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Effect of Plasma Activated Water, Hydrogen Peroxide, and Nitrates on Lettuce Growth and Its Physiological Parameters

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Abstract: Cold plasma generated by atmospheric pressure air discharge is a source of various gaseous reactive oxygen and nitrogen species (RONS). When the plasma is generated in a contact with water, the RONS dissolve into water, change its chemical composition, while producing so-called plasma activated water (PAW). The PAW has the potential to be effectively used in various agricultural applications, as the long lived liquid RONS (H_2O_2, NO_2^-, NO_3^-) may act like signaling molecules in plant metabolism or serve as nutrients. We studied the effect of the PAW on lettuce plants and compared it with the effect of H_2O_2 and/or NO_3^- solutions of various concentrations to assess their role in the PAW. The PAW was generated from tap water by DC driven self-pulsing transient spark discharge. Pre-grown lettuce plants were cultivated in pots with soil and irrigated with the PAW or solutions of H_2O_2 and/or NO_3^-. After 5 weeks the growth parameters, number and quality of leaves, fresh and dry weight of plants, photosynthetic pigment (chlorophyll a + b) content, photosynthetic rate, and activity of antioxidant enzymes (superoxide dismutase, SOD) were evaluated. Lettuce plants irrigated with the PAW in comparison with chemically equivalent solution of H_2O_2 and NO_3^- had similar dry weight; however, the PAW induced higher photosynthetic pigment content, higher photosynthetic rate, and lower activity of SOD. The NO_3^- mainly contributed to the increase of dry weight, photosynthetic pigment content, photosynthetic rate, and overall better appearance of plants. The H_2O_2 contributed to an increase of dry weight and induced SOD activity. In general, H_2O_2 and NO_3^- in proper concentrations can stimulate plant growth and affect their physiological properties.

Keywords: plasma activated water; plasma agriculture; hydrogen peroxide; nitrates; lettuce; growth enhancement; pigment contents; photosynthesis; antioxidant enzymes

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1. Introduction

The production of fruits and vegetables is an economically important segment in many regions of the world. Fresh and high-quality agriculture products with adequate content of macro and micronutrients are of a great demand as they offer good quality of life. To support the growth of the fruits and vegetables, various organic and inorganic fertilizers are used that provide plant nutrients necessary for their growth [1]. Besides the use of fertilizers, several physical methods have been used too, e.g., static magnetic fields or pulsed electric fields [2,3]. Another promising and still novel physical method represents a cold (nonthermal, non-equilibrium) plasma.

The cold plasma generated by various kinds of atmospheric pressure air discharges produces highly reactive environment of high energy electrons, radicals, and various gaseous reactive species, including $^\bullet OH$, H_2O_2, NOx, HNOx. When the plasma is in a contact with water, these species dissolve into water and produce so-called plasma activated water (PAW). The PAW is a mixture of long-lived reactive oxygen and nitrogen species (RONS), such as H_2O_2, NO_2^- or NO_3^- and it is considered as a clean and sustainable alternative to chemical fertilizers. Cold plasma and plasma activated water has a potential to be effectively used in various agricultural applications [4,5]. Most of the existing studies deal with direct plasma treatment of seed for seed disinfection, germination improvement, plant growth, etc. [6–14]. Besides that, indirect plasma treatment, i.e., the use of PAW or plasma activated fertilizer on seeds and plants, has recently become an interest too [15–22].

The plasma generated RONS in the PAW (H_2O_2, NO_2^-, NO_3^-) may be easily transported into plant cells and act as signaling molecules in plant metabolism or can be a source of nutrients for a plant. H_2O_2 (hydrogen peroxide) can be transported through cell membrane by free diffusion or by membrane proteins facilitating water transport (aquaporins) [23]. In the cell it can be degraded by antioxidant enzyme peroxidase or by catalase with oxygen and water as products. H_2O_2 is the most stable reactive oxygen species (ROS) and, therefore, plays a crucial role in intracellular signaling in many physiological processes. The transport of NO_3^- (nitrate) into the plant cell is slower and a concentration-dependent process happening through specialized membrane transport proteins or by simple diffusion [24,25]. NO_3^- is a major source of nitrogen as an essential macronutrient for plants. Moreover, NO_3^- may also act like signaling molecule and induce expression genes for self-transport and assimilation in the plant cell. Absorbed nitrogen could be efficiently used especially for active photosynthesis and assimilation in favor of biomass production. At last, some plants are also able to utilize NO_2^- (nitrite) as a nitrogen source, although, for many other plants, it can be potentially toxic [26].

To better understand the process in plants, it is necessary to focus on the effects of individual RONS components in plasma activated water (PAW). In the past, the effect of RONS was mainly investigated with respect to general visual appearance of plants and their growth parameters. However, recently, the research shifted toward analyses on microscopic and molecular level to further understand the physiological mechanisms that RONS induces in seeds and plants. For example, Kang et al. treated various liquids with microwave plasma and used them to support plant vitality and sanitation effect [27]. Their results suggest that NO and NO_3^- contribute to the enhancement of plant growth and stress tolerance and may also play role in antimicrobial effect. Adhikari et al. [28] observed that the PAW irrigation of tomato plants improved their growth, endogenous RONS, defense hormones, and expression of key pathogenesis related genes. Gierczik et al. [29] showed that PAW improves tolerance against low temperature and hypoxia stresses during germination through the changes in carotenoids, cysteine, and γ-glutamylcysteine content. They attributed the effect to H_2O_2 and NO. H_2O_2 can activate various defense mechanisms through the redox signaling pathway, while NO formed from NO_2^- and NO_3^-, can activate the protective processes under the stress conditions. The PAW possessed outstanding abilities in improving seed germination, seedling growth, and microbial decontamination. Zhou et al. treated mung bean seedlings with the PAW and found changes in enzymatic and hormone activities indicating that PAW can reduce oxidative damage, increase antioxidant enzyme activities and promote seed germination and seedling growth [17]. Our group investigated the effect of PAW composition (activity), i.e., concentrations of H_2O_2, NO_2^-, and NO_3^-, on wheat seeds and plants [18]. We found that seeds cultivated in the PAW interact mainly with H_2O_2 in the early growth stages during imbibition and germination, while NO_3^- and NO_2^- are metabolized once the germination starts and during the early plant growth. The experiments showed that, for prospective application of the PAW, further optimization of water composition is necessary.

Encouraged by the previous results, we decided to further investigate the effect of PAW. The objective of this work was to compare the effects of PAW and solutions of H_2O_2 and NO_3^-, in order to better understand the role and contribution of individual RONS to plant growth and physiology. The experiments were performed with pre-grown lettuce plants and the evaluated parameters were visual appearance and growth parameters of plants, photosynthetic pigment content, photosynthetic rate, and activity of antioxidant enzymes. So far, the effect of cold plasma relevant to lettuce plants has been mostly mentioned in context of improved food quality and inactivation of pathogen microorganisms from fresh-cut leaves. The plasma was applied to lettuce leaves directly [30], indirectly as plasma activated water [31–34], or as plasma activated air in the process for food sanitation [35]. Besides, only few papers have dealt with the effect of plasma or PAW on lettuce seeds, their germination and seedling growth parameters [36–38]. We are not aware of any other research papers discussing the effect of PAW on lettuce plants, neither comparing it with the effect of H_2O_2 and NO_3^- solutions. In this respect, we assume that the presented work investigating the effects of PAW and solutions of H_2O_2 and NO_3^- on lettuce growth and its selected physiological parameters is unique and original.

2. Materials and Methods

The experimental setup is depicted in Figure 1a. The plasma reactor of a point-to-plane geometry consisted of a high voltage needle electrode placed above a grounded electrode embedded inside an inclined polytetrafluoroethylene (PTFE) plane. The distance between the electrodes was ~1 cm. Tap water was placed in a test tube and, by a peristaltic pump, (Masterflex L/S, Vernon Hills, IL, USA) pumped down the inclined electrode and back to the tube with the constant flow rate of 14 mL·min^{-1}. Self-pulsing transient spark (TS) discharge generated in atmospheric pressure air [39] was applied to the tap water circulating through the discharge zone to generate the PAW. The test tube was immersed in an ice bath to prevent the unwanted heating of the produced PAW. The TS discharge was driven by positive DC power supply (Technix RS20–R–1200, Créteil, France) and its electrical characteristics were monitored by a high voltage probe (Tektronix P6015A, Berkshire, UK) and a Rogowski type current probe (Pearson Electronics 2877, Palo Alto, CA, USA) connected to an oscilloscope (Tektronix TDS 2024, Berkshire, UK). The typical electrical characteristics of TS discharge in our experiments was as follows: the amplitude of the applied voltage U ~ 10–13 kV, amplitude of discharge current pulses I_{max} ~ 8–10 A, frequency of the discharge current pulses f ~ 2–3 kHz, and average discharge power was ~ 6 W. The characteristic voltage and current waveforms of the TS discharge are depicted in Figure 1b. The upper one presents the voltage waveform in ms timescale, while the bottom one presents both voltage and current waveforms in μs scale.

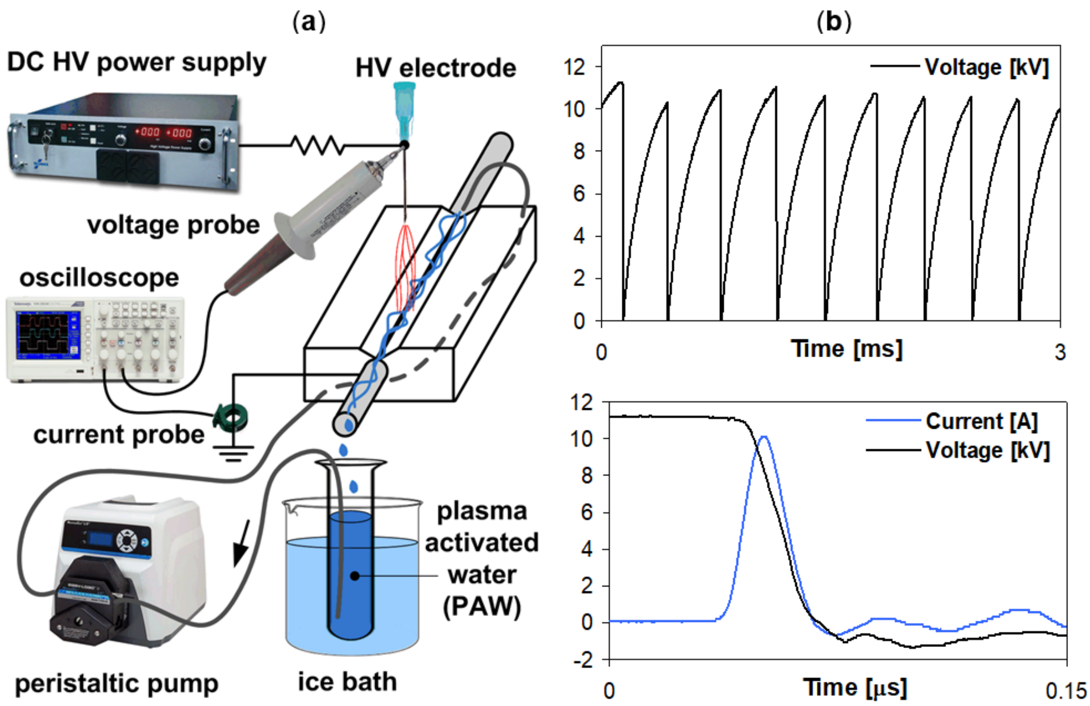

Figure 1. (**a**) The scheme of experimental setup and (**b**) characteristic voltage and current waveforms of self-pulsing transient spark discharge in two different timescales.

The PAW was produced from tap water. Unlike deionized or distilled water, it is more physiological for plants and it has a natural ability to preserve constant pH thanks to hydrocarbon buffer system. Therefore, the PAW produced from tap water by TS discharge treatment/activation does not significantly changes its pH. The water activation time by plasma was set to 1 min·mL^{-1} (i.e., every 1 mL of water was activated by plasma for 1 min), and was constant for all experiments. The chemical composition of the PAW was measured by established UV/Vis spectrophotometric methods (Shimadzu UV–1800, Kyoto, Japan) to evaluate the concentrations of hydrogen peroxide (H_2O_2), nitrites (NO_2^-), and nitrates (NO_3^-). The H_2O_2 concentration was determined by its reaction with titanyl ions of $TiOSO_4$ resulting into a yellow-colored product with maximum absorbance peak at 407 nm [40]. The NO_2^- and NO_3^- concentrations were determined by the commercial kit using Griess reagents (Cayman Chemicals, Ann Arbor, MI, USA) forming a pink-colored azo-product with maximum absorbance peak at 540 nm [41,42]. The pH and temperature were monitored with portable pH meter (WTW 3110, Weilheim, Germany). Additional information on the experimental system, TS discharge and the chemical analysis can be found in our previous papers [18,43].

As a model plant, we used lettuce (*Lactuca sativa* L. var. capitata cv. Král máje I). The plants were pre-cultivated in pots with soil at controlled conditions: 24/18 °C (light-dark), light intensity 120 µmol·m^{-2}·s^{-1} and 12 h photoperiod. Three week-old pre-grown plants that had 2–3 developed leaves were then separated—one plant per pot, four plants per each variant—and during the next 5 weeks, irrigated with the PAW or with solutions of the same and higher concentrations of H_2O_2 and/or NO_3^- as those in the PAW. The H_2O_2 solutions were prepared from 35% H_2O_2 (CentralChem, Bratislava, Slovakia) and diluted with tap water to the desired concentration. NO_3^- solutions were prepared from $NaNO_3$ salt (Penta, Praha, Czechia) that was dissolved and diluted with tap water to desired concentrations. The chemical composition and pH of all variants used for lettuce plant irrigation are listed in the Table 1. These variants include the PAW (0.42 mM H_2O_2 + 0.85 mM NO_3^-), solutions of various concentrations of H_2O_2 (0.4, 1, 10 mM), NO_3^- (0.85, 2, 20 mM), H_2O_2 + NO_3^- (0.4 mM + 0.85 mM, and 10 mM + 20 mM), and control. The table defines abbreviations for each solution that are further being used in the text and all figures. The control plants were irrigated with tap water only. The type of soil of low nutrient content (loam-sand soil) was used. The reason why we tested the effect of PAW and H_2O_2/NO_3^- solutions on pre-grown plants and not on seeds is that the stimulating effect of PAW is usually more pronounced in the later stages of vegetative plant growth, when plants need more nutrients. During the germination, seeds and young seedlings take nutrients mainly from seed supplies, while in later stages, from or soil or water.

Table 1. Chemical composition and pH of plasma activated water (PAW) and various H_2O_2/NO_3^- solutions. The respective concentrations are indicated in parentheses in abbreviated format that is used in text and figures.

Solution/Variant (Abbrev.)	H_2O_2 [mM]	NO_3^- [mM]	pH
Control (tap water)	-	~0.02	~7.5
PAW	~0.42	~0.85	~7.5
H_2O_2 (.4)	0.4	-	~7.6
H_2O_2 (1)	1.0	-	~7.7
H_2O_2 (10)	10.0	-	~7.8
NO_3^- (.85)	-	0.85	~7.9
NO_3^- (2)	-	2.0	~7.9
NO_3^- (20)	-	20.0	~7.9
H_2O_2 (.4) + NO_3^- (.85)	0.4	0.85	~7.9
H_2O_2 (10) + NO_3^- (20)	10.0	20.0	~7.4

After 5 weeks of irrigation with the PAW or with solutions of H_2O_2 and/or NO_3^-, the complex evaluation of visual appearance (number and quality of leaves), growth parameters (fresh and dry weight), photosynthetic pigment (chlorophyll a + b) content, photosynthetic rate, and activity of antioxidant enzymes (superoxide dismutase, SOD) in above-ground parts and roots of lettuce plants was performed.

Visual appearance was evaluated by counting the number of leaves per plant and qualifying them as healthy (green) or senescent (physiologically aged). The fresh weight of above-ground parts and roots of lettuce were measured separately. The dry weight was also determined separately, after plant parts were dried packed in aluminum foil at 60 °C for 3 days. The concentration of photosynthetic pigments, i.e., chlorophylls (a + b), in leaves were measured according to Lichtenthaler [44]. The method is based on absorbance of pigments in the UV/Vis region. The three repetitions per 1 g of fresh weight of average leaf sample per one variant were homogenized with sand, $MgCO_3$, and 80% acetone (v/v). The homogenized leaves were filtered and filled to a certain volume with acetone. The concentrations of chlorophyll a and chlorophyll b were calculated from the absorbance at 664 and 648 nm, respectively. Net photosynthetic rate was measured by infrared gas analyzer (PP-Systems CIRAS–2, Amesbury, MA, USA). The principle of the measurement is based on the CO_2 concentration changes estimated by absorption of infrared radiation. The concentration of CO_2 in gas passing into a cuvette surrounding the part of the leaf is compared to the CO_2 concentration leaving the chamber. The eighth fully developed leaf from the plant center was enclosed in cuvette (PLC6 cuvette) and the photosynthetic rate was evaluated in a controlled conditions—CO_2 concentration 380 $\mu mol \cdot mol^{-1}$, leaf temperature 23 ± 1 °C, relative air humidity 65–70%, and leaf–air vapor pressure difference 700–1000 Pa. The light intensity was decreased step-wise with irradiation periods of 3 min and subsequent saturation pulses were applied until 60 $\mu mol \cdot photon \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation (PAR) was reached. Then, the actinic light was switched off and after 10 min, the rate of respiration in the dark (RD) was recorded. The measurement was done in triplicates on three plants from each variant. The activity of representative antioxidant enzyme (superoxide dismutase, SOD) in above-ground parts of lettuce was measured. The antioxidant activity was measured by UV/Vis spectrophotometry according to standardized assay and normalized to soluble protein content in sample. The SOD activity was estimated as a decrease of absorbance at 560 nm, as it inhibits the photoreduction of thiazolyl blue tetrazolium bromide (MTT) with superoxide radicals $O_2^{\bullet -}$ that are produced by reaction of methionin with riboflavin under the white light [45].

The results are usually presented in the form of column charts containing three groups of columns. The first group consists of five columns, that present the PAW (1 $min \cdot mL^{-1}$), its chemically equivalent solution of H_2O_2 (.4) + NO_3^- (.85), separate solutions of H_2O_2 (.4) and NO_3^- (.85), and the control (tap water). The second group presents solutions of higher concentrations of H_2O_2 (1) and NO_3^- (2) that approximately correspond to water activation time of 3 $min \cdot mL^{-1}$. The third group presents solutions of the highest concentration of H_2O_2 (10), NO_3^- (20) and their combination H_2O_2 (10) + NO_3^- (20). The data are presented as mean values ± standard deviation. The one-way analysis of variance (ANOVA) and subsequent multiple range test by least significance difference method (LSD) were performed to judge the differences between the groups. The different lowercase letters represent statistically significant difference at probability $p < 0.05$.

3. Results and Discussion

In the experiments, we irrigated the lettuce plants with the PAW generated with 1 $min \cdot mL^{-1}$ water activation time. We decided to use this particular water activation time based on previous experiments, where we examined the effect of various water activation times in the range of 0.25–2.0 $min \cdot mL^{-1}$ on wheat [18] and several other species of pre-grown plants (radish, tomato, lettuce). Interestingly, we observed that various plant species reacted differently to the PAW irrigation. After 4 weeks of cultivation and irrigation with the PAW, tomato plants were found to be taller, no effect was found on radish plants, and the most pronounced effect was observed on lettuce plants. Therefore, we decided to further continue the experiments with lettuce plants and explore the role of two prominent

PAW species (H_2O_2 and NO_3^-) in the context of lettuce growth enhancement. The effect of PAW on lettuce plants was compared with the effect of H_2O_2 and/or NO_3^- solutions of various concentrations.

To understand the effect of PAW on lettuce plants, it is necessary to know its composition. The concentrations of H_2O_2, NO_2^-, and NO_3^- in the PAW were measured immediately after plasma activation and they were ~0.42 mM, ~0.38 mM, and ~0.85 mM, respectively. The pH of the PAW remained fairly constant (pH ~7.5) or changed very mildly with extended water activation time, thanks to a natural water hydrocarbon buffer system ($HCO_3^- + H^+ \leftrightarrow H_2CO_3$) [46].

3.1. Visual Appearance

The visual appearance of plants was evaluated based on leaf size, their number, and the stage of their physiological aging. We found that lettuce plants irrigated with the PAW had significantly larger leaf size compared to control, as can be seen in Figure 2; however, with similar number of leaves. While three week-old pre-grown lettuce plants had 2–3 developed leaves, after 5 weeks of cultivation, the plants had approximately 8–10 developed leaves, on average. A positive effect of the PAW on plant growth and the increase of biomass and leaf size have been found also in our previous study [18] and were also reported by other groups [15,16,47]. Plants irrigated with solutions containing NO_3^- had slightly higher number of green than senescent leaves compared to control. On the other hand, high concentration of H_2O_2 negatively affected the overall appearance of plants, i.e., leaf size, shape, and color. We observed an increased number of senescent leaves in plants irrigated with high H_2O_2 (1, 10) concentrations. However, when H_2O_2 was combined with high NO_3^- concentration, the positive effects of NO_3^- prevailed, as plants with proper nutrition could better handle the stress created by higher concentrations of H_2O_2. Overall, the premature physiological aging of leaves (senescence), as also evident in Figure 2, can be caused by lack of nitrogen, or plant stress (discussed in Section 3.5).

Figure 2. Photography of lettuce plants after 5 weeks of cultivation in soil irrigated with tap water (control, **left**) and the plasma activated water (water activation time 1 min·mL^{-1}, **right**).

3.2. Fresh and Dry Weight

The fresh and dry weight represents the amount of biomass produced by the plant, where the dry weight better reflects biomass change. The fresh and dry weight of lettuce above-ground parts and roots showed similar trends with respect to H_2O_2 and NO_3^- concentrations in solution. The fresh weight of above-ground parts of lettuce irrigated with the PAW was higher by 10% and the dry weight of above-ground parts by 16%, compared to the respective controls (Figure 3). Plants irrigated with a chemically equivalent solution of H_2O_2 (.4) + NO_3^- (.85) with a composition mimicking the PAW had very similar dry weight. On the other hand, plants irrigated with a solution containing only one of the species, i.e., either H_2O_2 (.4) or NO_3^- (.85), had slightly smaller dry weight, but still higher compared to control. In solutions with higher concentrations of H_2O_2 (1, 10) and NO_3^- (2, 20), the dry weight of above-ground parts of lettuce increased correspondingly. The NO_3^- is the main source of nitrogen for plant production of proteins and nucleic acids; therefore, it can be considered as the main component of the PAW responsible for biomass increase. On the other hand, H_2O_2 can also take part in weight increase through the process of plant tissue lignification. The H_2O_2 treatment promotes activities of enzymes in phenylpropaniod metabolism (PAL, C4H, 4CL) eliciting lignin accumulation and upregulating other enzyme (DNase, RNase, caspase 3-like) activities that finally contribute to the accelerated lignification [48]. Moreover, the H_2O_2 acts as co-substrate in oxidation reactions during lignin polymerization [49].

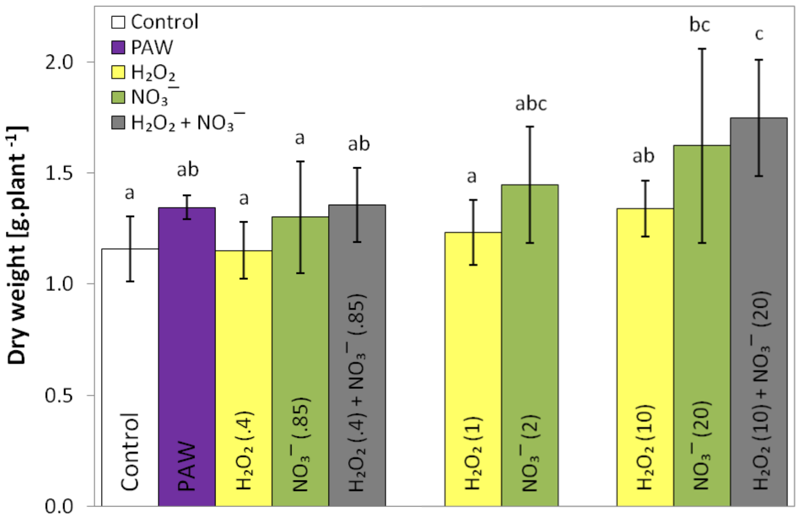

Figure 3. Dry weight of above-ground parts of lettuce plants after 5 weeks of soil cultivation irrigated with the plasma activated water (PAW), solutions of various concentrations of H_2O_2 (0.4, 1, 10 mM), NO_3^- (0.85, 2, 20 mM), $H_2O_2 + NO_3^-$ (0.4 mM + 0.85 mM, 10 mM + 20 mM) and control irrigated with tap water. The data are presented as mean values ± standard deviation from 3 independent repetitions. The lowercase letters represent statistically significant difference at probability $p < 0.05$.

The dry weight of roots showed a slightly different trend with respect to H_2O_2 and NO_3^- concentrations and with higher data uncertainty. The dry weight of roots increased with increasing NO_3^- concentration. On the other hand, the effect of H_2O_2 was found indistinctive, where the highest concentration of H_2O_2 (10) had similar effect as control. The highest dry weight of roots was observed for the lowest concentration of H_2O_2 (.4). A similar result was reported by Nazir et al. [50], who also observed stimulatory effect on roots weight for low concentrations of H_2O_2 (0.1, 0.5 mM). Overall, the stimulating effect of the PAW was observed mainly in above-ground parts and less in roots of the plants, which is in agreement with our previous study on wheat [18]. Likewise, Stoleru et al. also found no significant effect on the weight of roots, but an increase in the weight of above-ground parts (foliar weight) of lettuce irrigated with PAW [38].

3.3. Photosynthetic Pigment Content

The evaluation of pigment content in leaves is extremely important from a physiological point of view, because the chlorophyll pigments (a + b) found in the chloroplasts of the leaves play an important role in converting the light into energy through the photosynthesis process. Moreover, the amount of luminous energy taken by a leaf is determined by chlorophyll pigment contents, which directly affect both the process of photosynthesis and primary production. Chlorophyll pigment content indirectly also provides information about mineral nutrition, because chlorophyll stores a significant amount of nitrogen and content of pigments is a sign of total plant metabolism [37,51]

The concentration of photosynthetic pigments, i.e., chlorophylls (a + b), was higher by 2% in plants irrigated with the PAW compared to control (Figure 4). As the figure shows, solutions of NO_3^- had stimulating effect on chlorophyll content that increased with the increase of NO_3^- (.85, 2, 20) compared to control by 5, 9, and 13%, respectively. The result indicates that NO_3^- as a source of nitrogen can significantly contribute to the chlorophyll production in plants. On the other hand, the increasing concentration of H_2O_2 (.4, 1, 10) had almost no effect on chlorophyll content in lettuce leaves. The results confirm that H_2O_2 does not participate in chlorophyll biosynthesis, and that the lack of nitrogen can lead to their decrease [52]. Interestingly, a combination of the highest concentrations of H_2O_2 (10) + NO_3^- (20) induced higher chlorophyll content (18%) than separate solutions of H_2O_2 (10) or NO_3^- (20) that induced an increase by 2% and 13%, respectively. However, with the chemically equivalent solution of H_2O_2 (.4) + NO_3^- (.85) mimicking the PAW, we observed an opposite effect, i.e., the effect of separate solutions to chlorophyll content was stronger. The lettuce irrigated with the PAW had similar effect on chlorophyll content as the solution containing the same concentration of NO_3^- as it is in the PAW. The positive effect of the PAW on pigments content was also previously reported in other studies [16,18] that claimed 12–17% increase of pigments after 2–4 weeks of wheat cultivation. Significant increase in chlorophyll content was recently also observed by Stoleru et al. with lettuce irrigated by the PAW [38].

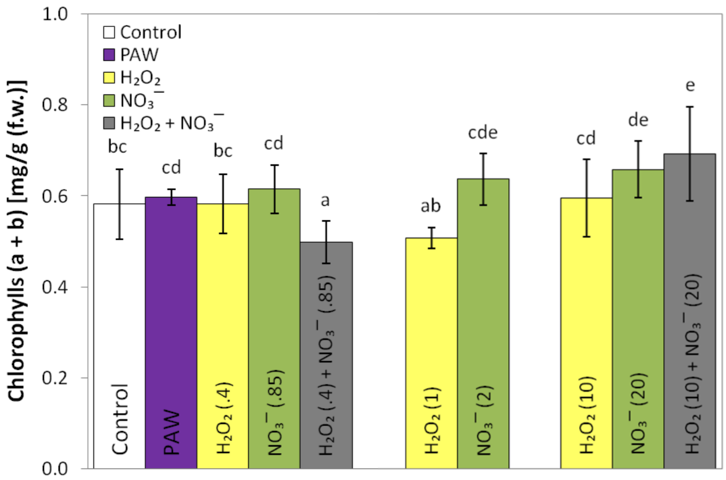

Figure 4. Chlorophyll (a + b) content in above-ground parts of lettuce plants after 5 weeks of soil cultivation irrigated with the plasma activated water (PAW), solutions of various concentrations of H_2O_2 (0.4, 1, 10 mM), NO_3^- (0.85, 2, 20 mM), H_2O_2 + NO_3^- (0.4 mM + 0.85 mM, 10 mM + 20 mM), and control irrigated with tap water. The data are presented as mean values ± standard deviation from 3 independent repetitions. The lowercase letters represent statistically significant difference at probability $p < 0.05$.

3.4. Net Photosynthetic Rate

Autotrophic organisms like plants photosynthesize and assimilate CO_2 using the energy of light. High levels of chlorophylls can be attributed to increased physiological activity and photosynthesis in plants. Enhanced leaf photosynthesis results in increased phloem sap movement and plant growth [53]. The photosynthetic rate is given by photosynthetic pigment content, but may decrease when plants experience a stress [54]. The environmental stress may cause reduction in the chlorophyll content. This reduction could be due to destruction of the chloroplast and instability of chlorophyll protein complex [55]. The plasma or PAW can potentially also induce stress or otherwise affect the plant metabolism. Therefore, we measured the photosynthetic rate of lettuce irrigated with the PAW and correlated it with the chlorophyll contents.

The photosynthetic rate was positively affected by the PAW irrigation (Figure 5). The highest photosynthetic rate was observed for plants irrigated with solutions with the highest concentration of NO_3^- (20) and the solution of H_2O_2 (10) + NO_3^- (20). On the other hand, the highest concentration of H_2O_2 (10) had no effect on photosynthetic rate. This again confirms that NO_3^- plays a very important role in photosynthesis and pigment process compared to H_2O_2. However, under special conditions even H_2O_2 can promote photosynthesis (e.g., under the stress) [50]. A comparison of the effects of PAW and the chemically equivalent solution mimicking the PAW, i.e., H_2O_2 (.4) + NO_3^- (.85), showed slightly better results for the PAW. The result on net photosynthetic rate correlates with the results on photosynthetic pigment content. It indicates that the PAW had a positive effect on both the chlorophyll content and the photosynthetic rate.

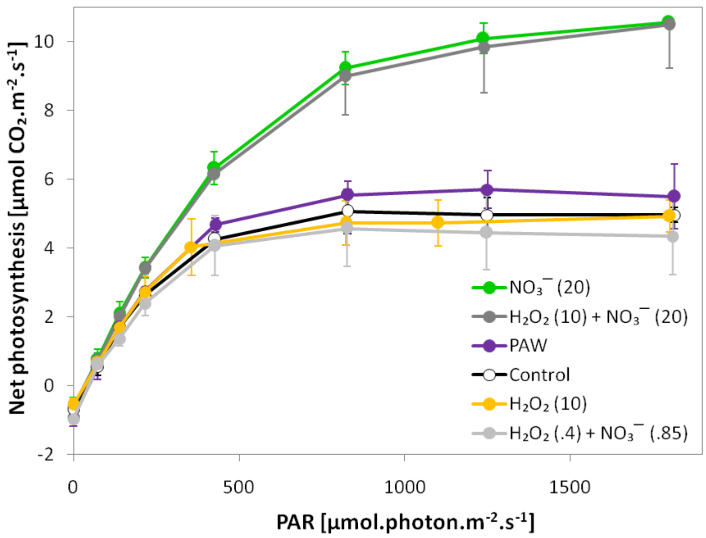

Figure 5. Net photosynthetic rate in above-ground parts of lettuce plants after 5 weeks of soil cultivation irrigated with the plasma activated water (PAW), solutions of various concentrations of H_2O_2 (10 mM), NO_3^- (20 mM), H_2O_2 + NO_3^- (0.4 mM + 0.85 mM, 10 mM + 20 mM), and control irrigated with tap water. The data are presented as mean values ± standard deviation from 3 independent repetitions.

3.5. *Activity of Antioxidant Enzymes*

The activity of antioxidant enzymes reflects the level of oxidative stress that a plant is exposed to and depends on many factors, e.g., direct or indirect plasma treatment, application on seeds, type of seedling or plant, etc. Here, we present the results on superoxide dismutase (SOD) activity as a representative enzyme that is a part of a complex specialized system protecting plant cells against oxidation. SOD is an intracellular antioxidant that decomposes superoxide to hydrogen peroxide in a reaction $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$. The PAW can be a potential source of oxidation as it contains various reactive oxygen species and free radicals. However, in our experiments, we found a decrease of activity of antioxidant enzymes in the PAW irrigated plants by 15% when compared to control (Figure 6). It indicates that the PAW did not increase the oxidative stress in plant cells. However, it can be also an evidence of elevated oxidation stress in plants irrigated with tap water (control) with a deficit of nutrients. In comparison to the effect of PAW, the effect of various H_2O_2/ NO_3^- solutions on the activity of antioxidant enzymes was a little more complicated. The SOD activity in above-ground parts of plants decreased with concentration of NO_3^- (20) by 42% and increased with concentration of H_2O_2 (10) by 60% (Figure 6). The H_2O_2 can contribute to elevated oxidative stress by its oxidation–reduction potential, but also as the result of nutrient deficit. On the other hand, NO_3^- works well against the antioxidant enzymes activity through its nutrient function and the increase of NO_3^- (.85, 2, 20) led to a decrease of SOD activity, as also shown in our previous paper [18].

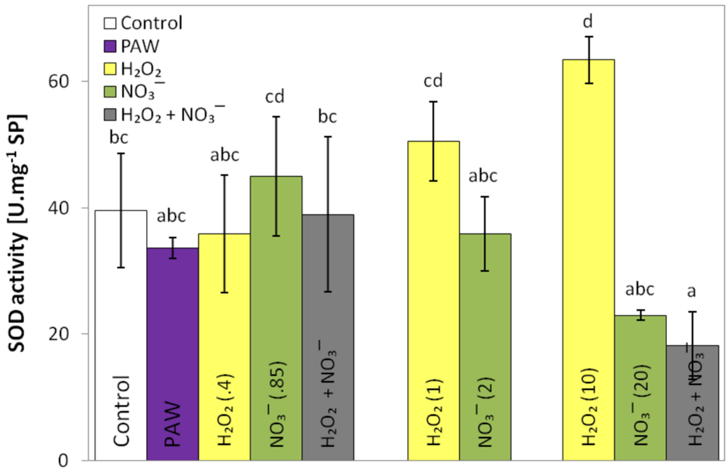

Figure 6. Activity of superoxide dismutase (SOD) in the above-ground parts of lettuce plants after 5 weeks of soil cultivation irrigated with the plasma activated water (PAW), solutions of various concentrations of H_2O_2 (0.4, 1, 10 mM), NO_3^- (0.85, 2, 20 mM), $H_2O_2 + NO_3^-$ (0.4 mM + 0.85 mM, 10 mM + 20 mM), and control irrigated with tap water. The data are presented as mean values $\pm$ standard deviation from 3 independent repetitions. The lowercase letters represent statistically significant difference at probability $p < 0.05$.

The results reported by various groups are disparate. Švubová et al. found increased concentration of radicals and activation of antioxidant enzymes in seeds exposed to air plasma [13,56]. Zhou et al. reported that PAW applied on mung bean seedlings possessed higher SOD activity indicating reduction of oxidative damage [17]. Zhao et al. also found the antioxidant enzyme activity level to be higher after the PAW treatment in fresh-cut kiwi fruit than when treated by HNO_3 or H_2O_2 solution [57]. Guo et al. found that dielectric-barrier discharge plasma treatment enhanced the antioxidant activity of wheat seeds under drought stress by regulating the functional and regulatory genes [58]. On the other hand, our current as well as previous results [18] indicate a decrease of SOD activity with an increase of PAW activity. Yemeli et al. studied the effect of PAW generated by various air discharges in about-ground parts of barley and maize plants and found similar effect. They also observed a decrease of SOD, especially for PAW, but at the same time an increase of other antioxidants enzymes, e.g., guajacol peroxidase and catalase [20]. Overall, the activity of antioxidant enzymes can be affected by various parameters, e.g., growth phase of plants, soil type, or activity and composition of PAW. Our results showed that H_2O_2 stimulate SOD activity, while NO_3^- suppresses it. Thus, total SOD activity depends on their actual balance in the PAW and may decrease despite the fact that the PAW contains RONS that are eventual oxidative stressors.

4. Conclusions

The effect of PAW generated by transient spark discharge on visual appearance, growth parameters, and important physiological and biochemical parameters of lettuce plants were investigated. The effect of irrigation with the PAW was compared and correlated with the effect of various H_2O_2 and/or NO_3^- solutions.

Lettuce plants irrigated with the PAW in comparison to lettuce irrigated with chemically equivalent solution of H_2O_2 (.4) + NO_3^- (.85) had similar dry weight of above-ground parts and roots. However, the PAW induced higher photosynthetic pigment content (chlorophyll a + b), higher photosynthetic rate, and lower activity of antioxidant enzymes (superoxide dismutase, SOD). The NO_3^- in the solution mainly contributed to the increase of dry weight, photosynthetic pigment content, photosynthetic rate, decrease of SOD activity, and overall better visual appearance of plants. The H_2O_2 in the solutions had usually negative effect on the development of plants. Although it induced an increase of dry weight, it did not contribute to photosynthetic pigment content and photosynthetic rate and even increased SOD activity. The NO_3^- and H_2O_2 are, without a doubt, the most important RONS in the PAW that affects the development and quality of the plants. Their joined effect usually results in an improvement of the plant growth and their physiological parameters; however, their ratio must be optimized for further improvement. Based on the results obtained in this study, we can conclude that PAW may positively affect several physiological parameters of plants. However, the overall effect of PAW on plant growth in this study was rather limited. Thus, optimizing the PAW composition must be performed prior to its eventual applications in agriculture.

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