https://doi.org/10.31925/farmacia.2022.6.10 **ORIGINAL ARTICLE**

EFFECTS OF NON-THERMAL PLASMA ACTIVATED WATER AND LOW TEMPERATURE ON WHEAT SPROUTS: A FOCUS ON PHOTOSYNTHETIC PIGMENTS, PROTEIN AND PHENOLIC CONTENTS, ANTIOXIDANT ACTIVITY, ANTIOXIDANT AND PROOXIDANT ENZYMES ACTIVITY

ALEXANDRU MANDICI [1], CRAITA ROSU [2], RADU BURLICA [3], DANIEL EUSEBIU CRETU [3], DRAGOS ASTANEI [3], OANA BENIUGA [3], DENIS CONSTANTIN TOPA [4], TUDOR GEORGE AOSTACIOAEI [4], ANCA MIRON [1]*

[1]*Department of Pharmacognosy-Phytotherapy, Grigore T. Popa University of Medicine and Pharmacy, 16 Universității, Iași, Romania*
[2]*Department of Experimental and Applied Biology, Institute of Biological Research Iasi, 47 Lascăr Catargi, 700107 Iași, Romania*
[2]*Department of Electrical Engineering, Gheorghe Asachi Technical University, 71 D. Mangeron, 700050 Iași, Romania*
[4]*Department of Pedotechnics, University of Life Sciences, 3 Mihail Sadoveanu, 700490 Iași, Romania*

**corresponding author: anca.miron@umfiasi.ro*

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Abstract

The study aimed to evaluate the effects of non-thermal plasma activated water (PAW) in combination with low temperature (15°C) on the biosynthetic potential, antioxidant activity and enzymes activity in wheat (*Triticum aestivum* L. cv. Glosa) sprouts. The exposure to three different types of PAW (PAW1, PAW2 or PAW3) and low temperature (15°C) had positive influence on wheat sprouts. Content in proteins and photosynthetic pigments and activity of superoxide dismutase increased in wheat sprouts at day 3 and shoots at day 8. Peroxidase was stimulated in wheat sprouts (day 3) while catalase activation was detected in shoots (day 8). Polyphenol oxidase, responsible for enzymatic browning, was inhibited in shoots at day 8. PAW1 boosted free phenolic content in shoots at day 8. An increment in the antioxidant activity of bound phenolic fraction of shoots (day 8) under treatment with PAW1 was also detected. Overall, non-thermal PAW in combination with low temperature (15°C) is an efficient approach to enhance the quality of wheat (*Triticum aestivum* L. cv. Glosa) sprouts.

Rezumat

Studiul a avut drept scop evaluarea efectelor apei activate cu plasmă non-termică (PAW) în asociere cu temperatura scăzută (15°C) asupra capacității de biosinteză, acțiunii antioxidante și activității enzimatice în germenii de grâu (*Triticum aestivum* L. cv. Glosa). Expunerea la trei tipuri diferite de PAW (PAW1, PAW2 sau PAW3) și temperatura scăzută (15°C) a avut efecte favorabile asupra germenilor de grâu. Conținutul în proteine și pigmenți fotosintetici, precum și activitatea superoxid dismutazei au crescut în germenii de grâu în ziua a 3-a și în frunze în ziua a 8-a. Peroxidaza a fost stimulată în germenii de grâu (ziua 3) în timp ce catalaza a fost activată în frunze (ziua 8). Polifenoloxidaza, răspunzătoare de brunificarea enzimatică, a fost inhibată în frunze în ziua a 8-a. PAW1 a determinat o creștere a conținutului în compuși fenolici liberi în frunze în ziua a 8-a. A fost observată o creștere a activității antioxidante a fracțiunii polifenolice legate din frunze (ziua 8) tratate cu PAW1. În general, apa activată cu plasmă non-termică (PAW), în asociere temperatura scăzută (15°C), reprezintă un procedeu eficient pentru creșterea calității germenilor de grâu (*Triticum aestivum* L. cv. Glosa).

Keywords: non-thermal plasma activated water, wheat sprouts, biosynthetic potential, superoxide dismutase

Introduction

Germination (sprouting) enhances the nutritional quality and medicinal properties of seeds by increasing the content in soluble fibre, proteins, vitamins, anti-oxidants and improving the bioavailability of amino acids and sugars [8, 27, 29, 34, 35]. There are numerous ways to stimulate the biosynthesis and accumulation of bioactive compounds during germination such as exposure to stress (cold/hot environments, hypoxia) and various physical factors (light, pressure, ultrasounds, magnetic fields, non-thermal plasma) [10]. Temperature during soaking and sprouting is the most important factor affecting germination. For wheat, a temperature of 20 - 25°C significantly increases the protein digestibility, tocopherols, niacin, riboflavin and total phenolics and reduces phytic acid [1, 45, 55]. γ-Aminobutyric acid (GABA) increases in plants exposed to thermal stress as a consequence of intracytosolic Ca^{2+} increase and stimulation of glutamic acid decarboxilase (GAD),

enzyme involved in GABA biosynthesis. Ca^{2+}-calmodulin (CaM)-dependent activation of GAD was demonstrated in cold-exposed plant tissues [9, 19]. Anthocyanin content increases in tartary buckwheat (*Fagopyrum tataricum* L.) seeds following cold stress, anthocyanins having antioxidant activity and acting as protectors against cold-induced tissue injuries [24]. An activation of heat-shock proteins was reported in wheat (*Triticum aestivum* L.) and broccoli (*Brassica oleracea* var. *italica*) seeds after exposure to heat and heat and hypoxia, respectively [16, 31]. Other studies reported an elevation in GABA levels due to hypoxia [41, 42]. In germinated black rice (*Oryza sativa* L.), exposed to hypoxia for 6 h, GABA levels increased 13-fold [11]. Ultrasound treatment was reported to have a positive influence on germination. Barley (*Hordeum vulgare* L.) seeds treated with 460 W ultrasounds had 30 - 45% reduction in germination period [52]. Sesame (*Sesamum indicum* L.) seeds treated for 10 and 20 min with 20 kHz ultrasounds showed an increase in the germination rate [40]. Ultrasounds were also reported to increase the germination rate of alfalfa (*Medicago sativa* L.), broccoli (*Brassica oleracea* var. *italica*), okra (*Abelmoschus esculentus* Moench) and zucchini (*Curcubita pepo* L.) seeds [17]. High hydrostatic pressure treatment caused enzyme inactivation and improved the organoleptic properties of seeds [40]. High hydrostatic pressure altered proteolysis and certain amino acids metabolic pathways in Brussels (*Brassica oleracea* L.) sprouts seedlings, increasing both aspartic and glutamic acids levels [3]. *In vitro* starch digestibility on wholegrain-germinated brown rice (*Oryza sativa* L.) was significantly improved after high hydrostatic pressure applied prior to germination; in addition, GABA levels were 25% higher in high hydrostatic pressure group compared to control [51]. Pulsed electric field increased glutathione content and antioxidant enzymes activity in seeds [12, 23]. There are few evidences regarding UV light influence on germination and accumulation of bioactive compounds in sprouts. One study reported an increase in ascorbic acid and total phenolic contents and antioxidant capacity of mung bean (*Vigna radiata* L.) sprouts exposed to UV light and pulsed electric field [15]. Exposure of groundnut (*Arachis hypogaea* L.) seeds to UV-C radiation (200 - 280 nm) for 1 h improved the germination rate by 83.3% and significantly elevated the seeds vigour [33]. UV-C exposure stimulated the growth and quality of cabbage (*Brassica oleracea* var. *capitata*) [5]. Non-thermal plasma (cold plasma) seed treatment was found to stimulate germination and plant growth, enhance accumulation of bioactive compounds in sprouts and lower the time frame for the highest compound accumulation [55]. Non-thermal plasma substantially increased the germination rate (by 24, 28 and 35.5%) of wheat caryopses after 4 min treatment [30]. Non-thermal plasma can be applied directly on seeds and indirectly, by using non-thermal plasma-activated water (PAW). Exposure to non-thermal PAW was reported to enhance the germination rate, seedling growth, accumulation of bioactive constituents and antioxidant activity of sprouts [18, 21, 26, 30, 32, 43, 44, 50, 54].

The aim of the present study was to investigate the potential influence non-thermal PAW in combination with low temperature (15°C) on wheat sprouts with respect to accumulation of proteins, photosynthetic pigments and total phenolics, antioxidant capacity and antioxidant and prooxidant enzymes activity.

Materials and Methods

Non-thermal plasma activated water: generation and analysis

Non-thermal PAW was generated by high voltage electric discharge in air in contact with distilled water at 60, 100 and 150 Hz and 2 ms pulse width (PAW1, PAW2 and PAW3, respectively). Both air and distilled water flows were constant (1.5 L/min and 15 mL/min, respectively). PAW was spectrophotometrically analysed for the content in hydrogen peroxide (H_2O_2) (titanium sulphate method), nitrates (NO_3^-) and nitrites (NO_2^-) (Visocolor Eco colorimetric test kits, Macherey-Nagel) [6, 7].

Treatment with non-thermal plasma activated water

After sterilization in 1.25% sodium hypochlorite (5 min), wheat caryopses (*Triticum aestivum* L. cv. Glosa) (0.9 kg) were soaked in PAW1, PAW2, PAW3 or distilled water for 4 h. Germination was conducted in controlled conditions (65% relative humidity, 15°C) in dark for 3 days followed by a light/dark cycle (12 h/12 h) for other 5 days (Weiss Gallenkamp climatic chamber), freshly prepared PAW and distilled water being changed at days 2, 4 and 6. Samples were collected at days 3 (sprouts) and 8 (shoots).

Quantification of total protein content

Total protein content was estimated using Bradford reagent [20]. The results were expressed as mg/g of fresh vegetal material.

Quantification of photosynthetic pigments

Photosynthetic pigments (chlorophyll a and b, carotenoids) were quantified spectrophotometrically according to Lichtenthaler's method as previously described [49]. The results were expressed as mg/g of fresh vegetal material.

Extraction of free and bound phenolic fractions

Free phenolic fractions were extracted with a mixture of methanol-water-glacial acetic acid (70:29.5:0.5, v/v/v), the remaining pellets being used for the isolation of bound phenolic fractions (alkaline hydrolysis followed by extraction with ethyl acetate) [8].

Quantification of total phenolic content

Total phenolic content in the free and bound phenolic fractions was determined using the Folin-Ciocâlteu method [28, 48, 53].

Assessment of antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, used to evaluate the antioxidant potential of the phenolic fractions, is based on the reduction of the free radical DPPH (violet) to diphenylpicrylhydrazine (yellow), with a significant reduction in absorbance at 517 nm [51, 53].

Determination of superoxide dismutase activity

Superoxide dismutase (SOD) activity was evaluated spectrophotometrically by the riboflavin/nitro blue tetrazolium (NBT) assay which is based on SOD ability to inhibit the reduction of NBT triggered by the superoxide anion radical generated from light-excited riboflavin. SOD activity was expressed as U/mg protein/min, one unit (U) representing the amount of SOD causing 50% inhibition of NBT reduction [2, 39].

Determination of peroxidase activity

Determination of peroxidase (POX) activity was based on its ability to reduce the oxidation of *o*-dianisidine in the presence of hydrogen peroxide and consequently, the absorbance of the reaction mixture at 540 nm. PO activity was expressed as U/mg protein/min, one unit (U) decomposing 1 μM H_2O_2 in 1 min (20°C) [2, 47].

Determination of catalase activity

Catalase (CAT) activity was estimated on the basis of H_2O_2 decomposition rate at 240 nm over 2 min. 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) [39, 47].

Determination of polyphenol oxidase activity

Polyphenol oxidase (PPO) activity was evaluated on the basis of its property to oxidize catechol with a consequent increase in absorbance at 420 nm over 2 min interval. PPO activity was expressed as U/mg protein/min [13].

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 (95% confidence interval). The paired t-test was used to test if the means of two paired measurements are significantly different. A p value lower than 0.05 was considered to be statistically significant.

Results and Discussion

Among the methods used to boost germination and enhance the health promoting value of sprouts, non-thermal PAW attracted significant interest in the last years. Generated by electric discharges in water/gas in contact with water, PAW contains reactive oxygen and reactive nitrogen species which have a major role in enhancing seed germination, biomass accumulation, biosynthesis of metabolites with antioxidant properties and phytohormones, expression of stress protection and defence related genes. These reactive species are also responsible for the antimicrobial effects of PAW [20, 37]. Some reactive species have a short half-life (singlet oxygen $^1O_2$, hydroxyl radical $HO^{\cdot}$, superoxide anion radical $O_2^{\cdot-}$, nitric oxide radical $NO^{\cdot}$, peroxynitrite anion $ONOO^-$) whereas others are stable (H_2O_2, NO_3^-, NO_2^-, NO, O_3). H_2O_2 and NO_3^- are the major species impacting germination, growth and biosynthetic potential of plants [14, 20, 46]. In addition to its antimicrobial effects, H_2O_2 was reported to alter seed inactivity and enhance germination and protein synthesis. Abscisic and gibberellic acids are key phytohormones in regulating seed dormancy and germination; abscisic acid promotes dormancy whereas gibberellic acid promotes germination. H_2O_2 reduced abscisic acid level *via* up-regulating its catabolism genes (*CYP707A* genes) in *Arabidopsis thaliana cyp707a2* seeds (mutant lacking (+)-abscisic acid 8'-hydroxylase, enzyme involved in abscisic acid catabolism). Additionaly, H_2O_2 enhanced gibberellic acid levels *via* up-regulating its biosynthesis genes (*GA3ox* and *GAw20ox* genes) [25]. H_2O_2 was reported to activate *CAT* genes, responsible for protein synthesis, and also enhance germination in *Paulownia tomentosa* seeds [38]. Similar to H_2O_2, NO_3^- was reported to change abscisic acid to gibberellic acid ratio towards elevated levels of the former, thus enhancing germination. NO_3^- was also found to stimulate phytochrome A activity, a pigment regulating germination, in *Arabidopsis thaliana* seeds [4, 46]. Although NO^-_2 is toxic for some plants, wheat tolerates NO_2^-. In addition to NO_3^-, NO_2^- is an important nitrogen source, contributing to plant growth and development. Nitrogen is an essential element for amino acid, protein and chlorophyll synthesis, being involved in biocatalytic processes, electron transport and various metabolic pathways; it also facilitates the uptake of other essential plant elements such as potassium and phosphorous [4, 20, 22].

A recent study reported that low temperature (15°C) differently impacts the phenolic content in sprouts depending on plant species/genotype. Germination conducted at 15°C (for 3 days) increased the free phenolic content in two wheat varieties (*Triticum aestivum* L., Gentil Rosso and Wheat Mix) while the content of bound phenolics was inconsistently influenced. The same germination conditions induced an inconsistent trend in the accumulation of both free and bound phenolics in millet (*Panicum miliaceum* L., Millet) [8].

The present study investigated the combined effects of three different types of PAW and low temperature (15°C) on the germination of *Triticum aestivum* L. cv. Glosa caryopses regarding protein, photosynthetic pigments and total phenolic contents, antioxidant capacity and antioxidant and prooxidant enzymes activity. The wheat variety used in this study, *Triticum*

aestivum L. cv. Glosa, has a good productivity and resistance and therefore, it is largely cultivated and used in Romania.

Non-thermal plasma activated water

Three types of PAW were generated and analysed in the present study. The concentrations of NO_3^-, NO_2^- and H_2O_2 determined in PAW1 - 3 are given in Table I.

Total protein content

All PAW treatments positively influenced the total protein content in wheat sprouts (day 3) and shoots (day 8), with the highest increase being noted in PAW2 treated sprouts (5.69 mg/g at day 3 and 5.18 mg/g at day 8 *vs.* 2.93 mg/g at day 3 and 2.43 mg/g at day 8 in the control group). PAW3 induced a decrease in the total protein content in comparison with PAW2 (Figure 1).

Table I

Chemical composition of PAW1 - 3

PAW	NO_3^- (mg/L)	NO_2^- (mg/L)	H_2O_2 (mg/L)
PAW1	25.0 ± 2.5	4.0 ± 0.4	6.0 ± 0.5
PAW2	35.0 ± 3.5	5.0 ± 0.5	7.5 ± 0.7
PAW3	45.0 ± 4.5	6.0 ± 0.6	8.0 ± 0.8

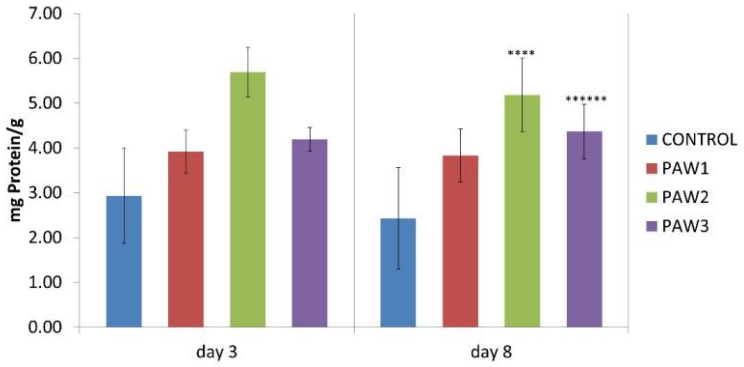

Figure 1.

Effect of PAW treatment on the total protein content in wheat sprouts (day 3) and shoots (day 8)
****significant difference ($p < 0.05$) between PAW1 and PAW2;
******significant difference ($p < 0.05$) between PAW2 and PAW3

Photosynthetic pigments content

The carotenoid content in wheat shoots increased in the following order: control < PAW1 < PAW2 < PAW3, PAW2 and PAW3 enhancing the carotenoid content by 26.92% and 32.3%, respectively in comparison with control. With respect to chlorophylls, PAW1 determined 100.3% increase in total chlorophyll content compared with control, followed by PAW3 (29.41% increase) and PAW2 (9.95% increase). Chlorophyll a and chlorophyll b contents were also enhanced by ~ 73% and ~ 145%, respectively, in PAW1 treated group (Figure 2).

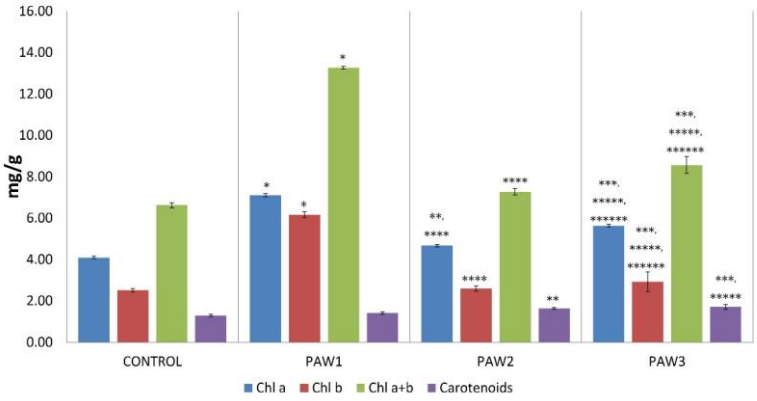

Figure 2.

Effect of PAW treatment on the photosynthetic pigments content in wheat 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 control and PAW3; ****significant difference ($p < 0.05$) between PAW1 and PAW2; *****significant difference ($p < 0.05$) between PAW1 and PAW3;
******significant difference ($p < 0.05$) between PAW2 and PAW3

Total phenolic content

Bound and free phenolic contents in wheat sprouts (day 3) and shoots (day 8) were also influenced by PAW treatment. At day 8, PAW1 induced an important increase (19.18%) in the free phenolic content in comparison with control whereas PAW2 was more efficient in enhancing the bound phenolic content (9.51% increase in comparison with control) (Figure 3).

Antioxidant activity

Antioxidant effects of both free and bound phenolic fractions were slightly influenced by PAW treatment, except PAW1 which induced a substantial increase in the antioxidant activity of the bound phenolic fraction isolated from wheat shoots at day 8 (82.74 *vs.* 63.95% for control). PAW1 and PAW2 enhanced the antioxidant activity of the free phenolic fractions at day 8 (52.65 and 52.20%, respectively *vs.* 36.02% for control) (Figure 4).

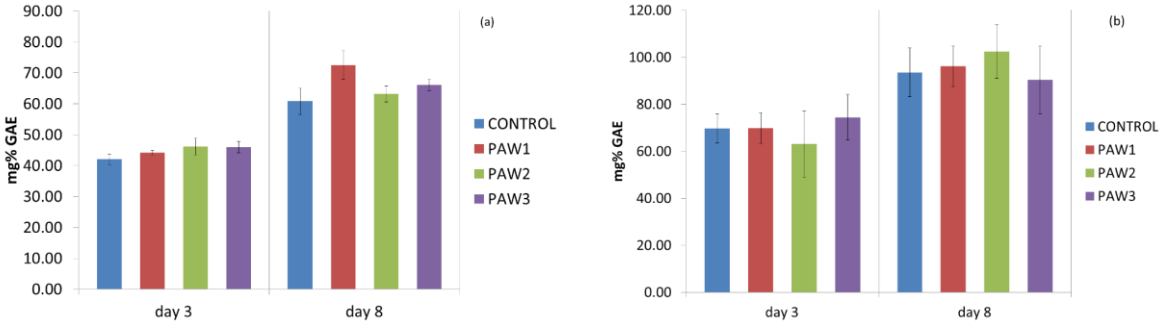

Figure 3.

Effect of PAW treatment on the free (**a**) and bound (**b**) phenolic contents in wheat sprouts (day 3) and shoots (day 8)

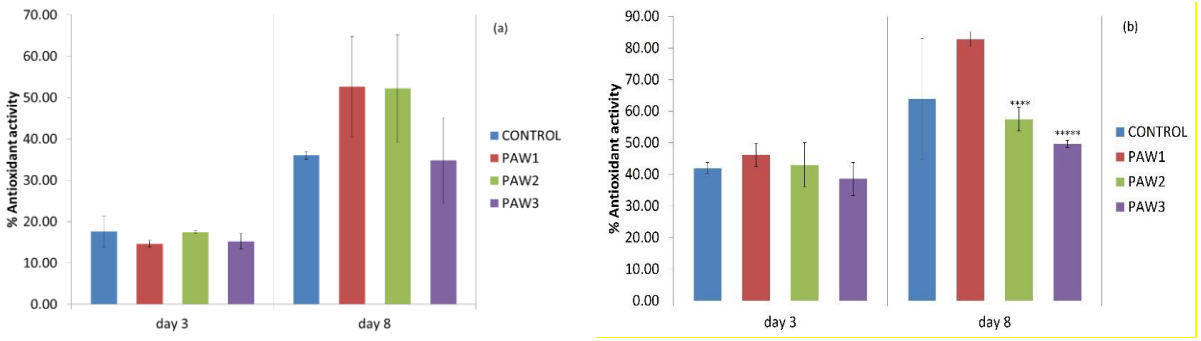

Figure 4.

Effect of PAW treatment on the antioxidant activity of free (**a**) and bound (**b**) phenolic fractions isolated from wheat sprouts (day 3) and shoots (day 8)

****significant difference ($p < 0.05$) between PAW1 and PAW2;

*****significant difference ($p < 0.05$) between PAW1 and PAW3

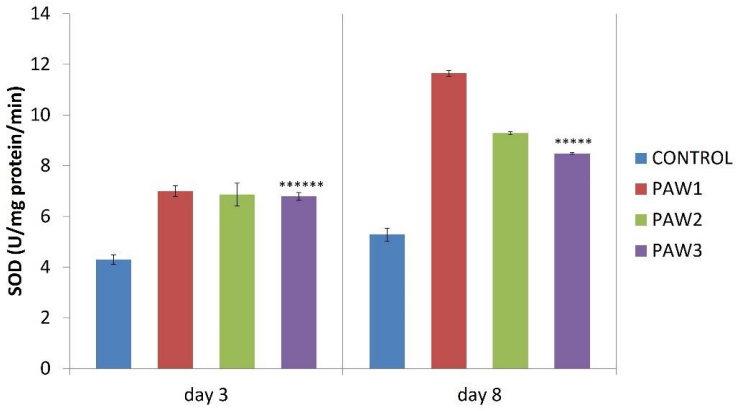

Figure 5.

Effect of PAW treatment on SOD activity in wheat sprouts (day 3) and shoots (day 8)

*****significant difference ($p < 0.05$) between PAW1 and PAW3;

******significant difference ($p < 0.05$) between PAW2 and PAW3

Superoxide dismutase activity

PAW treatment stimulated SOD with more pronounced increments in activity at day 8 (11.66, 9.28 and 8.49 U/mg protein/min in PAW1, PAW2 and PAW3 treated group, respectively *vs.* 5.28 U/mg protein/min in the control group). At day 3, PAW1, PAW2 and PAW3 showed comparable effects while at day 8, PAW1 exerted the most prominent effect, PAW2 and PAW3 being less active (Figure 5).

Peroxidase activity

PAW treatment significantly elevated POX activity in wheat sprouts at day 3 (3.70, 7.30 and 6.95 U/mg protein/min in PAW1, PAW2 and PAW3 treated group, respectively *vs.* 1.36 U/mg protein/min in the control group). Surprisingly, PAW decreased POX activity in shoots at day 8, the most pronounced reduction being registered for PAW3 (48.15, 45.25 and 43.75 U/mg protein/min in PAW1, PAW2 and PAW3 treated group, respectively *vs.* 52.55 U/mg protein/min in the control group) (Figure 6).

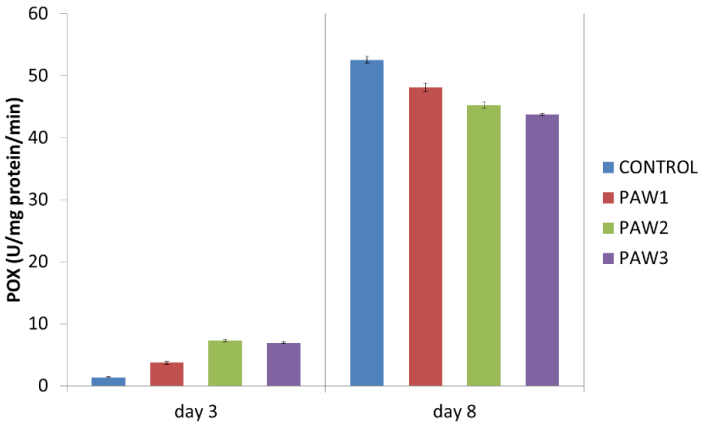

Figure 6.

Effect of PAW treatment on POX activity in wheat sprouts (day 3) and shoots (day 8)

Catalase activity

PAW significantly elevated CAT activity in wheat sprouts at day 3 (6.34, 7.95 and 7.97 U/mg protein/min in PAW1, PAW2 and PAW3 treated group, respectively *vs.* 10.84 U/mg protein/min in the control group) and reduced it in shoots at day 8 (30.00, 31.24 and 32.79 U/mg protein/min in PAW1, PAW2 and PAW3 treated group, respectively *vs.* 26.92 U/mg protein/min in the control group) (Figure 7).

Polyphenol oxidase activity

PAW treatment slightly activated PPO in wheat sprouts (day 3), but lowered its activity in shoots (day 8), PAW1 inducing the most pronounced inhibition (4.90 *vs.* 6.19 U/mg protein/min in the control group) (Figure 8).

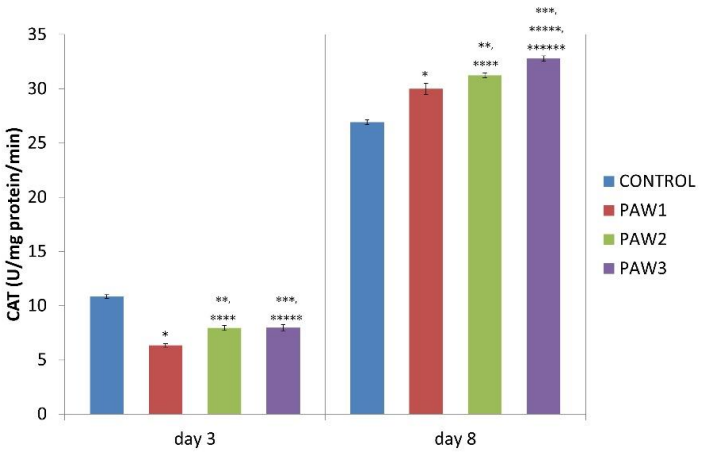

Figure 7.

Effect of PAW treatment on CAT activity in wheat 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 control and PAW3; ****significant difference ($p < 0.05$) between PAW1 and PAW2; *****significant difference ($p < 0.05$) between PAW1 and PAW3; ******significant difference ($p < 0.05$) between PAW2 and PAW3

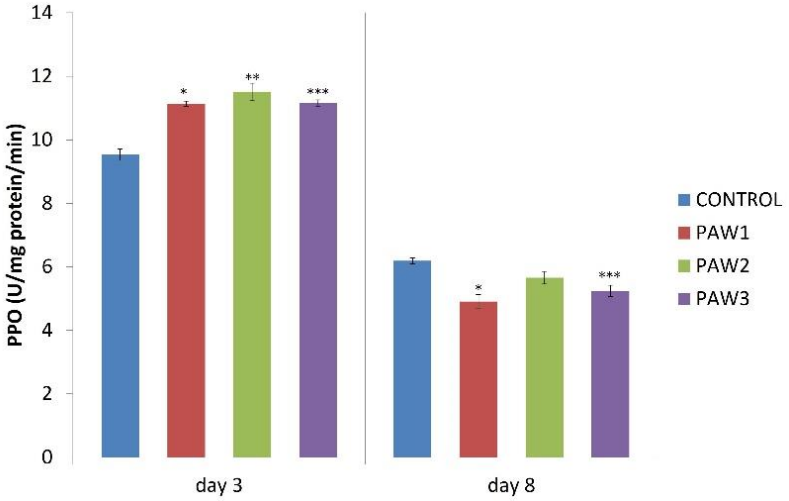

Figure 8.
Effect of PAW treatment on PPO activity in wheat sprouts (day 3) and shoots (day 8)
*significant difference ($p < 0.05$) between control and PAW1; **significant difference ($p < 0.05$) between control and PAW2; ***significant difference ($p < 0.05$) between control and PAW3

Overall, all PAW treatments in combination with low temperature (15ºC) increased the protein, total chlorophyll and carotenoid contents in wheat sprouts. Reactive species in PAW generate a defensive response by enhancing the accumulation of secondary metabolites with antioxidant properties (phenolics, carotenoids) and expression/activity of antioxidant enzymes (SOD, POX, CAT) which reduce the effects of reactive oxygen species ($O_2^{\cdot-}$, H_2O_2) [37, 49]. PAW1 induced a remarkable increase in free phenolic content and also in antioxidant potential of bound phenolic fraction in shoots at day 8. PAW1-3 also elevated the activity of antioxidant enzymes (SOD at days 3 and 8, POX at day 3, CAT activity at day 8). PPO is responsible for the enzymatic browning (conversion of polyphenols into quinones, followed by polymerization and/or interaction of quinones with amino acids and proteins). Enzymatic browning lowers the organoleptic quality and nutritional value of vegetal products [36]. PAW1-3 slightly affected PPO activity at day 3 and lowered it at day 8, indicating that PAW treatment at 15ºC could be useful to preserve the quality of wheat sprouts [14, 38, 46].

Most of the recent studies reported conflicting results regarding PAW impact on sprouts depending on the procedure used to generate PAW, PAW physico-chemical characteristics, treatment protocol and plant species/cultivar [18, 21, 26, 30, 32, 43, 44, 50, 54]. Wang *et al.* explored the influence of PAW treatment on tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.) sprouts and observed an increase in germination rate, stem length, shoot weight, total amino acids, reducing sugars, flavonoids, total phenolics and GABA along with a decrease in proteins, carbohydrates and crude fat. Antioxidant activity of tartary buckwheat sprouts was enhanced under PAW exposure. In this study, Wang *et al.* used electric discharge to generate PAW (time: 20 min, power: 450 W, gas flow: 40 L/ min, action spacing: 0.3 cm) [50]. Similar results were reported by Kim *et al.* who found PAW (generated by 10, 20 or 30 min plasma discharge in water) to improve germination, glucosinolate and phenolic contents in radish (*Raphanus sativus* L.) sprouts [18]. Puač *et al.* showed various effects of PAW (generated by using atmospheric pressure plasma jet for distilled water treatment) on CAT in *Paulownia tomentosa* Steud. seeds depending on the treatment time of distilled water (10, 20 or 30 min) and time the analyses were done (immediately, 1, 2, or 3 days after induction of germination) [38]. It is obvious that each application of PAW should be optimized with respect to type and parameters of generation process which strongly affect the physicochemical characteristics of PAW and also treatment protocol.

Conclusions

Our study indicates that PAW treatment may be considered a promising strategy to improve the quality of *Triticum aestivum* L. cv. Glosa sprouts. PAW content in reactive species should be optimized for a better impact on accumulation of phytochemicals having nutritional and medicinal properties.

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Conflict of interest

The authors declare no conflict of interest.

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