of electrical discharges and it is often utilized in numerous environmental and biomedical applications.[8–11] Recently, the CP has also found its applications in food processing, packaging, and agriculture.[12–14]

The application of the CP in agriculture is either direct (seeds or plants are in a direct contact with plasma) or indirect (seeds or plants are exposed to plasma treated/activated gas or water).[14] The plasma in direct contact with the seed can stimulate its germination and growth[15–18] and subsequent fruit yield,[19] change enzymatic activity in developing seeds,[20–22] change secondary metabolites content,[23,24] and also reduce phytopathogenic microflora from the seed surface.[25–28] Plasma can also modify seed surface and change its affinity towards water to either hydrophilic or hydrophobic, that both can be beneficial.[25] When using the plasma, it is very important to know and optimize the plasma exposure/activation time. Short exposures may induce weak effects, while long exposures may result in inhibition and can negatively affect the seed germination and development of the plants.[15,29–31]

The indirect plasma treatment/activation of seeds and plants by the CP, that is, the effect of plasma activated gas or plasma activated water (PAW) is far less studied than the direct plasma effect even though it can also enhance seed germination and plant growth. The PAW can be generated by operating electric discharges directly in water[32,33] but more often in gas in a contact with water,[34] usually above the water surface, for example, gliding arc,[35–37] glow discharge,[38] dielectric barrier discharge,[39–42] or plasma jets.[43,44] Besides pure water also other liquid media, such as drainage water from pots,[32] nutrient solution,[33] or fertilizer[43] have been activated and used. The common characteristic of these discharges is they produce various gaseous and aqueous reactive oxygen and nitrogen species (RONS). The RONS affect and control numerous processes in seeds and plants, including their germination, growth, development, and response to a stress. Among these RONS, hydrogen peroxide H_2O_2 and nitrates NO_3^- are considered being the main species responsible for the enhancement of seed germination and growth of plants irrigated with the PAW. Maniruzzaman et al.[33] found slightly better effect of the PAW when compared to chemical solutions of H_2O_2 or NO_3^- and attributed it to the short-lived and intermediate species ($^{\bullet}OH$, NO). The effect of the PAW on seeds and plants is however very complex and depends on the activity of the PAW usually determined by the concentration of the RONS. The quality/activity of the PAW can be affected by various parameters, such as discharge type and power, composition of gas mixture and gas flow rate, and type of water. However the PAW effect also depends on plant species, their requirements on water and nutrients.[35] Therefore it is always necessary to study and describe the effect of the PAW with respect to the particular discharge plasma properties and plant species, as the efficiency and mechanism of action can be different.

The objective of the present study is to investigate the effect of the PAW generated by a transient spark discharge in ambient air in contact with water on germination and growth of wheat (*Triticum aestivum* L.). We analyzed water uptake by seeds, germination of seeds, and growth parameters of seedlings and plants grown in different cultivation conditions (in vitro and in vivo). The effect of the PAW on seeds and seedlings was investigated along with the monitoring of RONS concentration in the PAW during cultivation as it was in the contact with seeds. To our knowledge such experiment has not been done before. From the physiological parameters we determined the content of photosynthetic pigments and soluble proteins in plants irrigated with the PAW. Moreover, we monitored the antioxidant enzymes activity that also has not been yet measured in plants irrigated with the PAW.

2 | EXPERIMENTAL SECTION

2.1 | Experimental setup

The experimental setup is schematically depicted in Figure 1a. Plasma reactor consisted of high voltage hollow needle electrode placed above the inclined grounded plane electrode in point-to-plane geometry. The distance between the electrodes was 1 cm. Deionized water (DW) or tap water (TW) was supplied via narrow channel in the inclined plane electrode and circulated by a peristaltic pump with a constant flow rate (14 ml/min) to provide repetitive contact of water with the discharge.[45] Transient spark discharge (TS), a self-pulsing repetitive streamer to spark transition discharge was used to activate water.[46] The discharge was driven by positive DC power supply (Technix RS20-R-1200) and its electrical characteristics were monitored by high voltage probe (Tektronix P6015A) and Rogowski type current probe (Pearson Electronics 2877) connected to an oscilloscope (Tektronix TDS 1012). Its characteristic voltage and current waveforms are depicted in Figure 1b. As the figure shows, the TS discharge is typical with current pulses of high amplitude (order of several tens of A) and very short duration (10–100 ns).[46] The typical amplitude of the applied voltage used in our experiments was

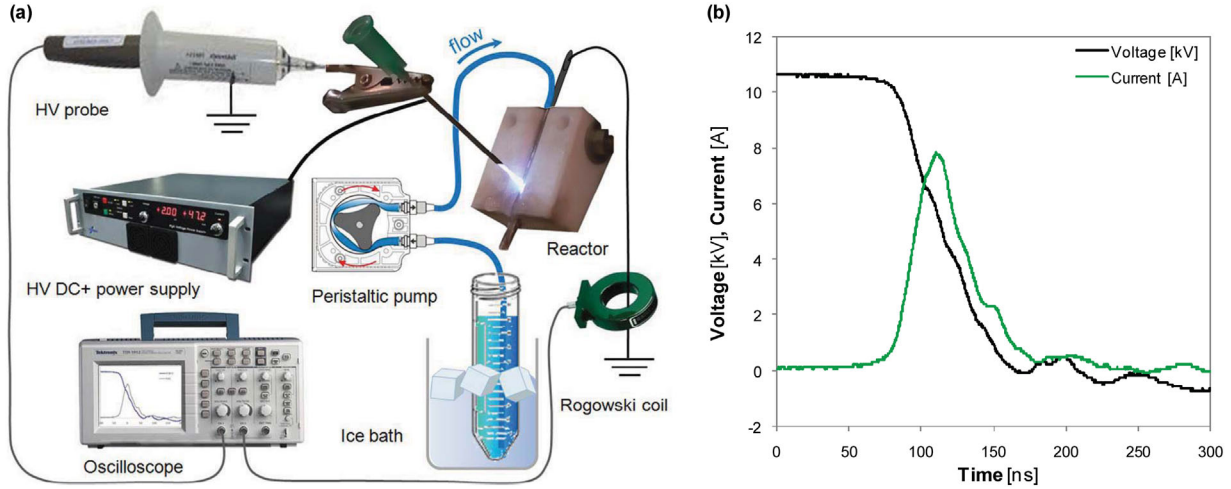


FIGURE 1 (a) The scheme of experimental setup and (b) characteristic voltage and current waveforms of transient spark discharge.

$U_{app} = 16$ kV, amplitude of the breakdown voltage $U_{br} =$ 10–13 kV, average discharge power 6 W, amplitude and frequency of the discharge current pulses were $I_{max} = 6$–8 A and $f = 2$–3 kHz, respectively. The activation time was in the range 1–40 min and water volume in the range 5–40 ml. To compare the results obtained with different activation times and water volumes we use the term water activation time expressed in units of min/ml. It represents the ratio between the activation time and the water volume (e.g., 5 ml of water activated for 10 min by plasma gives water activation time of 2 min ml^{-1}). While operating the TS with long activation times an erosion of the electrodes can be observed. In our previous paper[47] we discussed a possible contamination of the PAW by impurities (e.g., metallic nanoparticles) originated from electrodes that may play a role in bacterial inhibition. Subsequent analysis of the PAW showed the presence of particles with the size from nm to several μm,[48] although no effect on bacteria was observed. Therefore, we assume the effect of metallic particles in the PAW on the seeds and plants may also be insignificant.

2.2 | Chemical analysis of plasma activated water

The activation of deionized water (DW) (< 3 μS cm^{-1}, pH ~ 5.5) or tap water (TW) (~550 μS cm^{-1}, pH ~ 7.5) by the TS discharge plasma resulted into a production of plasma activated deionized water (PADW) and plasma activated tap water (PATW), respectively. The analysis of various RONS generated by the TS discharges in activated water was performed by colorimetric methods using UV/Vis absorption spectroscopy (Shimadzu UV-1800). We measured concentrations of various aqueous RONS, namely hydrogen peroxide H_2O_2, nitrites NO_2^-, and nitrates NO_3^-. The H_2O_2 concentration in water was evaluated by titanium sulfate colorimetric method.[49] Immediately after the water activation we stabilized the sample by sodium azide NaN_3 to prevent the reaction of H_2O_2 with NO_2^-, and subsequently added titanium oxysulfate $TiOSO_4$. The H_2O_2 reacts with titanyl ions of $TiOSO_4$ and produces yellow-colored product with maximum absorbance peak at 407 nm that is proportional to the H_2O_2 concentration. To determine NO_2^- and NO_3^- concentrations, we employed commercial colorimetric assay kit (Cayman Chemicals #780001) that uses Griess reagents to form pink-colored azo-product with maximum absorbance peak at 540 nm. We also monitored the pH (WTW 3110) and the temperature of the PAW. With the increasing activation time and decreasing water volume, that is, with the increasing water activation time (min ml^{-1}), the temperature of water as well as water evaporation increase. For example, water activation time of 2 min ml^{-1} led to a gradual increase of water bulk temperature by approximately 10 °C and a loss of 0.5 ml of water due to evaporation. As warm water may decrease or even inhibit the germination and viability of seeds, in all experiments the water container was immersed in the controlled ice bath in a way to keep the water bulk temperature constant and thus also to minimize water volume loss.

2.3 | Seeds

Seeds of wheat (*Triticum aestivum* L. cv. IS Gordius) were obtained from the Central Control and Testing Institute in Agriculture in Bratislava, Slovakia. The effect of the PAW on seeds germination and growth of seedlings was tested both in vitro and in vivo conditions by various methods.

2.4 | Water uptake

Water uptake by seeds was determined during the imbibition tests. 100 pcs of non-sterilized seeds in three repetitions were weighted and immersed in 20 ml of the PADW or the PATW (water activation times 0.5, 1, 2 min ml^{-1} and control). After 3, 6, or 24 h the seeds were taken out, blotted dried, and

weighted on an electronic balance (Sartorius BL-210S). The amount of water uptake was determined as the increase of seed weight.

2.5 | Cultivation in vitro

In vitro cultivation of seeds was performed on filter paper in Petri dishes. The seeds were sterilized for 2 min by 96% ethanol and washed several times with deionized or tap water. After sterilization, 110 seeds were imbibed in 20 ml of the PAW for 3 h. After that 25 seeds per dish were placed on filter paper moistened with 3 ml of freshly prepared PAW and cultivated for 3 days. On the 3rd day another 5 ml of freshly prepared PAW was added and cultivated for additional 3 days (6 days in total). Four Petri dishes per each variant were cultivated in dark at 22 °C for 6 days. At the end of cultivation the germination, fresh and dry weight of seedlings, length of roots and shoots and vigor indices were evaluated.

2.5.1 | Germination

Germination percentage was evaluated as a ratio between number of germinated seeds and total number of seeds per Petri dish. The seed was considered as germinated when the minimum length of 1 mm radicle and coleoptile emerged from the seed.

2.5.2 | Growth parameters

The length of the 10 randomly selected seedlings per one Petri dish was measured with mm scale. All germinated seedlings from each Petri dish were separately packed into aluminum foil of the known weight, the package was weighted and the fresh weight of one seedling was calculated. After drying at 60 °C for 3 days the package was weighted again and the dry weight of one seedling was determined. From the obtained data the vigor indices were calculated:

$$\text{Vigor index A} = \text{Germination [\%]} \times \text{Fresh weight [mg]}/100$$
$$\text{Vigor index B} = \text{Germination [\%]} \times \text{Dry weight [mg]}/100$$

2.6 | Cultivation in vivo

In vivo cultivation of wheat was performed in pots filled with a perlite. The perlite is a nutrient free inert solid substrate that provided only the mechanical support to plants. It was used to estimate the effect of the PAW itself on wheat growth without any potential influence or interference with natural nutrients present in common soils. The seeds were sown into the pots and after emergence unified on 5 plants per container in four repetitions per variant cultivated for 4 weeks in controlled conditions in growth chamber with a 12 h photoperiod, a temperature 24/18 °C (day/night) and 120 μmol m^{-2} s^{-1} light intensity. The plants were irrigated either with the TW (control) or with a combination of the TW and the PATW (samples) of different activity (0.5 and 2 min ml^{-1} PATW). The reason why the samples were not irrigated only with the PATW was that the plant demand for water was higher than the volume of the PATW we were able to generate within a reasonable time. Instead the samples were irrigated with the PATW only every second day and with the TW on the remaining days. The total volume of water irrigated on the samples for 4 weeks was 170 ml of the PATW and 443 ml of the TW (i.e., the PATW-to-TW ratio was 0.38). The same total volume (613 ml) was used for the control. As the last variant, we used Hoagland nutrition solution (HNS) that provides all macro and micro nutrients necessary for growth of plants. The HNS was used the same way as the PATW, that is, in the combination with the TW and using the same procedure and the same volumes. For physiological and biochemical analysis we used total 20 plants per one variant and verified four variants – control, 0.5 min ml^{-1} PATW, 2 min ml^{-1} PATW and the HNS as a nutritional standard.

2.6.1 | Growth parameters

We counted number of leaves per plant and qualified them as healthy (green) or senescent (physiological aging). The length and width of second fully developed leaf was measured to estimate a leaf area by simple equation $Y = 0.75$ LW (cm^2), where Y represents the leaf area, L is the length (cm) and W is the maximal width (cm) of the leaf measured at the half of the leaf length.[50] Fresh and dry weight from in vivo experiments were evaluated separately for root and above-ground part of the plants in the same manner as for in vitro experiments.

2.6.2 | Photosynthetic pigments content

Photosynthetic pigments (chlorophylls and carotenoids) were determined in the leaves. Average sample of leaves (0.5 g of fresh weight (f. w.)) in three repetitions per variant was homogenized with sand, $MgCO_3$ and 80% acetone in mortar. The filtered solution was filled to certain volume and diluted to the linear absorbance range 0.3–0.7 a.u. The concentration of chlorophyll *a*, chlorophyll *b* and carotenoids (xanthophylls and carotenes, $x + c$), were evaluated based on the absorbance measured by UV/Vis spectrophotometer (Jenway 6400) at 664, 648, and 470 nm, respectively.[51]

2.6.3 | Soluble protein content

The extraction and determination of soluble proteins was done separately for root and for above-ground part. Samples (0.5 g of fresh weight (f.w.)) 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 (Roche cOmplete). 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 proteins and enzymes analysis. Total soluble protein content was measured using bovine serum albumin (BSA) as a standard via the specific reaction of Coomassie Brilliant Blue G-250 dye with maximum absorbance at 595 nm.[52]

2.6.4 | Antioxidant enzymes activity

Activity of antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (G-POX) were measured according to standardized assays, with minimum three measurements per sample. This group of antioxidant enzymes is capable selectively scavenge reactive oxygen species ($^{\bullet}OH$, $O_2^{\bullet-}$, H_2O_2). The SOD is the most effective intracellular antioxidant enzyme, as it decomposes $O_2^{\bullet-}$ to H_2O_2. The SOD activity was measured as a decrease in absorbance at 560 nm, as the SOD inhibits the photoreduction of thiazolyl blue tetrazolium bromide (MTT) induced by superoxide radicals produced by reaction of methionine with riboflavin under white light. One enzyme unit of SOD is defined as the amount of protein causing a 50% of inhibition.[53] The H_2O_2 is degraded by peroxidase using reducing compound as ascorbate or by the CAT in a pure catalytic reaction $2H_2O_2 \rightarrow O_2 + 2H_2O$. The CAT activity was estimated based on the decomposition rate of H_2O_2 in time, which is proportional to the absorbance decrease at 240 nm.[54] The G-POX activity was measured as increase in absorbance at 440 nm in time as the G-POX uses guaiacol as a substrate for H_2O_2 decomposition with yielding red-brown tetraguaiacol.[55] All antioxidant enzymes activities were normalized to soluble protein content in sample. The measurement of absorbance for enzymes activity evaluation was done by UV/Vis spectrophotometer (Jenway 6705).

2.7 | Statistical analysis

Data obtained from the measurements are presented as mean values ± standard deviations (SD), if not stated otherwise. The data were analyzed by one-way analysis of variance ANOVA and subsequent multiple range test by least significance difference method (LSD) using appropriate software (Statgraphics 18–X64 and Microsoft Excel). The groups of data in the figures marked with lowercase letters indicate significant difference at probability level of $p < 0.05$.

3 | RESULTS AND DISCUSSION

3.1 | Chemical analysis of plasma activated water

To understand the effect of the PAW on seeds germination and plant growth it is necessary to know chemical changes induced by plasma discharge in water. When the TS discharge is generated in ambient air, it mainly produces NO and NO_2.[56] In contact with water the hydroxyl radicals $^{\bullet}OH$ are produced that rapidly recombine into hydrogen peroxide H_2O_2 (Eq. 1). The gaseous NO and NO_2 dissolve in water, produce nitrites NO_2^- and nitrates NO_3^- and cause acidification of water (Eqs. 2, 3). In acidic pH, NO_2^- can be oxidized to NO_3^- (Eq. 4) or reacts with H_2O_2 to form intermediate peroxynitrous acid ONOOH (Eq. 5), that is unstable and eventually decomposes into NO_3^- or into $NO^{\bullet}_2$ and $^{\bullet}OH$ radical (Eqs. 6, 7).[57,58]

$$^{\bullet}OH + {}^{\bullet}OH \rightarrow H_2O_2 \quad (1)$$

$$NO_{2(aq)} + NO_{2(aq)} + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (2)$$

$$NO_{(aq)} + NO_{2(aq)} + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (3)$$

$$3NO_2^- + 4H^+ + H_2O \rightarrow 2NO + NO_3^- + 3H_2O^+ \quad (4)$$

$$NO_2^- + H_2O_2 + H^+ \rightarrow O = NOOH + H_2O \quad (5)$$

$$O = NOOH \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}OH \quad (6)$$

$$O = NOOH \rightarrow NO_3^- + H^+ \quad (7)$$

Figure 2 shows pH and concentrations of the selected RONS (H_2O_2, NO_2^-, NO_3^-) in the PADW and the PATW as function of the water activation time. In tap water pH remained fairly constant (pH ~ 7.5) and changed very mildly with water activation time thanks to natural hydrocarbon buffer system.[59] On the other hand, in deionized water the pH decreased rapidly with increasing water activation time from initial pH 6.0 down to pH 2.7 for 3 min ml^{-1}. The increase of the water activation time also resulted in an increase of concentration of measured reactive species H_2O_2, NO_2^- and NO_3^-. In the PADW the RONS concentrations roughly saturated after 1 min ml^{-1} of water activation time due to their mutual reactions in acidic environment (Eqs. 4, 5). On the contrary, in the PATW the concentrations of RONS increased almost linearly with the increasing water activation time as the natural buffer system of tap water preserve the pH and restrain the chemical reactions that occur in the PADW. For 3 min ml^{-1} water activation time the concentrations of H_2O_2, NO_2^-, NO_3^- in the PADW were ~ 0.85 mM, 0.27 mM, 0.57 mM and in the PATW ~ 1 mM, 0.97 mM, 1.62 mM, respectively. The concentrations of the RONS generated by the TS discharge are relatively high compared to other plasma discharges. In comparison, Zhang et al.[43] used He plasma jet and generated ~ 0.16 mM of H_2O_2 in the PAW, while Park et al.[35] used transferred arc discharge and generated

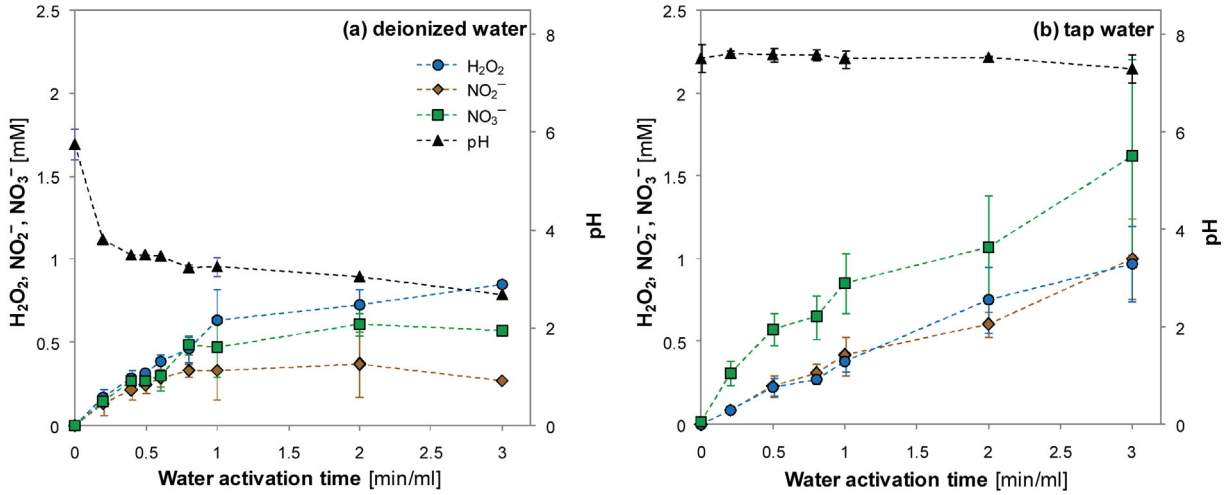


FIGURE 2 Concentrations of H_2O_2, NO_3^-, NO_2^- and pH in (a) plasma activated deionized water (PADW) and (b) plasma activated tap water (PATW) as function of water activation time. Values are shown as mean ± SD.

concentrations of $H_2O_2 \sim 0.08$ mM, $NO_2^- \sim 0.26$ mM and $NO_3^- \sim 0.9$ mM.

3.2 | Ageing of plasma activated water and its interaction with seeds

The concentration of the RONS determines the activity of the PAW and the effects induced in seeds and plants. Once the plasma discharge is stopped, the activity of the PAW decays over time. Therefore it is also very important to know the activity and stability of the PAW after activation. Figure 3 shows post activation time development of H_2O_2, NO_2^- and NO_3^- concentrations in the PATW, that is, the aging of PATW without seeds or in a contact with seeds. Without seeds the concentration of H_2O_2 in the PATW decreased slowly and after 6 days still its residual concentration was detected, as spontaneous H_2O_2 decomposition is a slow process.[60] On the contrary, the concentration of H_2O_2 in the PATW in contact with seeds decreased rapidly during several minutes and 18 h after activation no H_2O_2 was detected. The concentration of NO_3^- in the PATW without seeds remained relatively stable for several days, while with seeds the concentration started to decrease mainly after 24 h, when seeds started to germinate. The concentration of NO_2^- in the PADW without seeds decreased rapidly during the first hour after plasma activation as NO_2^- reacts with H_2O_2 in acidic environment (Eq. 5). However, in the PATW, where pH remained neutral and the reaction (Eq. 5) does not apply, NO_2^- concentration was stable and did not change with post activation time. A slight increase of its concentration is probably caused by the water evaporation from sample during cultivation. When the PATW was in contact with seeds NO_2^- likewise NO_3^- degraded over time as they both were probably metabolized by seeds once they started to germinate.

The use and transport of the RONS into the seeds is usually a very complex process. There are several possible explanations of the RONS effect on the seeds. H_2O_2 in the PAW can be transferred through plant cell membrane by free diffusion that is dependent on membrane lipid composition, and by aquaporins, that is, membrane proteins that facilitate water transport.[61] On the other hand, the transport of NO_3^- is concentration dependent and slower process that is governed by specialized membrane transport proteins, beside simple diffusion.[62,63] In agreement with the above, our results also showed that H_2O_2 decays much faster than NO_2^- and NO_3^-. Moreover the seeds have enhanced capacity to detoxify H_2O_2 during their imbibition and germination.[64] Thus, the effect of the PAW on seed germination is firstly driven by response

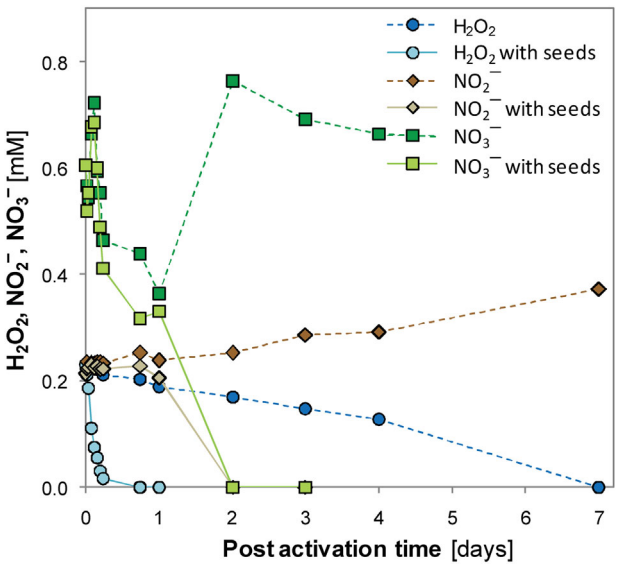


FIGURE 3 Post activation time development of H_2O_2, NO_2^- and NO_3^- concentrations without and in contact with seeds in the 0.5 min ml^{-1} PATW.

of seed metabolism to H_2O_2, namely antioxidant enzymes activity, and/or cellular signaling. NO_3^- as a major source of nitrogen and nutrient for seedlings[65] was effectively metabolized by the seeds but in longer time scale compared to H_2O_2. According to Prochádzka et al.,[66] NO_3^- can also act as a signaling molecule and induce expression of genes for NO_3^- transport proteins through membrane and for nitrate/nitrite reductase genes for the NO_3^- assimilation. Besides NO_3^-, also NO_2^- could be considered as a potential source of essential nitrogen. However, NO_2^- is also found as being toxic to plant growth and metabolism,[67] although some plants (including wheat, cucumber, barley, tomato) are NO_2^- tolerant or even able to utilize it for their growth.[68]

3.3 | Water uptake

Along with the chemical analysis of the PAW and changes of its composition during and after activation, we also monitored the water uptake by seeds during the imbibition. We investigated whether there is a difference in the uptake of the DW/TW and the PADW/PATW by seeds. Faster water uptake may result into accelerated germination and subsequent faster growth. The water uptake by seeds was monitored for 24 h based on the changes in the weight of seeds. The most intense water uptake was observed during the first 3 h of imbibition. Figure 4a shows the water uptake as a function of water activation time during this period, when approximately 0.2–0.3 g of water per 100 seeds was absorbed. Seeds imbibed in 0.5 min ml^{-1} PADW and PATW absorbed 2 and 5% more water than their respective controls. However, with further increase of water activation time the amount of water absorbed decreased and the seeds imbibed in 2 min ml^{-1} PADW and the PATW absorbed 10 and 8% less water than their respective controls. Water uptake after 6 h and 24 h of imbibition (not shown in this paper) was much smaller, with only 0.1 and 0.05 g of water absorbed per 100 seeds, respectively and there was no significant difference between the variants. These results suggest that seeds imbibed in 0.5 min ml^{-1} PAW (both the PADW and the PATW) reached water saturation slightly faster compared to seeds imbibed in other PAWs or controls that may lead subsequently to their faster germination.[69] The increased absorptive ability of seeds cultivated in 0.5 min ml^{-1} PAW might be accompanied by their increased ability to absorb nutrients, which promotes the subsequent growth of seedlings.

A process of germination starts with water imbibition by seeds that results in the enhancement of key enzymes involved in the catabolism of seed storage reserves. Faster hydration of seeds observed for 0.5 min ml^{-1} water activation time can cause quicker changes in enzymatic state of the germinating seeds, especially in earlier amylase and protease activity, as it was also found under the influence of magnetopriming in cucumber seeds.[70] Although the seeds imbibed faster in the DW, the TW is more suitable for the seedling growth and metabolism. The small concentrations of minerals in the TW are more beneficial than none in the DW, as minerals are the essential part of plant chelates and enzymes. The difference between uptake of the DW and the TW by seeds shown in Figure 4a can be also due to differences in their osmotic potentials. The osmotic potential of the DW is equal to zero, while the TW potential is negative. As the water uptake is determined by water diffusion and decreases with the decreasing osmotic potential, the TW is absorbed less than the DW. The osmotic potential could also explain the decrease of water uptake observed for long water

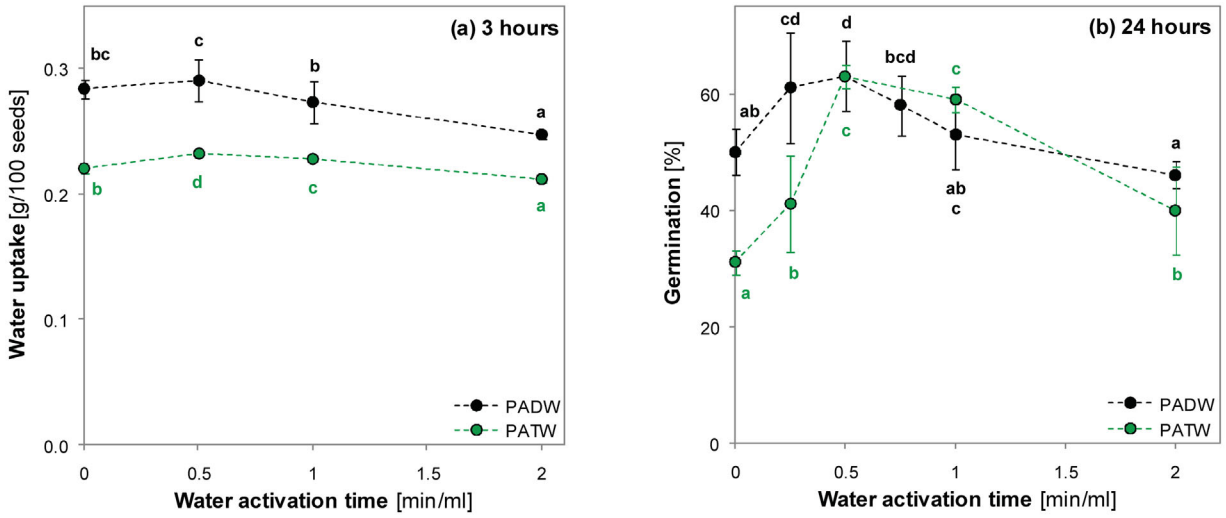


FIGURE 4 (a) Water uptake during the first 3 h of imbibition of seeds in the PADW and the PATW and (b) germination of seeds cultivated in the PADW and the PATW as function of water activation time after 24 h. Values are shown as mean ± SD. Lowercase letters represents statistically significant difference at $p < 0.05$.

activation times (1 min ml^{-1} and more). The extensive plasma activation of water lead to formation of high concentrations of various RONS in water resulting into lower and hence more negative water potential than that of untreated water. Moreover, soaking seeds in water with high concentration of NO_3^- could delay the imbibition time and had deleterious effect on seed germination percentage.[71] Nevertheless the osmotic potentials cannot explain the increase of water uptake for 0.5 min ml^{-1} PAW compared to the other variants. We assume that in this case other factors such as seed surface, selectivity of ions uptake by seeds, presence of salts or temperature may play a role and affect the imbibition process.[69]

Similar to our observations, Naumova et al.[34] found increased swelling of rye seeds compared to control for seeds imbibed for 72 h in the PATW generated by 5 min treatment with underwater discharge, however gave no explanation of this effect. On the contrary, Peethambaran et al.[37] observed 30% reduction in water uptake by *Arabidopsis thaliana* plants irrigated with the PADW generated by gliding arc. Despite reduced water uptake, they reported larger growth of plants and higher plant yield. These results suggest that seeds and plants can react differently to the PAW.

3.4 | Cultivation in vitro

3.4.1 | Germination

In vitro cultivation of seeds was performed in Petri dishes. The used seeds were characterized by a relatively high natural germination 95–96% (0 min ml^{-1}, Table 1). The germination percentage of seeds cultivated in the PADW and the PATW as function of water activation time and evaluated after 24 h is depicted in Figure 4b. For both the PADW and the PATW the maximum stimulation effect was observed for water activation time ~0.5 min ml^{-1}, where germination increased by 26 and 103%, respectively (Figure 4b). Table 1 shows data on seed germination after 6 days of cultivation in vitro in the PADW or the PATW. The comparison of Figure 4b with data in Table 1 shows that increase in germination was more significant after 24 h than after 6 days as most of seeds germinated during the first 2 days and later the differences slightly attenuated toward the 6th day. After 6 days of cultivation the maximal increase in germination of 4% was observed for seeds cultivated in 1 min ml^{-1} PADW and 0.5 min ml^{-1} PATW (Table 1). The profile of germination (Figure 4b) matches the trend of water uptake as function of activation time (Figure 4a) and indicates that improvement of hydration of seeds results into faster germination, as discussed in previous section.

The stimulating effect of the PAW on germination was probably due to the preferential intake of H_2O_2 from the PAW during seed imbibition and subsequent cultivation. The accumulation of H_2O_2 in seeds in the early stages of imbibition[72,73] and positive effect of H_2O_2 on seed germination[74–77] was already reported for several plant species. H_2O_2 may stimulate respiration and metabolic activities by production of O_2 during catalase scavenging of H_2O_2, it can etch the seed's coat and help the water diffusion or it may oxidize germination inhibitors.[76,78] The positive effect of the PAW can also be caused by additive effect of other reactive species, such as $O^{\bullet}_2^-$ or $^{\bullet}OH$, that were also reported to accumulate in germinating seeds.[78] Also reactive nitrogen species, such as NO or NO_2^-,[79,80] and NO_3^-, can stimulate germination in broad range of plant species and break seed dormancy.[81–83] Several models had been proposed to determine NO_3^- role in germination and conclude that the RONS signaling involve interactions with endogenous phytohormones by affecting abscisic acid levels in imbibed seeds or by enhancing gibberellic acid synthesis.[80,21] In order to clarify the mechanism of the PAW effect on germination in more details, it would be necessary also to analyze activity of hydrolytic enzymes affecting germination rate and initial plant development. The effect of the PAW on germination also depends on type of plant species, type of discharge, type of plasma treatment (direct or indirect), type of water (deionized or tap), and experimental conditions (in vitro or in vivo), etc. For example, Lindsay et al.[38] found non-significant difference in germination rate of radish, tomato, or marigolds grown in pots with soil and irrigated with the PADW compared to control, while Zhang et al.[43] observed 50% improvement in germination of lentil seeds irrigated in vitro with the PAW produced by atmospheric pressure He plasma jet. These results indicate that, in general it is very difficult to predict the effect of the plasma as different plant species could respond in different manner.

3.4.2 | Growth parameters

Faster water uptake together with faster germination can lead to pronounced growth of seedlings. Table 1 summarizes the main growth parameters of wheat seedlings after 6 days cultivation, for example, their fresh and dry weight, average length, and vigor indices A and B. As the table shows the most pronounced effect was observed for 0.5–1 min ml^{-1} PADW and PATW. The average length of seedlings increased by ~6–7% for 0.5–1 min ml^{-1} PATW. For longer water activation time (2 min ml^{-1}) the average length was shorter compared to control. We observed ~19% and ~13% increase of fresh weight of seedlings cultivated in 0.5 min ml^{-1} PATW and PADW, respectively. In comparison, Maniruzzaman et al.[33] in similar experiment with wheat seedlings grown for 7 days in soil-free system irrigated with PADW observed a 35% increase of fresh weight. However, their PADW contained much higher concentrations of NO_3^- (~1.6 mM), compared to our PADW (0.3 mM). Therefore with respect to the NO_3^-

TABLE 1 Germination, fresh and dry weight, length, and vigor indices A and B of wheat seedlings cultivated for 6 days in vitro in the PADW or the PATW

PADW [min/ml]	Germination [%]	Fresh weight [mg/seedling]	Dry weight [mg/seedling]	Total length [cm/seedling]	Vigor index A	Vigor index B
0	95.00 ± 1.52^{a}	129.44 ± 1.47^{a}	30.55 ± 0.27^{a}	13.35 ± 0.40^{a}	123.01 ± 3.23^{a}	29.02 ± 0.45^{a}
0.25	97.33 ± 1.33^{a}	132.51 ± 2.20^{a}	31.11 ± 0.73^{ab}	13.48 ± 0.07^{a}	128.97 ± 2.48^{a}	30.26 ± 0.48^{ab}
0.5	97.33 ± 1.33^{a}	$\mathbf{146.67 \pm 0.96^{b}}$	$\mathbf{34.17 \pm 0.48^{c}}$	$\mathbf{14.00 \pm 0.25^{a}}$	$\mathbf{142.73 \pm 1.22^{b}}$	$\mathbf{33.24 \pm 0.23^{c}}$
1	$\mathbf{98.67 \pm 1.33^{a}}$	$\mathbf{145.70 \pm 0.97^{b}}$	$\mathbf{32.92 \pm 1.04^{bc}}$	$\mathbf{13.96 \pm 0.24^{a}}$	$\mathbf{143.77 \pm 2.75^{b}}$	$\mathbf{32.49 \pm 1.28^{bc}}$
2	96.00 ± 2.30^{a}	144.38 ± 3.06^{b}	32.50 ± 0.47^{abc}	13.37 ± 0.42^{a}	138.53 ± 3.23^{b}	31.08 ± 1.15^{abc}
PATW [min/ml]	**Germination [%]**	**Fresh weight [mg/seedling]**	**Dry weight [mg/seedling]**	**Total length [cm/seedling]**	**Vigor index A**	**Vigor index B**
0	96.00 ± 0.00^{ab}	135.00 ± 1.27^{a}	30.56 ± 0.55^{a}	13.47 ± 0.07^{b}	129.60 ± 1.22^{a}	29.33 ± 0.53^{a}
0.25	94.00 ± 1.15^{a}	151.72 ± 1.72^{b}	33.05 ± 1.68^{ab}	13.99 ± 0.34^{bc}	142.63 ± 2.70^{b}	31.11 ± 1.96^{ab}
0.5	$\mathbf{100.00 \pm 0.00^{c}}$	$\mathbf{160.14 \pm 2.84^{c}}$	$\mathbf{34.17 \pm 0.48^{c}}$	$\mathbf{14.23 \pm 0.18^{c}}$	$\mathbf{160.14 \pm 2.84^{d}}$	$\mathbf{34.17 \pm 0.48^{c}}$
1	98.67 ± 1.33^{bc}	$\mathbf{164.44 \pm 2.27^{c}}$	33.89 ± 0.55^{c}	$\mathbf{14.39 \pm 0.04^{c}}$	$\mathbf{162.26 \pm 2.67^{d}}$	33.44 ± 0.87^{c}
2	99.00 ± 1.00^{c}	151.94 ± 1.00^{b}	33.61 ± 0.28^{c}	11.74 ± 0.13^{a}	150.41 ± 1.10^{c}	33.27 ± 0.06^{c}

Maximal values are highlighted in bold. Data are listed as mean ± SE. Lowercase letters represents statistically significant difference at $p < 0.05$.

concentration difference the result obtained with our PADW/PATW are promising. In general, overall better performance (germination, fresh, and dry weight and total seedling length) was observed for PATW rather than for PADW, therefore the following experiments were done solely with the TW.

3.5 | Cultivation in vivo

In vivo cultivation of wheat plants was performed in pots filled with a solid substrate. After the cultivation the number of leaves, leaf area, fresh and dry weight, photosynthetic pigments (chlorophylls $a + b$, carotenoids $x + c$), soluble protein content and activity of antioxidant enzymes were evaluated for all four studied variants – control (TW), 0.5 min ml^{-1}, 2 min ml^{-1} (PATW), and Hoagland nutrition solution (HNS). In previous experiments (data not shown) we observed that the effect of the PAW in vivo strongly depended on the type of solid substrate (soils). In the soil with low nutrient content (e.g., loam-sand soil) the effect of the PAW was more significant, as here the PAW was the dominant source of signaling and growth stimulating RONS. In nutrient rich soils, the effect of the PAW was negligible as here the most of the nutrients come from soil itself. The stimulation effect of the PAW could be more pronounced in the later stages of vegetative growth of wheat, where plants needs more nitrogen supply, that the PAW is able to provide in the form of NO_2^- and NO_3^-. Therefore, we decided to perform in vivo experiments in perlite, a nutrient free substrate, and cultivate the wheat plants for prolonged period of 4 weeks to observe the effect of the PATW. After 4 weeks of cultivation no obvious differences between control and plants irrigated with the PATW was found. Subsequent detailed analysis of plants, however, showed several differences in their growth parameters (leaf area and number of green and senescent leaves), photosynthetic pigments, soluble protein content, and antioxidant enzymes activity.

3.5.1 | Growth parameters

The control irrigated with the TW and variants irrigated with 0.5 and 2 min ml^{-1} PATW had similar number of leaves; however they differed in the leaf area and number of green leaves. Figure 5 shows, the leaf area and number of green and senescent leaves of wheat plants irrigated with four different solutions. Plants irrigated with 0.5 and 2 min ml^{-1} PATW had higher leaf area by 22 and 23% respectively compared to control. In addition, plants irrigated with the PATW with higher RONS concentrations had greener and less senescent leaves. The premature physiological aging of leaves could be caused not only by lack of nitrogen but also by deficiency of other macro elements needed for intense growth. The most significant effect on the plant growth was found for the HNS that contains all necessary macro and micro elements. The plants irrigated with the HNS had more leaves in average, higher leaf area and nearly no senescent leaves. The leaf area and the number of green leaves of plants irrigated with the HNS was 1.5 times higher than the effect of the PATW. However, the concentration of NO_3^- in the HNS solution is two orders of magnitude higher compared to the PATW. Taking this into account, the effect obtained with the PATW is not so bad after all and it can be further improved by optimizing water activation times and with longer cultivation period than 4 weeks.

The effect of the PAW was also studied as the change in the fresh and dry weight of root and above-ground part of plants. The dry weight is being presented more often as it better represents the amount of produced biomass and is not loaded with an error from water content compared with the fresh weight. Plants irrigated with 0.5 min ml^{-1} PATW shown no significant difference in dry weight when compared to control irrigated with the TW only (data not shown). To achieve significant effect of the PAW on total biomass production would probably require longer cultivation period or the PAW generated with different water activation times. The stimulating effect of the PATW as well as the HNS was observed mainly in the increase of above-ground part of the plant and less in its root. This finding shows that nitrogen absorbed by plants is efficiently used especially for active photosynthesis and assimilation in favor of biomass production. Positive effect of the PAW on plant growth and the increase of biomass and leaves length have been previously reported also by other groups. Maniruzzaman et al.[33] in experiments with wheat seedlings found NO_3^- rich PAW more effective for above-ground part growth, while the root biomass increased more with the H_2O_2 rich PAW. Takaki et al.[32] observed improvement of growth rate of *Brassica rapa* irrigated with the PAW and also reported increase in concentration of nitrogen in leaves and increase of leaves length with increasing activation time.

3.5.2 | Photosynthetic pigments content

The content of photosynthetic pigments (chlorophylls and carotenoids) also increased with the PAW activity, that is, with the increasing RONS concentrations in the PATW (Figure 6). Compared to control, the plants irrigated with 2 min ml^{-1} PATW showed 17 and 12% increase of chlorophylls $(a+b)$ and carotenoids $(x+c)$, respectively. Chlorophylls are important photosynthetic pigments and their increase is a sign of increased rate of photosynthesis and total plant metabolism.[84] However, nitrogen deficiency can cause decrease in chlorophylls and carotenoids content.[85] Carotenoids have also additive role in photosynthesis and protective role against photo-oxidation. Their increase usually indicates the increase of oxidative stress in plant cells. Our results showed increased content of carotenoids for both PATW (6 and 12% for 0.5 and 2 min ml^{-1}, respectively) and the HNS (41%). As the HNS should not create any oxidative stress in the plants we assume that the increase of carotenoids in plants irrigated with the PATW is not a sign of elevated oxidative stress, but rather the result of higher concentration of the RONS in the PATW compared with the TW. In comparison, Maniruzzaman et al.[33] observed similar chlorophyll increase ~14% of wheat plants after 14 days of growth in soil-free system irrigated with the PADW.

3.5.3 | Soluble protein content

High protein content is one of the reasons why wheat is widely grown as one of the major crop plants in the world. Nitrogen is one of the essential elements for the protein production and NO_3^- is one of the main forms of nitrogen that plants can assimilate. It is known, that the soluble proteins play an important role in the growth of the plants and are very important component of numerous plant enzymes that reflect

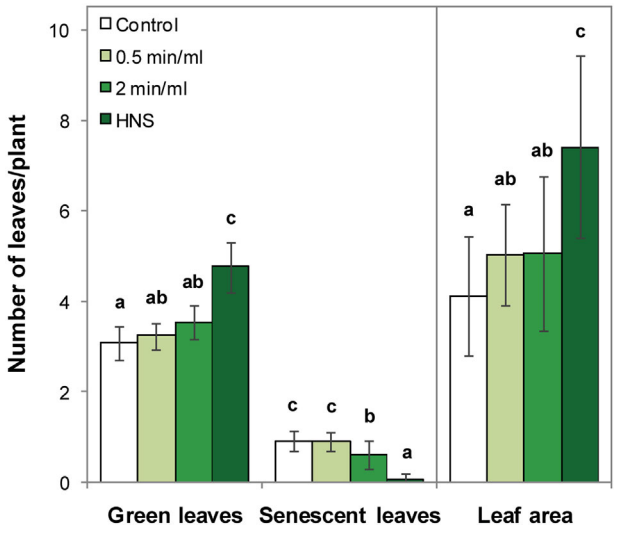


FIGURE 5 Area of the second fully developed leaf [cm^2] and number of green and senescent leaves of wheat plants grown in perlite substrate after 4 weeks of cultivation, irrigated with TW (control), PATW (0.5 min ml^{-1}, 2 min ml^{-1}), and Hoagland nutrition solution (HNS). Values are shown as mean ± SD. Lowercase letters represents statistically significant difference at $p < 0.05$.

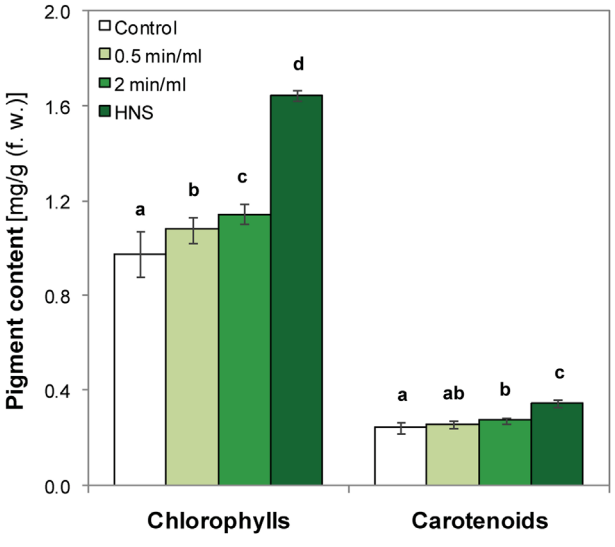


FIGURE 6 Chlorophylls $(a+b)$ and carotenoids $(x+c)$ content in leaves of wheat plants grown in perlite substrate after 4 weeks of cultivation and irrigated with TW (control), PATW (0.5 min ml^{-1}, 2 min ml^{-1}), and Hoagland nutrition solution (HNS). Values are shown as mean ± SD. Lowercase letters represents statistically significant difference at $p < 0.05$.

the overall plant metabolism.[86] Soluble proteins that we analyzed represent all proteins that are not incorporated in the cell or organelle membrane and are soluble in the water solutions (not only enzymes, but also pathogen related proteins or RuBisCo protein).[66] Figure 7 presents the results on total soluble protein content in root and above-ground part of wheat plants grown in perlite substrate after 4 weeks of cultivation and irrigated with the TW, the PATW and the HNS. We observed significant increase ~43% and ~69% in proteins content in root of wheat plants irrigated with 0.5 min ml^{-1} and 2 min ml^{-1} PATW, respectively. In above-ground part of plants we observed smaller increase in proteins content ~19% and ~14% for 0.5 min ml^{-1} and 2 min ml^{-1} PATW, respectively. The increase of soluble proteins in wheat plants is probably mainly caused by NO_3^- as a source of nutrient. However, the effect cannot be assigned only to NO_3^-, as also other RONS may affect the protein concentration. Our results clearly show that the use of the PATW increases protein content mainly in roots. Henselová et al.[20] found similar effect in maize roots after direct plasma treatment of seeds with CP and irrigated with water. However, it is difficult to compare our results with these results in detail as they were obtained with direct plasma treatment of seeds and not for indirect PAW mediated treatment, as it is in our case.

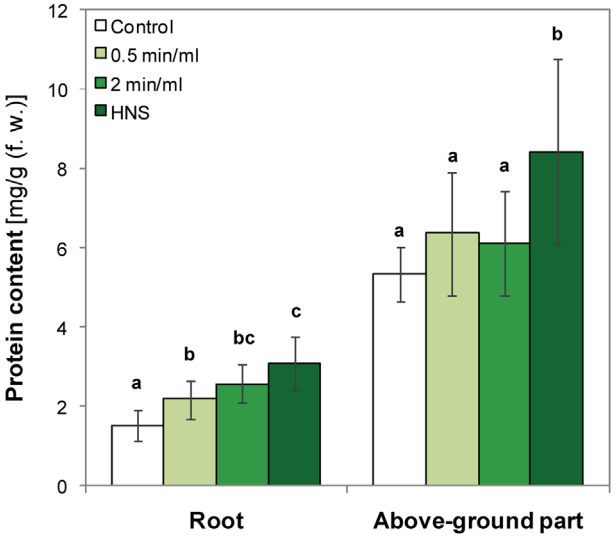


FIGURE 7 Soluble protein content in root and above-ground part of wheat plants grown in perlite substrate after 4 weeks of cultivation and irrigated with TW (control), PATW (0.5 min ml^{-1}, 2 min ml^{-1}), and Hoagland nutrition solution (HNS). Values are shown as mean ± SD. Lowercase letters represents statistically significant difference at $p < 0.05$.

3.5.4 | Antioxidant enzymes activity

Finally, the activity of antioxidant enzymes was evaluated to find whether the PAW can cause oxidative stress to plants. The increase in oxidative stress usually induces increased expression of antioxidant enzymes.[87] Plant resistance to stress is determined by the activity of antioxidant enzymes and the ability to rapidly increase these activities.[65] The PAW can potentially increase the oxidation stress as it contains various reactive oxygen species and highly reactive free radicals. We measured the activity of several antioxidant enzymes (SOD, CAT, G-POX) to find whether species present in the PAW can be the source of oxidative stress to plants. Figure 8 shows the activity of antioxidant enzymes SOD, CAT, and G-POX in root and above-ground part of plants irrigated with the TW, the PATW, and the HNS as a nutritional standard. The highest activity of enzymes signaling the highest oxidation stress was found in the control, that is, in plants with nitrogen deficiency. This is in an agreement with findings that showed increase of antioxidant enzymes activity (SOD, CAT, POD) in nitrogen-deficient plants compared with plants irrigated with complete nutrient solution.[65,85] The observed elevated oxidation stress is the result of long lasting deficit of nutrients as the control was grown in nutrient free substrate and irrigated with the TW only. The activity of the individual enzymes decreased with the increasing PATW activity, that is, with the increasing concentration of the RONS, probably mainly due to NO_3^-. Significant reduction of SOD activity to 92 and 79%, CAT activity to 75 and 54% and G-POX activity to 76 and 61% in above-ground part of wheat was found for 0.5 and 2 min ml^{-1} PATW, respectively. With respect also to other reported physiological parameters of the plants that were improved after irrigation with the PATW, for example, leaf area, pigment content, etc., we may assume that our PATW did not induce the oxidative stress to the plants under conditions in which they were grown. The lowest activity of enzymes was determined in plants grown in the HNS corresponding to the lowest stress state of the plants. We observed higher CAT activity in above-ground part than in the root and higher G-POX activity in the root than in the above-ground part of wheat plants. This observation is in agreement with a literature, as CAT is the most important H_2O_2 scavenging enzyme in leaves, while the G-POX seems to play a key role in roots.[88] Several papers reported antioxidant enzymes activity and concluded that plasma in direct contact with plant cell increases the activity.[40,89] On the contrary, seeds germinated in the PAW[44] or plants irrigated with the PAW, as it is in our experiments, showed decrease of antioxidant enzymes activity. However, the activity can also be affected by others parameters, for example, growth phase of plants.[20,44] Unfortunately, there are no studies dealing with the monitoring of antioxidant enzymes activity in plants irrigated with the PAW (related to indirect plasma treatment) to make more specific conclusion.

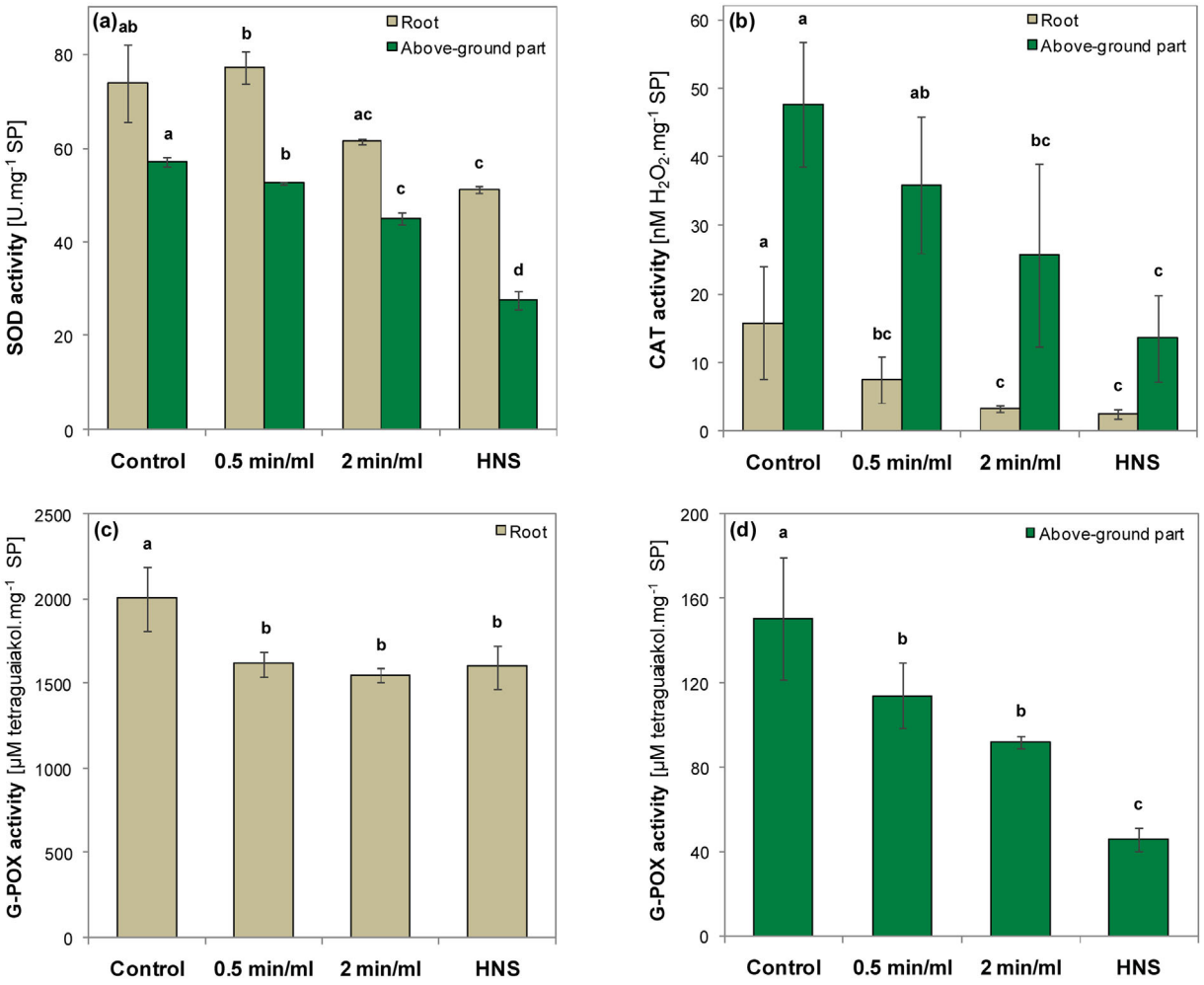


FIGURE 8 Activity of antioxidant enzymes (a) SOD, (b) CAT, and (c, d) G-POX in wheat plants after 4 weeks of cultivation in perlite substrate and irrigated with TW (control), PATW (0.5 min ml^{-1}, 2 min ml^{-1}), and Hoagland nutrition solution (HNS). Values are shown as mean ± SD. Lowercase letters represents statistically significant difference at $p < 0.05$.

4 | CONCLUSION

Atmospheric pressure cold plasma represents a promising method that is often utilized in various applications in medicine and agriculture. Direct plasma treatment or indirect plasma treatment mediated by the plasma activated media (gases or liquids) can stimulate the germination of seeds and growth of the plants. We investigated the effect of plasma activated water (PAW) generated by the transient spark on wheat seeds and plants (*Triticum aestivum* L.). Water uptake, germination, various growth parameters, and vigor indices, as well as photosynthetic pigments, content of soluble proteins and activity of antioxidant enzymes were studied and evaluated with respect to the type of the PAW and its activity both in vitro and in vivo conditions. The maximum improvement of seed germination, fresh, and dry weight and length of seedlings analyzed in vitro were observed for ~0.5 min ml^{-1} water activation time. The improvement in response to the use of the PAW is probably due to the increase in water uptake that induces faster seed's nutrition reserve utilization and metabolization of nitrogen species during vegetative growth of wheat. 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, while NO_2^- and NO_3^- are metabolized once the seeds start to germinate. The plants irrigated with the PAW and cultivated for 4 weeks in nutrient free substrate in vivo showed more green leaves, higher chlorophyll and carotenoids content and lower activity of antioxidant enzymes. The results demonstrate that NO_3^- as well as other RONS in the PAW serves not only as nutrients but they may also act like signaling molecule and have the potential to enhance the germination of wheat seeds and subsequent growth of the seedlings. For prospective

application of the PAW for irrigation of seeds and plants further optimization of water activity with respect to the specific plant growing conditions is necessary. We assume that the use of the PAW in the crop production process can be prospective in the future mainly in nitrogen deficient soils or hydroponic cultivation.

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