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Preliminary Study on the Impact of Non-Thermal Plasma Activated Water on the Quality of *Triticum aestivum* L. cv. Glosa Sprouts

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Abstract: The present study aimed to investigate the effects of non-thermal plasma-activated water (PAW) on *Triticum aestivum* L. cv. Glosa sprouts. Two types of PAW were generated by exposing distilled water to a high-voltage electric discharge. Wheat caryopses were treated either with PAW1 (25 mg/L NO_3^-, 4 mg/L NO_2^-, and 6 mg/L H_2O_2) or PAW2 (35 mg/L NO_3^-, 5 mg/L NO_2^-, and 7.5 mg/L H_2O_2) for 8 days, with samples being collected at days 1, 2, 3, and 8. The germination rate, growth parameters, protein, photosynthetic pigments, total phenolic contents, antioxidant activity of free and bound phenolic fractions, and activity of antioxidant enzymes were evaluated. Both PAW1 and PAW2 had positive effects on *Triticum aestivum* L. cv. Glosa sprouts. PAW2 had a better impact on sprouts with respect to growth parameters, free phenolic content, and antioxidant activity of the free phenolic fraction (day 3), the accumulation of proteins, and photosynthetic pigments (day 8). In conclusion, exposure to PAW increases the quality of *Triticum aestivum* L. cv. Glosa sprouts. Further optimization of PAW treatment is required for the most favorable impact on the accumulation of biomass and health-promoting compounds in sprouts.

Keywords: wheat sprouts; germination; seedling growth; proteins; photosynthetic pigments; phenolic compounds; antioxidant activity; antioxidant enzymes

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Citation: Mandici, A.; Cretu, D.E.; Burlica, R.; Astanei, D.; Beniuga, O.; Rosu, C.; Topa, D.C.; Aostacioaei, T.G.; Aprotosoaie, A.C.; Miron, A. Preliminary Study on the Impact of Non-Thermal Plasma Activated Water on the Quality of *Triticum aestivum* L. cv. Glosa Sprouts. *Horticulturae* **2022**, *8*, 1158. https://doi.org/10.3390/horticulturae8121158

Academic Editors: Ivan Salamon and Charalampos Proestos

Received: 22 October 2022
Accepted: 29 November 2022
Published: 7 December 2022

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1. Introduction

A healthy diet improves quality of life and prevents chronic diseases [1]. In this respect, the consumption of whole grains has overwhelming health benefits [2]. Among cereals, whole grain wheat is an excellent source of nutritional and bioactive compounds. Dietary fiber is an important component of the whole grain wheat (11.6–17.0%). Almost half of all dietary fiber is represented by non-starch polysaccharides, with arabinoxylan (70% of wheat bran) as a major component [3]. Whole grain wheat is also an important dietary source of starch (60–70%), composed of rapidly digestible starch (23.9–34.4%), slowly digestible starch (3.4–23.4%), and resistant starch (1.8–16.9%) [4]. The latter acts as a functional fiber undergoing colonic bacterial fermentation with the production of short-chain fatty acids (acetate, propionate, butyrate) which are essential for colon health [5]. Whole grain wheat is also a source of proteins (7–22%) [6] and lipids (2–2.5%, free fatty acids and acylglycerols being predominant) [7]. Phytosterols, sterol glycosides and esters, alkylresorcinols, vitamin E, and carotenoids are important lipid soluble constituents in wheat grains [8,9]. The average total content of tocopherols and tocotrienols in wheat

grains was found to vary from 27.6 to 79.7 μg/g, alpha-tocopherol and beta-tocotrienol being the most abundant ones [10]. Vitamin E is a potent antioxidant playing a major role in the protection against atherosclerosis, arthritis, cataracts, and cancer [11], with the cardioprotective potential being mostly attributed to tocotrienols [12]. Whole grain wheat also contains carotenoids, with large variations in contents (0.94–13.6 mg/kg) depending on the cultivar [13]. As antioxidants and vitamin A precursors, carotenoids have numerous health benefits in the treatment of degenerative eye disorders, cardiovascular diseases, Alzheimer's disease, colorectal cancer, type 2 diabetes, and obesity [14]. Whole grain wheat is also a source of gamma aminobutyric acid (GABA) (3.4 mg/100 g dry weight) [15], betaine (291.2 mg/100 g dry weight), and choline (14.4 mg/100 g dry weight) [16]. GABA, a non-protein amino acid, is an inhibitory neurotransmitter with many other physiological effects in humans such as regulation of blood pressure and heart rate and stimulation of insulin secretion [15]. Betaine and choline reduce hyperhomocysteinaemia, a major risk factor for cardiovascular and neurological diseases [17]. Whole grain wheat also contains phenolic acids with strong antioxidant properties, localized mainly in bran [18]. Ferulic acid is the predominant phenolic acid (269.2–744.7 μg/g), followed by *p*-coumaric (10.4–92.4 μg/g), syringic (7.9–29.5 μg/g), and vanillic (5.6–19.5 μg/g) acids [18–22].

Overall, whole grain wheat contains valuable nutritional and bioactive compounds which provide significant health benefits in the treatment of cardiovascular disease, type 2 diabetes, and cancer [9]. The germination process enhances the nutritional and medicinal value of grains. Contents in soluble dietary fiber, proteins, free lipids, vitamins, and polyphenols increase during germination. Moreover, germination activates different enzymes such as alpha-amylase and proteases which degrade starch and proteins, respectively, resulting in an improved digestibility of grains and an enhanced availability of sugars and free amino acids. Germination was also found to increase the antioxidant capacity of sprouts [23–26]. Therefore, sprouted whole grains are important functional foods, being consumed as part of a diet for the prevention of chronic diseases [15].

There are various methods to control germination and enhance the production and accumulation of nutritional and bioactive compounds in sprouts. The most common ones are the exposure to stress (cold/heat, hypoxia) and use of different forms of physical energy such as pressure, light, ultrasounds, magnetic field, or non-thermal plasma [24]. Non-thermal (cold) plasma, usually generated by electrical discharge in a high-velocity gas flow, is a complex mixture of ions, atoms, molecules, free radicals, and electrons. Non-thermal plasma treatment was reported to enhance the seed germination and accumulation of bioactive compounds in sprouts, reduce the germination time required to accumulate the highest content of phytochemicals, promote plant growth, and prevent microbial contamination of plant material [24,27,28]. Non-thermal plasma can be applied not only directly, but also as non-thermal plasma-activated water (PAW), usually produced by spraying water into the plasma zone. The process generates reactive oxygen and nitrogen species, with hydrogen peroxide and nitrates being considered the major species affecting the seed germination, seedling growth, biosynthetic pathways, and stress response in plants. Nitrates are a source of nitrogen which is essential for the synthesis of proteins; the latter are important not only for growth, but also as components of enzymes with different metabolic functions. Reactive oxygen and nitrogen species trigger a defensive response in the plant tissue consisting in an enhanced production and accumulation of phytochemicals with antioxidant properties such as phenolics, vitamin E, and carotenoids [29]. Treatment with PAW is a promising strategy to improve the nutritional and medicinal potential of sprouts and, at the same time, a promising alternative to chemical fertilizers. Two major advantages of using non-thermal PAW, such as low costs of production and environmental safety, need to be emphasized.

In this respect, we have initiated research studies aiming to boost the nutritional and bioactive potential of sprouts by treatment with non-thermal PAW. The present study reports on the effects of non-thermal PAW on *Triticum aestivum* L. cv. Glosa caryopses regarding germination, growth, accumulation of various metabolites, antioxidant capacity, and antioxidant enzyme activity in sprouts (collected at days 1–3) and shoots (collected at day 8).

2. Materials and Methods

2.1. Chemicals

Folin–Ciocalteu's phenol reagent, gallic acid, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, Bradford reagent, protein standard (2 mg/mL), *o*-dianisidine, and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Steinheim, Germany). Nitro blue tetrazolium (NBT) chloride and riboflavin were from Merck KGaA (Darmstadt, Germany). 1,4-Dithiothreitol and ethylenediaminetetraacetic acid (EDTA) were from Scharlau Chemie (Sentmenat, Spain). Acetone, ethyl acetate, and methanol were from Riedel-de Haën (Seelze, Germany). Titanium sulfate solution was purchased from Fisher Chemical (Fisher Scientific, Loughborough, UK). Sodium hypochlorite and sodium hydroxide were provided by FEELXKIM SRL (Tomesti, Romania) and Chimreactiv SRL (Bucharest, Romania), respectively. Ultrapure water was obtained using the SG Water Ultra Clear TWF water purification system (Barsbüttel, Germany).

2.2. Vegetal Material

Wheat caryopses (*Triticum aestivum* L. cv. Glosa) were supplied by the Territorial Inspectorate for Seed and Propagating Material Quality (Iasi, Romania). Prior to the experiments, the caryopses were immersed in 1.25% sodium hypochlorite solution for 5 min and further rinsed with sterile demineralized water until no chlorine odor was detected [30].

2.3. Generation of Non-Thermal Plasma-Activated Water

Table 1 and PAW2, were obtained in the Department of Electrical Engineering, *Gheorghe Asachi* Technical University (Iasi, Romania). The non-thermal plasma reactor for PAW production, the factors affecting the chemical composition of PAW, and the whole process of PAW production have been previously described [31,32]. Briefly, in our study, a high voltage pulsed power supply generated a low power electrical discharge between two cylindrical point-to-point stainless steel electrodes (1.6 mm diameter). Thus, plasma was produced between the high voltage electrode and ground electrode in a 3.1 mm inner diameter glass cylinder, at a gap of 3 mm. Distilled water was obtained using a Biobase WD-A5 water distiller (Shandong, China) and pumped at a flow of 15 mL/min. Air (carrier gas) was generated by a commercial compressor (2.5 atm). A mass flow regulator MASS-VIEW MV-304 (Bronkhorst High-Tech B.V., The Netherlands) kept the air flow constant at 1.5 L/min. PAW was generated by a pulse electrical discharge between the high voltage electrode of the reactor and ground electrode, in a gas flow (Figure 1). Distilled water was mixed with air in a nozzle with two inlets arranged perpendicularly. The mixture was further sprayed into the plasma area in the reaction chamber, resulting in the formation of various reactive species such as hydrogen peroxide (H_2O_2), nitrates (NO_3^-), and nitrites (NO_2^-) [31]. For the experiments described in the present study, PAW was generated at 60 and 100 Hz for 2 ms pulse width, the distilled water and air flows being constant (15 mL/min and 1.5 L/min, respectively). The electrical parameters of the non-thermal plasma reactor for the frequencies used in our study are shown in Table 1. The current and voltage measurements were carried out using a shunt resistance of 100 Ω and a TT-HVP-2739 high voltage probe (divider voltage ratio 1000:1) (Testec Elektronik GmbH, Dreieich, Germany), respectively [31,33].

Table 1. Electrical parameters of the non-thermal plasma reactor.

Frequency (Hz)	I_{max} (mA)	I_{avg} (mA)	U_{max} (V)	U_{avg} (V)	P_{avg} (W)
60	58	2.5	6049	153	3
100	59	2.8	7200	250	4

Imax—maximum discharge current, Iavg—average current, Umax—maximum discharge voltage, Uavg—average discharge voltage, Pavg—discharge average power.

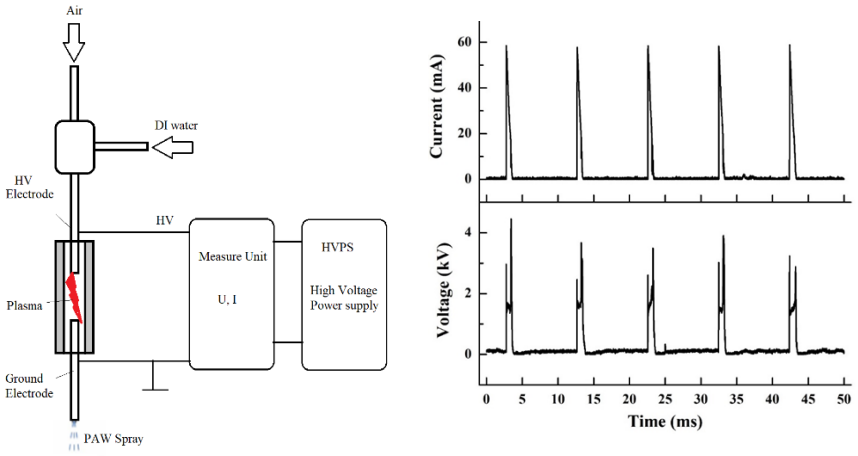


Figure 1. The experimental set-up (**a**) and the current and voltage waveforms (**b**) of the discharge (frequency: 100 Hz, pulse width: 2 ms, distilled water flow: 15 mL/min, air flow: 1.5 L/min).

The current and voltage waveforms were recorded using a WaveSurfer 454 digital oscilloscope (LeCroy, New York, NY, USA) [31,34]; the waveforms corresponding to the frequency of 100 Hz, 2 ms pulse width, distilled water flow of 15 mL/min, and air flow of 1.5 L/min are illustrated in Figure 1. The energy efficiency (EEf), representing the amount of a reactive species (g) produced in PAW per kWh, was calculated as the amount produced (n) divided by the energy consumption (E) for the process, which is n/E, where the value of E is equal to the product of the average power (P_{avg}) and treatment time (t) [31].

2.4. Non-Thermal Plasma-Activated Water: Composition and Analysis

Some of the major chemical reactions occurring in the non-thermal plasma generation area are listed below. As air was used as a working gas to produce plasma, nitrogen oxides were also generated [31,33].

$$H_2O \rightarrow H\cdot + \cdot OH \quad (1)$$

$$H_2O \rightarrow H^+ + e^-_{aq} + \cdot OH \quad (2)$$

$$\cdot OH + \cdot OH \rightarrow H_2O_2 \quad (3)$$

$$N_2 + e^- \rightarrow 2N + e^- \quad (4)$$

$$O_2 + e^- \rightarrow 2O + e^- \quad (5)$$

$$N + O \rightarrow NO \quad (6)$$

$$NO + O \rightarrow NO_2 \quad (7)$$

$$NO_2 + OH \rightarrow HNO_3 \quad (8)$$

$$O_2 + NO_2 + H_2O \rightarrow NO^-_2 + NO^-_3 + 2H^+ \quad (9)$$

H_2O_2 concentration of PAW was determined spectrophotometrically using titanium sulfate. 1 mL of titanium sulfate was mixed with 2 mL of PAW. A yellow color indicated

the presence of H_2O_2 in PAW. The absorbance of the reaction mixture was determined at 410 nm (Shimadzu UVmini-1240 spectrophotometer) [33]. A calibration curve for H_2O_2 was also plotted. The levels of NO_3^- and NO_2^- in PAW were measured using the Visocolor Eco colorimetric test kits (Macherey-Nagel, Germany) and a compact photometer PF-3 (Macherey-Nagel, Germany). The quantification of NO_3^- (Visocolor Eco nitrate colorimetric test kit) involved its reduction to NO_2^- in an acidic medium, followed by the reaction of NO_2^- with an aromatic amine to generate an orange-yellow azo dye. The quantification of NO_2^- (Visocolor Eco nitrite colorimetric test kit) was based on the diazotization of sulfanilamide in an acid solution followed by coupling with naphthylamine to produce a reddish-purple azo dye [33,35,36].

2.5. Treatment and Germination of Wheat Caryopses

Approximately 0.9 kg of disinfected wheat caryopses was divided in three groups which were soaked for 4 h in PAW1, PAW2, and distilled water (control), followed by germination in a Weiss Gallenkamp climatic chamber (Weiss Technik UK Ltd., Loughborough, UK) under controlled conditions (dark, 20 °C, and 65% relative humidity for 3 days). After 3 days, the sprouted caryopses underwent a light/dark cycle (12/12 h) until day 8. PAW1, PAW2, and distilled water were changed at days 2, 4, and 6 with fresh prepared solutions, the wheat caryopses in each group being soaked 2/3 in liquid. Germination rate (%) was determined for the wheat caryopses collected at day 1. Fifty randomly selected wheat caryopses were collected from each group and the germination percentage was calculated as the ratio between the number of germinated caryopses and total number of collected caryopses. Caryopses were considered germinated when the radicle and coleoptile had a minimum length of 1 mm [29]. Growth parameters, superoxide dismutase, catalase, and peroxidase activities were determined in fresh samples immediately after collection. For other assays, samples were stored at −80 °C until analysis.

2.6. Biometric Measurements

Fifty wheat caryopses were randomly collected at days 2, 3, and 8. The lengths of their roots and shoots were measured with a mm scale. The median lengths of roots and shoots were calculated.

2.7. Total Protein Content

Total protein content was determined in the sprouts collected at days 1, 2, and 3, and shoots collected at day 8. In brief, each sample (100 mg) was grinded in 50 mM potassium phosphate buffer (pH = 7) and centrifuged at 10,000 rpm and 4 °C for 10 min (Ortoalresa Digicen 20-R centrifuge, Alvarez Redondo, Madrid, Spain). An aliquot was taken out from supernatant and mixed with Bradford reagent (total volume 1 mL). The absorbance was measured at 595 nm (Specord 210 Plus spectrophotometer, Analytik Jena, Jena, Germany). A standard curve was created by mixing different volumes of protein standard solution with Bradford reagent [37]. The results were expressed as mg protein/g of sample (fresh weight).

2.8. Photosynthetic Pigments Content

Chlorophylls and carotenoids were quantified in the shoots collected at day 8. 0.05 g of shoots were homogenised with 3 mL of acetone and further centrifuged at 10,000 rpm and 4 °C for 15 min (Ortoalresa Digicen 20-R centrifuge, Alvarez Redondo, Madrid, Spain). The final volume was adjusted to 10 mL with acetone. The absorbances were recorded at 662, 645, and 470 nm (Specord 210 Plus spectrophotometer, Analytik Jena, Jena, Germany). The contents, expressed in mg/g of sample (fresh weight), were calculated using the following formulas: chlorophyll a = (11.24 × A662) − (2.04 × A645), chlorophyll b = (20.13 × A645) − (4.19 × A662), total chlorophylls (chlorophyll a + b) = (7.045 × A662) + (18.09 × A645), carotenoids = [(1000 × A470) − (1.9 × chlorophyll a) − (63.14 × chlorophyll b)]/214 [38,39].

2.9. Extraction of Free and Bound Phenolic Fractions

Free and bound phenolic fractions were extracted from the sprouts collected at days 1, 2, and 3, and shoots collected at day 8, according to previously described protocols with minor changes. In brief, free phenolic fraction was obtained as follows: the sample (1 g) was milled and suspended in 5 mL of methanol/ultrapure water/acetic acid mixture (70:29.5:0.5, *v*/*v*/*v*), vortexed for 1 min on ice (Velp Scientifica vortex mixer, Usmate, Italy), ultrasonicated for 40 min at 25 °C (Bandelin Electronic RK31 ultrasonic bath, Bandelin Electronic, Berlin, Germany), and centrifuged at 10,000 rpm for 10 min (Ortoalresa Digicen 20-R centrifuge, Alvarez Redondo, Madrid, Spain). The supernatant was collected, and the extraction was repeated twice. The supernatants were combined and vacuum dried (Büchi R-300 rotary evaporator, Büchi Laboratortechnik AG, Flawil, Switzerland). The residue was stored at −20 °C until further analysis. The pellets of the free phenolics extraction were used to isolate the bound phenolic fraction. Pellets were initially subjected to an alkaline hydrolysis with 4 M sodium hydroxide, followed by ultrasonication for 40 min at 25 °C (Bandelin Electronic RK31 ultrasonic bath, Bandelin Electronic, Berlin, Germany) and overnight rest. After adjusting the pH to a value of 2, each sample was extracted with 3 × 30 mL of ethyl acetate. The combined supernatants were vacuum dried (Büchi R-300 rotary evaporator, Büchi Laboratortechnik AG, Flawil, Switzerland) and stored at −20 °C until further use [26,40].

2.10. Total Phenolic Content

Total phenolic contents in both free and bound phenolic fractions were assessed using the Folin–Ciocalteu assay. The reaction mixture consisted of sample, Folin–Ciocalteu's phenol reagent, 20% sodium carbonate, and ultrapure water in a total volume of 4 mL. After 2 h incubation (dark, room temperature), the absorbance was determined at 765 nm (Specord 210 Plus spectrophotometer, Analytik Jena, Jena, Germany). A calibration curve was plotted using gallic acid [41–43]. The results were expressed as mg of gallic acid equivalents (GAE)/100 g of sample (fresh weight).

2.11. Antioxidant Activity

Antioxidant activity of the free and bound phenolic fractions was assessed by DPPH radical scavenging assay as previously described with minor modifications. Briefly, the sample (0.05 mL) was mixed with DPPH methanolic solution (0.075 mM, 2.95 mL), followed by vigorous shaking. After 30 min, the absorbance of the reaction mixture was determined at 515 nm. Additionally, the absorbance of DPPH solution (2.95 mL) and methanol (0.05 mL) was measured at 515 nm at 0 min (Specord 210 Plus spectrophotometer (Analytik Jena, Jena, Germany). DPPH radical scavenging activity was calculated using the formula: % DPPH scavenging activity = $[(A_{0\ min} - A_{30\ min})/A_{0\ min}] \times 100$ [23,42,44].

2.12. Superoxide Dismutase Activity

Superoxide dismutase (SOD, ***EC*** 1.15.1.1) was quantified in the sprouts collected at day 3 and shoots collected at day 8 according to previously described procedures. In brief, the enzyme was extracted by grinding the vegetal material in 5 mL of TRIS-0.1 M HCl buffer containing 1 mM dithiothreitol and 1 mM EDTA (pH = 7.8) at 4 °C, followed by centrifugation at 10,000 rpm for 15 min (Hettich Mikro 22R centrifuge, Tuttlingen, Germany). The supernatant was used for SOD quantification. The reaction mixture, consisting of 2.55 mL of potassium phosphate buffer (0.067 M, pH = 7.8), 0.2 mL of EDTA (0.1 M, pH = 7.8), 0.1 mL of supernatant, 0.1 mL of NBT (1.5 mM), and 0.05 mL of riboflavin (0.12 mM), was exposed to neon lamp illumination (24 °C, 5 min). The absorbance of the reaction mixture was further determined at 560 nm (Beckman Coulter DU 730 UV/Vis spectrophotometer, Beckman Coulter Inc., San Diego, CA, USA). SOD activity was expressed as U/mg protein/min; one unit (U) represents the amount of SOD causing 50% inhibition of light-induced NBT reduction [45,46].

2.13. Catalase Activity

Similarly, catalase (CAT, ***EC*** 1.11.1.6) activity was determined in the samples collected at days 3 and 8. The vegetal material was grinded in 5 mL of potassium phosphate buffer (0.05 M, pH = 7) and further centrifuged at 4500 rpm for 10 min (Hettich Mikro 22R centrifuge, Tuttlingen, Germany). In total, 1 mL of H_2O_2 (0.059 M) was incubated with 1 mL of phosphate buffer for 4–5 min at 25 °C, followed by the addition of 0.1 mL of supernatant (diluted to cause a decrease in absorbance of 0.03–0.07/min). The decrease in absorbance at 240 nm was measured for 2 min (Beckman Coulter DU 730 UV/Vis spectrophotometer, Beckman Coulter Inc., San Diego, CA, USA). CAT activity was expressed as U/mg protein/min, one unit (U) decomposing 1 μM H_2O_2 in 1 min (25 °C, pH = 7) [47].

2.14. Peroxidase Activity

Peroxidase (POX, ***EC*** 1.11.1.7) activity was assessed in the samples collected at days 3 and 8. First, samples were ground in 5 mL of TRIS—0.1 M HCl buffer containing 1 mM dithiothreitol and 1 mM EDTA (pH = 7.8) in an ice bath and further centrifuged at 4500 rpm for 15 min (Hettich Mikro 22R centrifuge, Tuttlingen, Germany). A volume of 0.1 mL of supernatant was mixed with 3 mL of buffered *o*-dianisidine and 0.2 mL of H_2O_2 0.05%, incubated at 20 °C for 5 min, followed by the addition of 5 mL of H_2SO_4 50% to block POX activity. The absorbance was read at 540 nm (Beckman Coulter DU 730 UV/Vis spectrophotometer, Beckman Coulter Inc., San Diego, CA, USA). PO activity was expressed as U/mg protein/min, one unit (U) decomposing 1 μM H_2O_2 in 1 min at 20 °C [48].

2.15. Statistical Analysis

All experiments were performed in triplicate, the results being presented as mean ± standard deviation (SD). Data were processed using SPSS software version 18.0 and Tukey HSD test (95% confidence interval). A p value lower than 0.05 was considered to be statistically significant.

3. Results

3.1. Generation and Analysis of Non-Thermal Plasma-Activated Water

Two types of PAW were produced in the present study. The concentrations of reactive species in PAW strongly depend on the electrical parameters of electrical discharge that generates PAW. The electrical parameters (current, voltage, frequency) as well as the physical ones (air and water flow rates) were selected in order to obtain the desired concentrations of reactive species in PAW: a level of NO_3^- (source of nitrogen for wheat sprouts) with a positive impact on sprouting and a level of H_2O_2 (strong oxidizing agent) which does not affect negatively the development of wheat sprouts. In PAW1, generated at 60 Hz, the concentrations of NO_3^-, NO_2^-, and H_2O_2 were 25.0 ± 2.5, 4.0 ± 0.4, and 6.0 ± 0.5 mg/mL, respectively. The higher frequency (100 Hz) used to produce PAW2 resulted in elevated levels of reactive species (35.0 ± 3.5 mg/L NO_3^-, 5.0 ± 0.5 mg/L NO_2^-, and 7.5 ± 0.7 mg/L H_2O_2) (Figure 2). PAW treatment was applied 2 h after preparation when measurements revealed that the concentrations of reactive species (NO_3^-, NO_2^-, H_2O_2) had stabilized. The EEf values used for the generation of H_2O_2, NO_2^-, and NO_3^- at both frequencies are illustrated in Table 2.

Table 2. Energy efficiency (EEf) values used for the generation of H_2O_2, NO_2^-, and NO_3^- in PAW1 and PAW2.

PAW	Frequency (Hz)	EEf (g/kWh)		
		H_2O_2	NO_2^-	NO_3^-
PAW1	60	1.7	1.4	8.6
PAW2	100	1.6	1.2	8.1

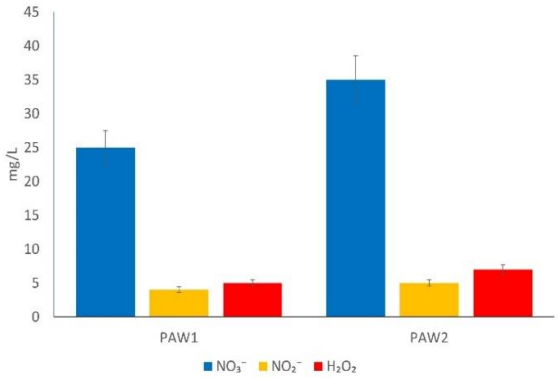


Figure 2. Concentrations of H_2O_2, NO_2^-, and NO_3^- in PAW1 and PAW2.

3.2. Germination Rate

PAW1 and PAW2 enhanced the germination of wheat caryopses by 4.66% and 5.33%, respectively, in comparison with the control (evaluation at day 1, Table 3), with no statistically significant differences between groups ($p > 0.05$).

Table 3. Effect of PAW treatment on the germination of *Triticum aestivum* L. cv. Glosa caryopses.

Treatment	Germination (%)
Control	42.67 ± 1.89
PAW1	47.33 ± 4.99
PAW2	48.00 ± 3.27

3.3. Growth Parameters

PAW treatment positively influenced the biometric characteristics of shoots and roots (Figure 3). At day 3, PAW2 induced the most pronounced increase in the lengths of shoots and roots (13.2 and 17.54 mm, respectively, vs. 1.81 and 8.14 mm, respectively, in the control group). At day 8, PAW1-treated caryopses showed the longest shoots (107.29 vs. 77.68 mm in the control group), whereas PAW2-treated ones had the most developed roots (97.02 vs. 75.05 mm in the control group). The shoot lengths in the PAW1-treated group at day 2 and the PAW2-treated group at days 2 and 3 were significantly different from the control group ($p < 0.05$). With respect to the root lengths, significant differences in comparison with the control were determined for the PAW2-treated group at days 2, 3, and 8 and the PAW1-treated group at day 2 ($p < 0.05$).

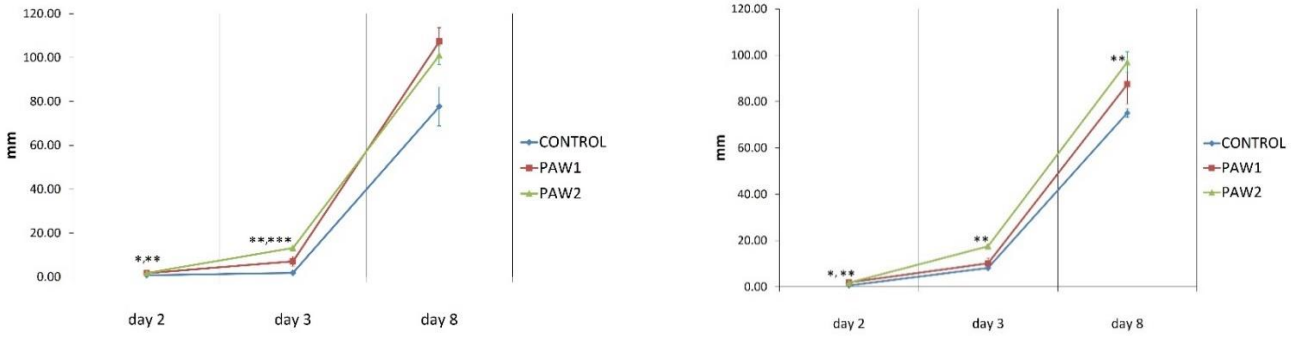


Figure 3. Effect of PAW treatment on the lengths of shoots (**a**) and roots (**b**) of *Triticum aestivum* L. cv. Glosa sprouts; * significant difference ($p < 0.05$) between control and PAW1; ** significant difference ($p < 0.05$) between control and PAW2; *** significant difference ($p < 0.05$) between PAW1 and PAW2. Each point represents the mean ± SD of three determinations.

3.4. Total Protein Content

Total protein content increased under PAW treatment (Figure 4). PAW1 and PAW2 caused important increase in the protein content of wheat sprouts at days 1 (~43 and ~31%, respectively) and 3 (~19 and ~18%, respectively). In the shoots at day 8, the increase in the protein content was smaller (~12% in the PAW1-treated group and ~17% in the PAW2-treated group). However, significant differences in comparison with the control were determined only for the PAW1-treated group at day 1 and the PAW2-treated group at day 8 ($p < 0.05$). There were no statistically significant differences between the PAW1- and PAW2-treated groups at all four collection days ($p > 0.05$).

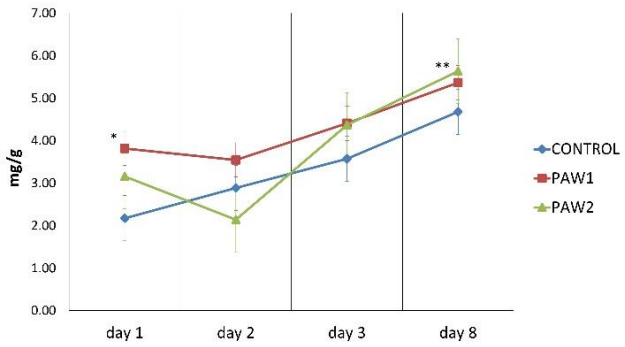


Figure 4. Effect of PAW treatment on the total protein content in *Triticum aestivum* L. cv. Glosa sprouts (days 1, 2, and 3) and shoots (day 8); * significant difference ($p < 0.05$) between control and PAW1; ** significant difference ($p < 0.05$) between control and PAW2. Each point represents the mean ± SD of three determinations.

3.5. Photosynthetic Pigments Content

Both chlorophylls and carotenoids in the shoots at day 8 increased under PAW treatment (Figure 5). PAW2 induced a more pronounced increase in chlorophyll a, chlorophyll b, total chlorophylls, and carotenoid contents in comparison with PAW1 (1.32-, 1.8-, 1.45-, and 2.18-fold, respectively vs. 1.24-, 1.13-, 1.21-, and 1.98-fold, respectively). The contents of chlorophyll a and total chlorophylls in PAW1- and PAW2-treated groups were significantly different from the control group ($p < 0.05$). With respect to the chlorophyll b content, a significant increase in comparison with the control was determined only for the PAW2-treated group ($p < 0.05$). Differences in chlorophyll b and total chlorophyll contents between the PAW1- and PAW2-treated groups were found to be statistically significant ($p < 0.05$). There were no statistically significant differences between the control and treated groups, on one hand, and between the PAW1- and PAW2-treated groups, on the other hand, with respect to the carotenoid content ($p > 0.05$).

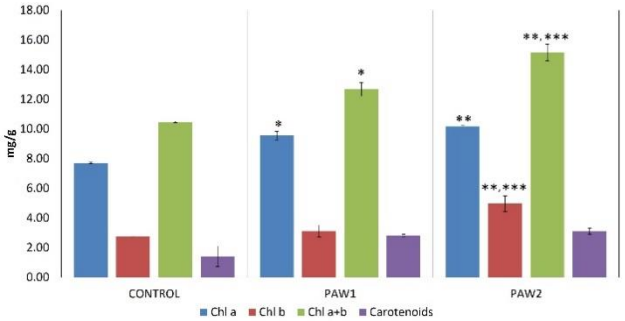
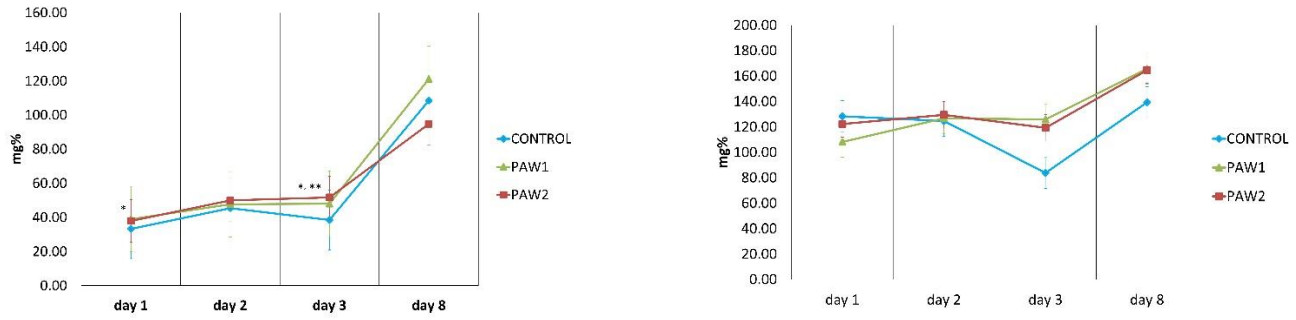


Figure 5. Effect of PAW treatment on the photosynthetic pigments content in *Triticum aestivum* L. cv. Glosa shoots at day 8; * significant difference ($p < 0.05$) between control and PAW1; ** significant difference ($p < 0.05$) between control and PAW2; *** significant difference ($p < 0.05$) between PAW1 and PAW2. Each point represents the mean ± SD of three determinations.

3.6. Total Phenolic Content

Our results revealed that PAW treatment enhanced the content in free and bound phenolic compounds (Figure 6). At day 3, both PAW1 and PAW2 induced a significant increase in the free phenolic contents in wheat sprouts in comparison with the control (48.05 vs. 38.49 mg% and 51.69 vs. 38.49 mg%, respectively) ($p < 0.05$). In addition, PAW1 enhanced the free phenolics in the sprouts at day 1 (39.02 vs. 33.27 mg% in the control group, $p < 0.05$) and shoots at day 8 (121.19 vs. 108.49 mg% in the control group, $p > 0.05$). PAW1 and PAW2 treatment also resulted in elevated bound phenolic contents in the sprouts at day 3 (125.94 and 119.52 mg% vs. 83.81 mg% in the control group) and shoots at day 8 (165.79 and 164.70 mg% vs. 139.41 mg% in the control group), with no significant differences between each treated group and control group, and also between PAW1- and PAW2-treated groups ($p > 0.05$).



(**a**) (**b**)

Figure 6. Effect of PAW treatment on the free (**a**) and bound (**b**) phenolic contents in *Triticum aestivum* L. cv. Glosa sprouts (days 1, 2, and 3) and shoots (day 8); * significant difference ($p < 0.05$) between control and PAW1; ** significant difference ($p < 0.05$) between control and PAW2. Each point represents the mean ± SD of three determinations.

3.7. Antioxidant Activity

PAW treatment positively influenced the antioxidant capacity of free phenolic fractions, except those isolated from PAW1-treated sprouts (day 1) and PAW2-treated shoots (day 8). A substantial increase in the antioxidant capacity was noticed for the free phenolic fractions obtained from PAW2-treated sprouts at days 2 (17.01 vs. 10.74% for the control group, $p > 0.05$) and 3 (18.32 vs. 10.15% for the control group, $p < 0.05$). At day 8, the antioxidant activity of the free phenolic fraction of PAW1-treated shoots was significantly higher in comparison with the one of PAW2-treated shoots (92.09 vs. 79.94%, $p < 0.05$). A burst in antioxidant capacity was noted for the bound phenolic fraction obtained from the shoots at day 8 under treatment with PAW1 (91.19 vs. 57.49% in the control group, $p < 0.05$) (Figure 7).

3.8. Antioxidant Enzymes Activity

PAW1 and PAW2 substantially stimulated SOD and CAT in the sprouts at day 3 and shoots at day 8, the differences between each treated group and control group and also between PAW1- and PAW2-treated groups being significant ($p < 0.05$). The increase in activity in both enzymes was more prominent at day 3, with a 143.72 and 91.53% increase in SOD activity under PAW1 and PAW2 exposure, respectively, and 50.21 and 36.34% increase in CAT activity under treatment with PAW1 and PAW2, respectively. On the contrary, PAW1 and PAW2 considerably elevated POX activity only on day 3 (by 207.32 and 244.72%, respectively) and reduced it at day 8 (by 39.02 and 18.98%, respectively) ($p < 0.05$ between each treated group and control group and between PAW1- and PAW2-treated groups) (Figure 8).

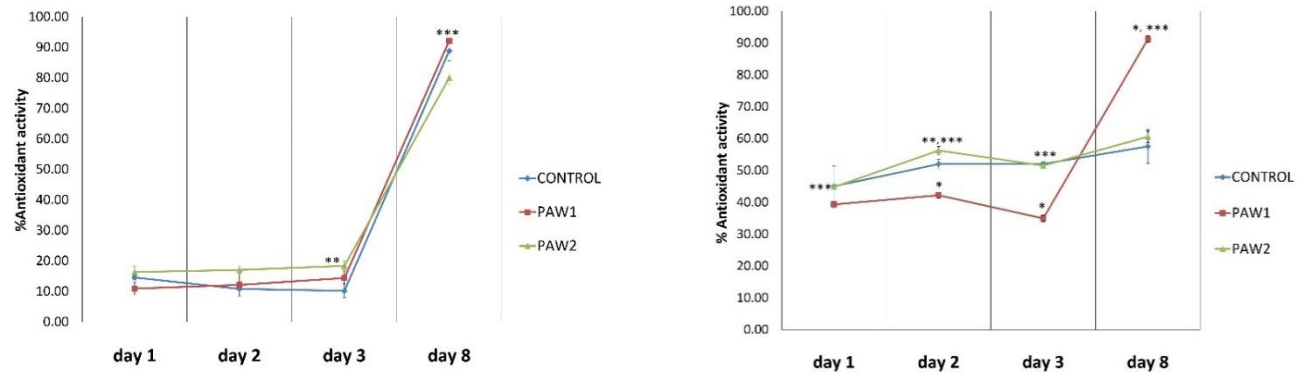


Figure 7. Antioxidant activity of the free (**a**) and bound (**b**) phenolic fractions isolated from *Triticum aestivum* L. cv. Glosa sprouts (days 1, 2, and 3) and shoots (day 8); * significant difference ($p < 0.05$) between control and PAW1; ** significant difference ($p < 0.05$) between control and PAW2; *** significant difference ($p < 0.05$) between PAW1 and PAW2. Each point represents the mean ± SD of three determinations.

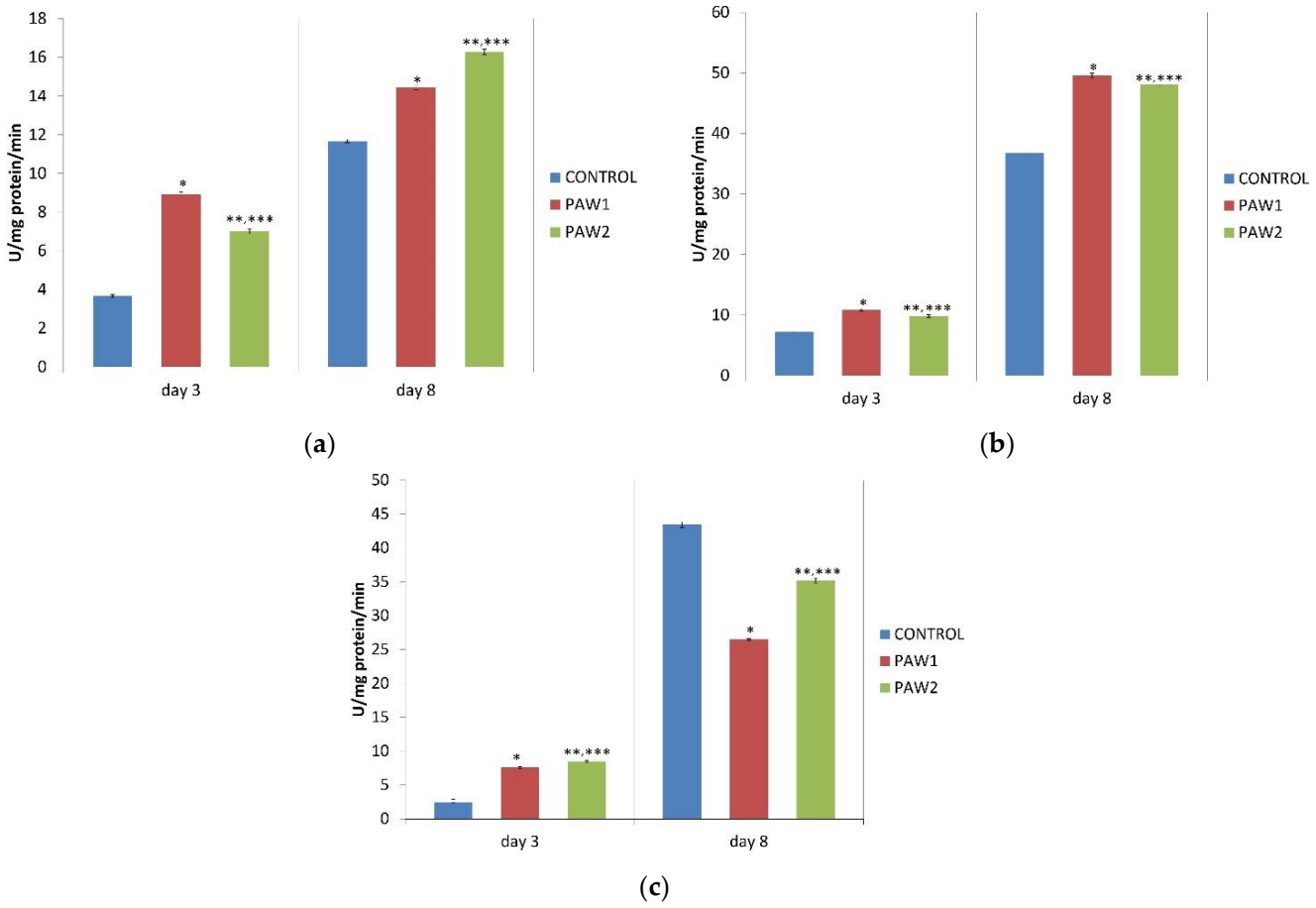


Figure 8. Activity of antioxidant enzymes SOD (**a**), CAT (**b**), and POX (**c**) in *Triticum aestivum* L. cv. Glosa sprouts (day 3) and shoots (day 8); * significant difference ($p < 0.05$) between control and PAW1; ** significant difference ($p < 0.05$) between control and PAW2; *** significant difference ($p < 0.05$) between PAW1 and PAW2. Each point represents the mean ± SD of three determinations.

4. Discussion

Non-thermal PAW has gained increasing attention in recent years as several studies showed that it significantly impacts germination, development of seedlings as well as accumulation of nutritional and bioactive compounds in plants [29,49–58]. In the present study, we investigated the impact of two types of PAW, generated by exposing distilled water to high-voltage electric discharge, on the caryopses of *Triticum aestivum* L. cv. Glosa, widely cultivated in Romania due to its productivity and resistance to various stress factors.

The influence of PAW1 and PAW2 on the germination of wheat caryopses was evaluated after 24 h as previous studies showed that the increase in germination is more pronounced after 24 h than after several days [29]. PAW1 and PAW2 slightly enhanced the germination of wheat caryopses (by 4.66% and 5.33%, respectively). The positive influence of PAW on germination is ascribed to its reactive oxygen and nitrogen species, especially H_2O_2 and NO. H_2O_2 decreases abscisic acid (hormone responsible for seed dormancy) while increasing gibberellic acid (hormone promoting germination) [48]. In addition, H_2O_2 is decomposed by CAT to water and O_2; O_2 enhances mitochondrial respiration and metabolic activities which results in a positive impact on germination. H_2O_2 oxidizes and inactivates inhibitors of germination present in pericarp [29]. On the other hand, nitrogenous compounds also enhance seed germination. NO_3^- and NO_2^- are a source of NO; the latter was reported to stimulate light-induced germination mediated by phytochrome A, downregulate abscisic acid signaling, and increase the expression of aquaporin genes, thus enhancing water absorption and sprout development [49]. Other studies also found a positive impact of PAW on germination. Kučerová et al. reported that PAW (produced by transient spark discharge) significantly influenced the germination of *Triticum aestivum* L. cv. IS Gordius caryopses depending on the type of water used to generate PAW (tap or deionized water), water activation time, and chemical composition of PAW. PAW obtained from deionized water enhanced germination by 26% whereas PAW produced from tap water, containing higher content of NO_3^- (~0.5 mM), induced 103% increase in germination [29]. The discrepancies between our results and the ones reported by Kučerová et al. could be attributed, in large part, to the cultivar (Glosa in our study, IS Gordius in Kučerová's study) and different physicochemical characteristics of PAW used in both studies. An increase in the germination rate was also reported by Wang et al. who investigated the effects of PAW generated by atmosphere pressure Ar–O_2 plasma jet (1–5 min activation time) on *Triticum aestivum* L. (Jimai 23) caryopses. After 7 days, PAW activated for 3 min (~2 mg/L NO_3^-, ~0.5 mg/L NO_2^-) induced 100% germination vs. ~98.5% germination rate in the control group (distilled water) [50]. Zhang et al. reported a significant increase in the germination rate of lentil (*Lens culinaris* Medik.) seeds (to almost 80% at day 14, the control showing a germination rate lower than 40%) when treated with PAW (~600 µM NO_3^-, 160 µM H_2O_2); PAW was generated from tap water in an atmospheric pressure plasma jet (He as carrier gas) [51]. In another study, Fan et al. prepared PAW by exposing distilled water to non-thermal plasma for 15, 30, 60, and 90 s in an atmospheric pressure plasma jet device. The concentrations of reactive oxygen and nitrogen species increased with exposure time, reaching 118.39 mg/L NO_3^-, 62.05 mg/L NO_2^-, and 0.90 mg/L H_2O_2 90 s after activation. After 30 h of treatment, only PAW produced with the shortest discharge time (15 s) increased the germination rate of the mung bean (*Vigna radiata* (L.) R. Wilczek, Zhonglv 5) seeds to 93.01% vs. 84.41% in the control (distilled water). PAW produced with a longer discharge time (30, 60, and 90 s) decreased the germination rate, most likely due to the toxic effects induced by the high levels of the reactive species [52]. Another previous report showed significant enhancement in the germination rate of radish (*Raphanus sativus* L.) seeds treated with two types of PAW containing 8.90 mg/L NO_3^- and 0.11 mg/L H_2O_2 and 16.22 mg/L NO_3^- and 0.10 mg/L H_2O_2, respectively. The germination rate was determined daily for 3 days after sowing. After 24 h, the germination rate of the treated seeds was significantly higher in comparison to control (60 and 100% vs. 40%) [56]. Lo Porto et al. observed an enhancement in the germination of soybean (*Glycine max* (L.) Merr) seeds exposed to two types of PAW containing various amounts

of NO_3^- and H_2O_2 (20 µM NO_3^-, 20 µM H_2O_2 and 170 µM NO_3^-, 100 µM H_2O_2), with significant differences between samples at day 2 [53]. PAW also increased the germination rate of black gram (*Vigna mungo* (L.) Hepper) seeds (10–15% increase after 24 h submersion in PAW, followed by 12 h treatment) [57] and rye seeds (by up to 50% at 72 h) [54]. On the contrary, Stoleru et al. found no significant increase in the germination rate of lettuce (*Lactuca sativa* L.) seeds exposed to PAW (containing 1.5 mg/L NO_3^- and 0.5 mg/L H_2O_2 or 3.0 mg/L NO_3^- and 1.65 mg/L H_2O_2) at 3 days after sowing [58].

In our study, the influence of PAW1 and PAW2 on the sprout growth was assessed at days 2, 3, and 8. PAW2 caused significant increases in the lengths of roots at days 2, 3, and 8 (185%, 115.48%, and 29.27%, respectively) and shoots at days 2 and 3 (58.66% and 629.28%, respectively). The growth promoting effects of PAW are mainly triggered by H_2O_2 via stimulation of sucrose phosphate synthase and modulation of NO and Ca^{2+} signaling pathways [59]. NO_3^- and NO_2^- also play an important role in plant growth and development. For example, NO_2^- upregulates genes encoding expansins (proteins inducing cell wall loosening and extension) and auxins (growth hormones) [60]. Kučerová et al. reported a weak effect of PAW (water activation time 0.5–1 min/mL) on the length of wheat seedlings (increase by only 6–7% after 6 days of cultivation). A longer water activation time (1.5–3 min/mL) produced PAW with higher reactive species content which caused a reduction in the seedling length [29]. Similar results were reported by Fan et al. and Sajib et al. PAW obtained with 15 s discharge time had positive effects on the growth of mung bean sprouts (8.09% increase in stem length) whereas PAW produced with longer exposure times (30, 60, and 90 s) negatively impacted the seedling growth [52]. After 7 days of cultivation, PAW, generated by 3 min high voltage discharge in deionized water, caused a pronounced elongation of black gram roots and shoots (to 5.46 vs. 4.10 cm in control and 7.67 vs. 4.83 cm in control, respectively), whereas PAW, produced by longer plasma treatment of deionized water (6–15 min), decreased the lengths of roots and shoots [57]. Other studies reported a positive impact of PAW treatment on seedling growth. PAW (1.5 mg/L NO_3^-, 0.5 mg/L H_2O_2) increased the lettuce average radicle length by 5.58% (53 vs. 50.2 mm in control) and the average hypocotyl length by 34.85% (8.9 vs. 6.6 mm in control) at day 8 after sowing [58]. PAW (20 µM NO_3^-, 20 µM H_2O_2) stimulated the growth of soybean stems, especially at days 10 and 20 [53]. PAW also induced the elongation of the roots and coleoptiles of rye seeds and radish seedlings [54,56]. In case of radish seedlings, a direct relationship between NO_3^- content in PAW and seedling length was observed [56].

Proteins are important components of plant enzymes and key players in plant growth [29]. In our study, PAW1 (25 mg/L NO_3^-) induced a significant increase in the protein content (76.04%) in the sprouted caryopses at day 1. At day 8, PAW2 (35 mg/L NO_3^-) was more efficient causing 20.51% enhancement in the protein content of shoots. The increase in protein content is due to NO_3^- and NO_2^- present in PAW; both species are important sources of nitrogen which is required for protein synthesis [29,61]. Kučerová et al. also observed an increase in the soluble protein content in the roots and above-ground parts of wheat plants after 4 weeks of cultivation in perlite substrate and irrigation with PAW produced from tap water (water activation time 0.5 and 2 min/mL). The increase in soluble protein content was higher in the roots in comparison with the above-ground parts (∼43% and ∼69%, respectively, vs. ∼19% and ∼14%, respectively) [29]. After 7 days of cultivation, Sajib et al. reported an increase in the total soluble protein content in the roots (19.18%) and leaves (33.28%) of black gram plants developed from the seeds treated with PAW (produced by 6 min exposure of deionized water to high voltage discharge). Longer discharge times (9–15 min) resulted in the reduction in soluble protein content [57].

Chlorophylls and carotenoids are involved in photosynthesis and other processes (metabolic reactions, protection against oxidative stress) [62]. In our study, both PAW1 and PAW2 enhanced the chlorophyll content in wheat shoots at day 8. PAW 2 was more potent increasing chlorophyll a, chlorophyll b, and total chlorophyll by 32.17, 80.07, and 44.93%, respectively. Moreover, PAW1 and PAW2 induced 2- and 2.2-fold increase in the carotenoid content, respectively, but with no significant differences between groups. H_2O_2 and NO_3^-

are responsible, to a great extent, for the boost in photosynthetic pigments. H_2O_2 increases the stomatal opening, thus enhancing CO_2 uptake, photosynthetic rate, and accumulation of photosynthetic pigments [58]. In case of NO_3^-, the effect is attributed to up-regulation of the genes encoding δ-amino levulinic acid dehydratase, enzyme involved in the biosynthesis of chlorophyll [63]. Kučerová et al. determined chlorophylls and carotenoids in the wheat plants after 4 weeks of cultivation (conditions mentioned above) and found that contents increased by 17 and 12%, respectively [29]. Moreover, Maniruzzaman et al. showed that PAW, generated using either Ar (containing mainly H_2O_2) or air (containing mainly NO_3^-), positively influenced the relative chlorophyll content in wheat seedlings grown in a potting mix up to 4 weeks or in a soil-free plant growth system up to 14 days [55]. A previous study conducted by Stoleru et al. showed that the exposure to PAW did not significantly affect the chlorophyll content in the lettuce leaves after transplanting in pots. Only treatment with PAW containing 3.0 mg/L NO_3^- and 1.65 mg/L H_2O_2, in a bigger volume of substrate (3200 cm^3), determined 10.71% increase in the chlorophyll content in lettuce leaves at day 64 after transplanting in pots [58]. Sajib et al. investigated the effects of PAW treatment of black gram seeds on the chlorophyll content of leaves after 7 days of cultivation. Treatment with PAW (produced by 6 min exposure of deionized water to high voltage discharge) enhanced the leaf chlorophyll level by 23.80% [57].

Reactive oxygen and nitrogen species in PAW stimulate the production and accumulation of antioxidant phytochemicals in treated plants [29]. Species in PAW, such as H_2O_2 and NO, are potent inductors of the genes involved in phenolic synthesis such as *pal* gene (encoding L-phenylalanine ammonia-lyase, enzyme which deaminates L-phenylalanine) and *chs* gene (encoding chalcone synthase, enzyme implied in flavonoid synthesis) [64]. In our study, exposure to PAW1 and PAW2 enhanced the phenolic content in wheat sprouts; the increase in the free phenolic content induced by PAW1 at days 1 (17.28%) and 3 (24.84%) and PAW2 at day 3 (34.29%) and also the increase in the bound phenolic content induced by PAW2 at day 3 (80.49%) were significant. In general, the impact of PAW1 and PAW2 on the antioxidant potential of phenolic fractions isolated from sprouts/shoots was negligible except the impact of PAW1 on the antioxidant effect of the bound phenolic fraction at day 8 (58.62% boosting effect). To the best of our knowledge, this is the first study reporting on the impact of PAW on the phenolic content and antioxidant effects of wheat sprouts/shoots. Previous investigations evaluated only the total phenolic content and antioxidant activity of the juice obtained from plantlets treated with PAW. Fourteen days of PAW application (generated by atmosphere pressure Ar–O_2 plasma jet, 1–5 min activation time) improved the total phenolic content (by 10.46%) and antioxidant effects (by 5.51–35.34%) of the wheat (*Triticum aestivum* L., Jimai 23) plantlet juice [50]. Data from the literature concerning the impact of PAW treatment on the phenolic content and antioxidant activity of other sprouts are scarce. Fan et al. reported an increase in the total phenolic and flavonoid contents (by 3.53 and 2.22%, respectively) in the mung bean sprouts exposed to PAW (15 s discharge time) with no important influence on the antioxidant potential of the sprouts. The authors observed that a prolonged discharge time reduced the total phenolic and flavonoid contents, and also the antioxidant activity [52].

Reactive oxygen species in PAW trigger not only the production of metabolites with antioxidant properties, but also the activity of antioxidant enzymes. H_2O_2 was reported to increase the expression and activities of SOD, CAT, and POX in various plants, including *Tritium aestivum* L. [65]. In the present study, the activity of these three enzymes was evaluated at the third and eighth days of germination. According to previous investigations, CAT and SOD activity does not change in the first 48 h of imbibition, but significantly increases with the third day, the increase in activity being accompanied by an intensive radicle protrusion. Concerning POX, its activity is related to early seedling development [66]. PAW1 and PAW2 enhanced the activity of all three enzymes in the sprouts at day 3 and SOD and CAT activity in the shoots at day 8. The increase in antioxidant enzymes activity indicates a defense response against PAW-induced oxidative stress. The reduction in POX activity at day 8 could be related to a lower predominance of the enzyme in the aerial parts in

comparison to roots [29]. Kučerová et al. investigated the effects of PAW produced from tap water (water activation time 0.5 and 2 min/mL) on the activity of SOD, CAT, and POX in the above-ground parts of wheat but after longer cultivation time (4 weeks) on a perlite substrate and found a reduction in the activity of antioxidant enzymes ranging from 8 to 46% [29]. Puač et al. reported a decrease in CAT activity in *Paulownia tomentosa* Steud. seeds imbibed with PAW immediately and 2 days after exposure to red light pulse (for inducing germination) with a significant increase in activity on the third day; PAW was obtained from distilled water in an atmospheric pressure plasma jet (5, 10, and 20 min treatment) [67]. It seems that the effect of PAW on the activity of antioxidant enzymes depends not only on the level of reactive species in PAW, but also on the germination phase.

Data from the literature clearly show huge differences in plant responses to PAW exposure, which are dependent on the procedure used to generate PAW, physicochemical characteristics of PAW, treatment protocol, and plant species or even cultivar. Therefore, PAW treatment should be optimized for each application [29,52,56].

To the best of our knowledge, this is the first study reporting on PAW impact on wheat sprouts during the short sprouting time (1–3 days) and wheat young shoots (8 days old). Previous investigations evaluated the effects of other types of PAW on wheat caryopses with reference to the growth parameters at different developmental stages [29,50,55], content in various compounds and activity of antioxidant enzymes in leaves after 4 week cultivation [29] and juice of plantlets after 14 days of cultivation [50], and antioxidant activity of the latter.

5. Conclusions

Our results indicate positive effects of PAW1 (25 mg/L NO_3^-, 4 mg/L NO_2^-, and 6 mg/L H_2O_2) and PAW2 (35 mg/L NO_3^-, 5 mg/L NO_2^-, and 7.5 mg/L H_2O_2) on *Triticum aestivum* L. cv. Glosa sprouts. Overall, exposure to PAW2 had a better impact on sprouts with respect to growth parameters, free phenolic content, and antioxidant activity of the free phenolic fraction (day 3), accumulation of proteins and photosynthetic pigments (day 8). The activity of antioxidant enzymes increased under exposure to PAW1 and PAW2. To conclude, PAW treatment increases the quality of *Triticum aestivum* L. cv. Glosa sprouts. Further investigations will focus on: (i) PAW2 impact on the biosynthesis of other nutritional and bioactive compounds (free amino acids, betaine, vitamins B, vitamin E) and (ii) optimization of PAW characteristics and treatment protocol for the most favorable effect on the accumulation of biomass and health-promoting compounds in *Triticum aestivum* L. cv. Glosa sprouts.

Author Contributions: Conceptualization, R.B. and A.M. (Anca Miron); methodology, A.M. (Alexandru Mandici), R.B., D.A., C.R., D.C.T. and A.M. (Anca Miron); software, R.B. and D.A.; validation, A.M. (Alexandru Mandici), D.A. and O.B; formal analysis, A.M. (Alexandru Mandici) and D.A.; investigation, A.M. (Alexandru Mandici), D.A., O.B., C.R., D.E.C. and T.G.A.; resources, R.B. and A.M. (Anca Miron); data curation, A.M. (Alexandru Mandici) and D.A.; writing—original draft preparation, A.M. (Alexandru Mandici), R.B., D.A., C.R. and A.M. (Anca Miron); writing—review and editing, R.B., D.C.T., A.C.A. and A.M. (Anca Miron); visualization, A.M. (Alexandru Mandici), D.A. and O.B.; supervision, R.B. and A.M. (Anca Miron); project administration, R.B. and A.M. (Anca Miron); funding acquisition, R.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a grant from the Romanian Ministry of Education and Research, CNCS-UEFISCDI, project PN-III-P2-2.1-PED-2019-0556 (contract 361PED/2020) within PNCDI III.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available upon request from the corresponding authors.

Acknowledgments: The authors would like to acknowledge Territorial Inspectorate for Seed and Propagating Material Quality (Iasi, Romania) for providing wheat caryopses (*Triticum aestivum* L. cv. Glosa).

Conflicts of Interest: The authors declare no conflict of interest.

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