

The impact of plasma activated water on spinach (*Spinacia oleracea* L.) growth, development and content of plant bioactive compounds

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ABSTRACT

The production of green leafy vegetables is an important segment in agriculture, because they are the source of functional compounds that are beneficial for human health. The main objective of this study was to determine the effects of plasma activated water (PAW) treatment on the morphological, qualitative and chemical properties of spinach under greenhouse and field growing conditions. The application of PAW has a significant influence on plant height, plant diameter, green mass and dry matter, as well as on the content of bioactive substances (phenols, flavonoids, proline) under both cultivation conditions. The results of this study show that the application of a higher dose of PAW (PAW 2) resulted in 46% higher green mass of spinach under field conditions and 57% higher green mass under greenhouse conditions compared with the control varieties. The application of PAW 2 increased the dry matter of the plants by 53% under field conditions and by 75% under greenhouse conditions compared with the control varieties. Similar results were obtained with respect to proline content under field conditions, which increased by 30% with the application of PAW 2 compared with the control varieties and by 50% in the greenhouse with the application of PAW 1. The results indicate that PAW treatment can be a useful method to improve the growth and nutritional quality in spinach cultivation under different climatic and growing conditions.

Keywords: bioactive compounds, growing conditions, morphological properties, plasma activated water, spinach

INTRODUCTION

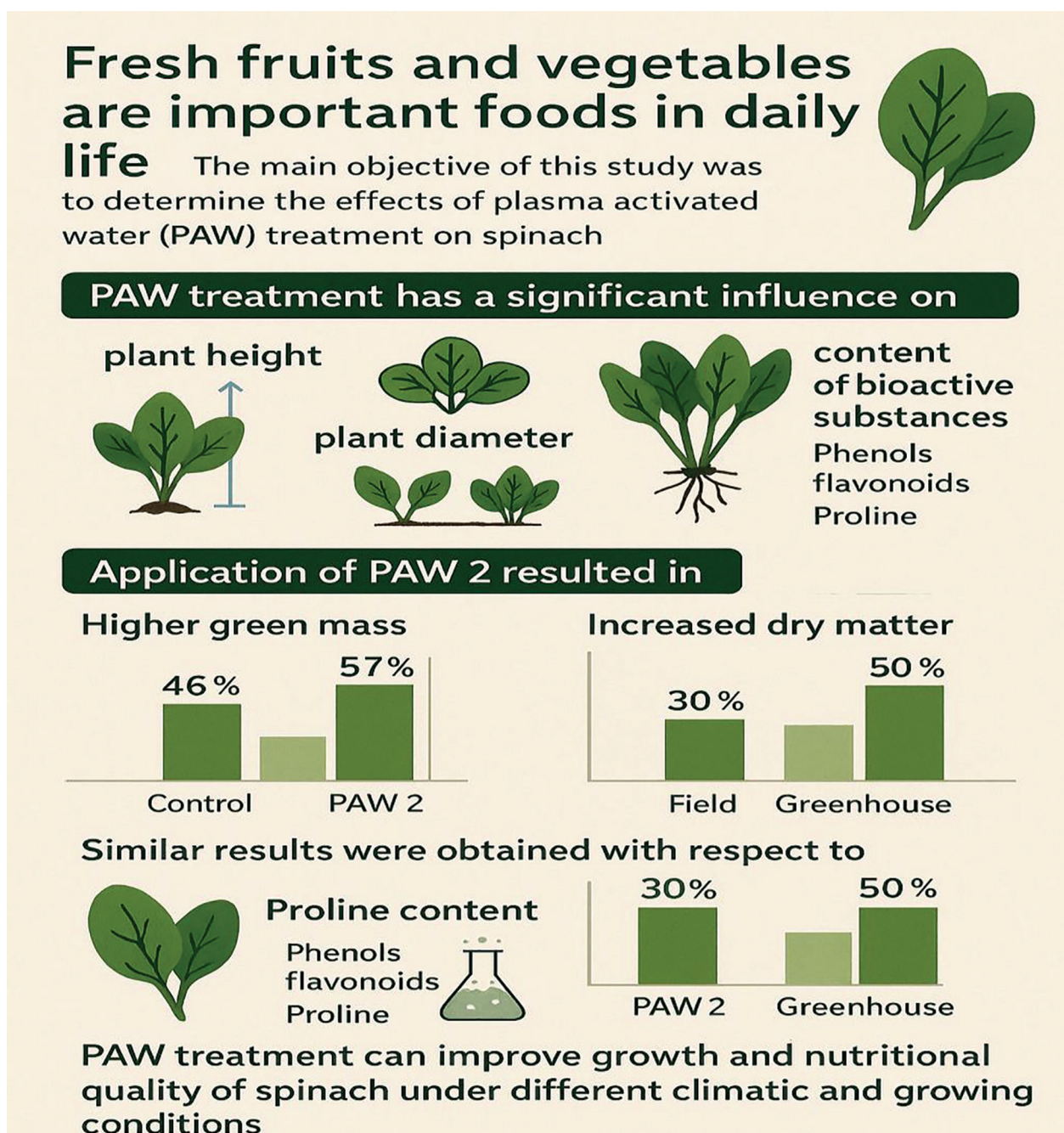
In recent years, the consumer demand for high-quality and healthy food has increased, making the production and consumption of fruits and vegetables an important segment of the daily diet. Fresh and high-quality agricultural products with adequate nutritional content are in high demand as they provide good quality of life (Randhawa et al., 2018; Kučerová et al., 2021). Leafy green vegetables (LGV), as a necessary and desirable source of nutrients in daily life, are also a major challenge

for agricultural production. The production of healthy food is linked to organic farming, which is a more sustainable form of food production and does not use synthetic pesticides and mineral fertilisers (Man et al., 2024). Spinach (*Spinacia oleracea* L.) is one of the most common leafy vegetables consumed worldwide as a healthy food. It is a vegetable with high nutritional value and a source of bioactive compounds that are beneficial for human health. Bioactive compounds in spinach

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GRAPHICAL ABSTRACT



include phenolic compounds and carotenoids (Roberts and Moreau, 2016; Park et al., 2023). The content of the major and minor constituents in spinach depends on various factors, including cultivar, growing conditions (GC), agricultural management and environmental conditions (Park et al., 2023). Agricultural practices are also affected by climatic fluctuations in the field, as sudden and unstable climatic fluctuations affect the efficiency and safety of agricultural production (Benković et al., 2021). For example, temperature fluctuations during spinach growth can affect spinach cultivation. The optimal air temperatures for spinach growth are between 16°C and 20°C; low temperatures

can damage the photosynthetic apparatus and thylakoid membranes and impair leaf growth (Ozrekin et al., 2018). Temperature and global radiation also have a strong influence on the nitrate and antioxidant content of spinach leaves, so that the growth and leaf development of spinach depends not only on the GC but also on the nitrogen supply. The increasing demand for healthy food is forcing the use of new methods to increase the nutrient content and crop yield in order to reduce the negative effects of excessive use of chemical fertilisers (Antar et al., 2021; Man et al., 2024). Recently, several physical methods have been proposed in agricultural practise as potential applications for crop fertilisation and hygiene

in food production (Kang et al., 2019). Plasma activated water (PAW) has emerged as an environmentally friendly and sustainable technology in agricultural production to ensure high-quality, healthy agricultural products (Kučerová et al., 2021). Plasma technology has a wide range of applications: decontamination of surfaces, safe food, clean water and improved yields in agriculture (Sivachandiran and Khacef, 2017; Gao et al., 2022). The activity of PAW generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Wang et al., 2023, 2024). The use of PAW to improve the growth of agricultural products has opened a new field for researchers (Abbaszadeh et al., 2021). PAW is an innovative approach in agriculture, with the potential to enhance plant growth and stress resistance without synthetic inputs. PAW contains reactive oxygen and nitrogen species (RONS), which can positively influence germination, growth and the antioxidant capacity in plants (Sivachandiran and Khacef, 2017; Thirumdas et al., 2018). Plasma-generated RNS can regulate resistance to abiotic and biotic stress factors as well as plant growth and development (Kang et al., 2019; Sami et al., 2021). Huda-Faujan et al. (2023) have reported about the antioxidant and antimicrobial properties of the natural and bioactive constituents in spinach. Hamed et al. (2025) have reported that application of biological gibberelin on spinach plants improves the growth dynamics, antioxidant capacities and leaf metabolites (prolines) in drought-stressed spinach plants. The application of PAW to food leads to deterioration of phytochemicals that stimulate antioxidant activity (Attri et al., 2020). Patra et al. (2022) have reported that PAW-reactive species (ROS and RNS) interact with the food matrix and alter its physical and chemical properties (increase or decrease in acidity, total soluble solids, antioxidant activity, total phenolic compounds and vitamins). The influence of PAW on antioxidant activity has also been demonstrated in reports by other authors. Švubová et al. (2021) found increased activation of antioxidant enzymes in seeds exposed to air plasma. Sharma et al. (2021) showed that PAW is a promising technology to increase the activity of antioxidant enzymes. Zhao et al. (2019) found that antioxidant enzyme activity was higher after PAW treatment of freshly sliced kiwifruit, and Guo et al. (2017) reported the same for wheat seeds. LGV have a high content of polyphenols, and the effects of these compounds are important for human health because they act as antioxidants to neutralise harmful free radicals (Randeniya and De Groot, 2015). In addition to their nutritional and health properties, these secondary substances, products of plant metabolism, are also elements of plant protection against environmental influences.

In this study, two different GC (open field and greenhouse) and two different PAW values were used for spinach cultivation. The main objective of this study was to determine the effects of PAW on the morphological characteristics, micro and macro element content and

bioactive compound formation in spinach. It also aimed to identify PAW as a potential fertiliser that can increase plant vitality, as a potential method of plant nutrition that makes plants more resistant to the stresses caused by climate change and reduces the use of N mineral fertilisers and CO₂ emissions in organic farming.

MATERIALS AND METHODS

Method of preparation of PAW

Plasma active species were generated in a plasma reactor based on an atmospheric pressure plasma jet with a single electrode. The atmospheric pressure plasma jet consisted of a quartz tube (outer and inner diameters of 1.5 mm and 1 mm) and a copper wire with a diameter of 100 µm, which served as an electrode and was inserted into the capillary. The electrode is operated with a sinusoidal voltage waveform of 28 kHz with a maximum voltage of 12 kV (PVM500-2500 Plasma Power Generator, Information Unlimited), and the power output is approximately 15 W. The discharge is generated in nitrogen gas (99.996% purity) supplied to the capillary at a flow rate of 500 sccm. The 800 mL sample was brought into contact with the plasma in a Berzelius beaker jet by placing the liquid at a distance of 5 mm from the capillary opening, which ensures comparable production of H₂O₂ and NO²⁻ in the PAW. Commercially purified pharmaceutical grade water (Pharmacopeia Europaea, Ph. Eur. 9) with a pH of 6.5 and a conductivity of 0.98 µS · cm⁻¹ was used. The treatment time with the nitrogen plasma jet was 70 min. The plasma-activated samples were analysed after treatment and several times during storage. The concentration of active nitrogen NO²⁻, NO³⁻, oxygen H₂O₂ and pH-value were measured with QUANTOFIX test strips. The strips were analysed using the QUAN-TOFIX Re-lax optical reader (Macherey-Nagel, GmbH, Düren, Germany), which enables quantitative analysis with high accuracy. The nitrate/nitrite strips were calibrated with known concentrations of NaNO₂ and NH₄NO₃ solutions and the calibration was checked with Ultraviolet–visible spectrophotometry (UV-VIS) absorption spectroscopy measurements. The monitoring of sample ageing by the strips with a time resolution of 1 min allows for determination without significant sample consumption, and the measurement error was <10%. The Mg ions were added by introducing a solid piece of magnesium (5 g) immediately during the plasma treatment and left in the liquid for 1 hr after the treatment. The physicochemical values of PAW measured after treatment correspond to the values at the time of water utilisation. PAW was prepared at the Institute of Physics in Zagreb, Croatia, according to Kutasi et al. (2019, 2021) and Gierczik et al. (2020).

Experimental design

Spinach seedlings of the Rembrandt variety were used to study the effects of PAW treatment on plant growth in the greenhouse and in the open field. The pot experiments

were conducted in the greenhouse and in the field at the Biotechnical Department of University of Slavonski Brod at the Slobodnica site (45°9'57"N 17°57'8"E). The two-factorial experiment in a randomised block design with three replicates was set up as follows: GC greenhouse (G) and open field (F), two PAW treatments (PAW 1 and PAW 2) and seedlings without treatment with PAW (control = PAW 0). Spinach seeds were sown in PVC containers (Pöpellmann TEKU BP 3153/60) with 60 sowing places (volume 76 mL) and filled with growing medium Potground P Klasman Deilman substrate. The sowing date was 11 March 2024, and the trays with the sown seeds were placed in a growth chamber (Memmert ICH2600L) under controlled conditions. One-month-old spinach seedlings were planted on 4 April 2024 in pots (Pöpellmann TEKU VCG 19) 19 cm × 14.9 cm (3.75 L) filled with garden soil. Chemical properties of the soil: pH-KCl 6.42; pH-H₂O 6.8; Al-P₂O₅ 23.57 mg · 100 g⁻¹ soil; Al-K₂O 23.09 mg · 100 g⁻¹ soil; 9.6 CaCO₃%, 1.21% humic content. After planting the seedlings in pots, the pots were divided into two groups: one group had 30 pots with the plants for greenhouse cultivation (i.e. 10 pots per each PAW treatment) and the other group had another 30 pots with seedlings for open field cultivation (Figure 1). Irrigation was applied after sowing, if necessary, depending on rainfall in an outdoor trial every 15 days (three times) during the trials. The plants were treated with 1.5 dL PAW pot⁻¹ (PAW 1) and 3.00 dL pot⁻¹ (PAW 2). The doses for PAW application were chosen based on previous experimental experience (Japundžić-Palenkić et al., 2022; Romanjek-Fajdetić et al., 2022). The control plants were irrigated with tap water at the same time.



Figure 1. Pots with spinach plants in greenhouse GC. GC, growing conditions.

Plant growth was measured four times at 2-week intervals on the following dates: 4 April, 19 April, 2 May and 16 May, until the spinach reached commercial maturity. The plant growth parameters measured were number of leaves, plant height, plant diameter, green mass yield and dry mass (Figure 2). Three plants were randomly selected from each replication. Samples were transferred to the Agroecological Laboratory, Biotechnical Department, University of Slavonski Brod, Croatia and stored in a cooler until used. All analyses were performed within 24 hr. The plant mass was determined using an analytical balance with an accuracy of 0.01 g (PL3002, Mettler-Toledo International Inc., Greifensee, Switzerland). In the first measurement on 4 April, the average weight of the plants was 0.44 g (range: 0.25–0.65 g). During the second measurement (19 April), the average weight of the samples was 1.25 g (range: 0.23–2.24 g). The weight of the plants, which was measured on 2 May, ranged from 3.74 g to 23.05 g (average 10.87 g), while in the final measurement the weight of the plants was 47.37 g (range: 21.59–79.99 g).

Statistical analysis

The collected research data were statistically processed with the TIBCO Statistica Version 14.1.0.8, using one-way Analysis of Variance (ANOVA) for first measurements, and factorial ANOVA for other measurements. In accordance with Fisher test of significant differences of variance analysis, the least significant differences (LSD) for statistical significance were calculated by comparing the mean values. As far as secondary metabolites are concerned, the analysis of statistically significant differences between the treated



Figure 2. Preparation of spinach plants material for measurement of plant growth parameters.

samples and controls was made by Student's *t*-test, (asterisks) corresponding to p -values $0.05 > p > 0.01$, $0.01 > p > 0.001$ and $p < 0.001$.

Chemical analysis of plants

The plant material was analysed for micro- and macroelements using standard analytical methods. Measurement of macroelements comprised total nitrogen content (HRN ISO 11261:2004, 1995), potassium content by flame photometry (AOAC, 2023) and phosphorus content by spectrophotometric method (AOAC, 2023). An atomic absorption spectrometer was used to determine the content of calcium, magnesium and trace elements (Fe, Zn, Mn, Cu) (AOAC, 2023).

Measurement of plant bioactive compounds in spinach

In this study, the total phenols, phenolic acid content, antioxidant capacity, flavonoid and proline content were determined in the plant material. The plant material was collected in the greenhouse and field conditions as well as in different PAW treatments; 30 mg of the dry plant material was taken for analysis. The analyses of total phenols, phenolic acids, antioxidant activity, flavonoids and proline were performed at the Ruđer Bošković Institute (Zagreb, Croatia). Total polyphenols were determined using the Folin-Ciocalteu method in 80% methanol (Singleton and Rossi, 1999). The content of total phenols is expressed in milligrams of gallic acid equivalents per unit mass of dry or fresh matter of the sample ($\text{mg gallic acid equivalents (GAE)} \cdot \text{g}^{-1} \text{ d.w.}$). The content of phenolic acids determined by the colorimetric method (European Pharmacopoeia, 2004) is expressed as milligrams of caffeic acid equivalents per gram of dry or fresh substance of the sample ($\text{mg caffeic acid equivalents (CAE)} \cdot \text{g}^{-1} \text{ d.w.}$). The antioxidant capacities of the samples were determined by *in vitro* DPPH (1,1-diphenyl-2-picrylhydrazyl) tests (Brand-Williams et al., 1995) and the results are expressed as Trolox equivalent per unit mass dry or fresh substances of the sample ($\mu\text{mol Trolox equivalent (TE)} \cdot \text{g}^{-1} \text{ d.w.}$). The content of total flavonoids was determined by the method of Zhishen et al. (1999) and expressed in milligrams of catechin equivalents per unit mass of the sample ($\text{mg catechin equivalents (CE)} \cdot \text{g}^{-1} \text{ d.w.}$). Proline content was determined by the protocol according to Carillo and Gibon (2011) and expressed as micromole proline per gram of dry or fresh matter ($\mu\text{mol proline} \cdot \text{g}^{-1} \text{ d.w.}$). All analyses were performed in triplicate, and values were expressed as mean values $\pm$ standard deviation (SD). Analysis of statistically significant differences between the treated and control sample conditions was done by Student's *t*-test.

RESULTS AND DISCUSSION

This research focuses on the evaluation of the application of PAW on the growth and development of spinach plants, the effect on the chemical composition (basic

micro- and macroelements) after the application of PAW and the content of bioactive substances, which are a form of defence mechanism against stress conditions. In agricultural practise, the response to PAW treatment depends on many characteristics, for example, plant variety, PAW concentration and GC (Attri et al., 2020). In addition, studies were conducted under different GC, namely under conditions where the environmental factor is reduced (greenhouse) and under conditions where the environmental factors change (temperature, humidity, precipitation, sun). Under the same GC, PAW treatments were applied to lettuce and pepper seedlings in 2021 and 2022 (Japundžić-Palenkić et al., 2022; Romanjek-Fajdetić et al., 2022). In this study, the effect of PAW was tested on pre-grown spinach plants and not on seeds. Kučerová et al. (2021) reported that the stimulating effect of PAW is usually more in the later stages of vegetative plant growth, when plants require more nutrients. During germination, seeds and young seedlings take up nutrients from seed stocks and at later stages from the soil or water.

Results of spinach growth and development in greenhouse and field conditions

The results of the growth and development measurements of spinach under two different GC and PAW treatments are shown in Tables 1–5. Since the LGV plants, like spinach, are grown for their above-ground green mass and the yield to be obtained, the morphological growth characteristics were evaluated by measuring the number of leaves, plant height, plant diameter, green mass and plant dry mass. The effects of PAW treatment and GC on the number of spinach leaves are shown in Table 1. The measurements showed that there were no significant differences in leaf development during plant growth under greenhouse and field conditions and during PAW treatment. Under greenhouse conditions, a higher number of spinach leaves was observed after the PAW 2 treatment than in the control and PAW 1 treatment in all the measured periods. This could be related to the higher nitrogen input and the stable ecological conditions. However, under field conditions, the PAW 2 treatment showed a higher number of leaves in the measurements after the first and second PAW applications. Although a statistically significant influence could not be detected at all measurement times, a positive influence of PAW was observed. The first measurement on 4 April was the starting point and no influence of PAW could be detected, as this was the day of the first PAW treatment.

The influence of PAW treatment and GC on plant height (Table 2) was only significant in the last measurement. The influence of PAW treatment was consistent. Taller plants were observed in pots with PAW 2 treatment under all GC (exception: plants in the greenhouse at the first measurement). In the last measurement, a significant interaction between the PAW treatment and the GC was observed. Statistically, the plants after PAW treatment were taller than the plants without PAW treatment in the field and the plants

Table 1. Number of spinach leaves under PAW treatment and different GC.

Number of leaves								
Sampling date	Measurement 1 4 April 2024		Measurement 2 19 April 2024		Measurement 3 2 May 2024		Measurement 4 16 May 2024	
	GC		GC		GC		GC	
PAW treatment	G	F	G	F	G	F	G	F
PAW 0	6.00	5.33	7.00	6.67	10.33	9.33	16.33	17.00
PAW 1			6.67	6.33	11.67	11.33	16.00	16.00
PAW 2			7.00	8.67	12.33	12.33	18.33	16.00
F_{PAW}			n.s. ($p < 0.05$, $F = 2.74$)		n.s. ($p < 0.05$, $F = 3.24$)		n.s. ($p < 0.05$, $F = 0.42$)	
F_{GC}			n.s. ($p < 0.05$, $F = 0.47$)		n.s. ($p < 0.05$, $F = 0.3$)		n.s. ($p < 0.05$, $F = 0.28$)	
Interaction								
$F_{\text{PAW}} - F_{\text{GC}}$			n.s. ($p < 0.05$, $F = 1.89$)		n.s. ($p < 0.05$, $F = 0.13$)		n.s. ($p < 0.05$, $F = 0.75$)	

PAW 1—treatment 1; PAW 2—treatment 2; PAW 0—control; G—greenhouse; F—field; F_{PAW} —F test PAW treatment; F_{GC} —F test growing conditions; $F_{PAW}-F_{GC}$ —F test interaction; n.s.—no statistical significance; Values between the same line followed by different lowercase letters are statistically significantly different (LSD) at $p < 0.05$; Values within the same columns followed by different capital letters are statistically significantly different (LSD) at $p < 0.05$.

GC, growing conditions; LSD, least significant differences; PAW, plasma activated water.

Table 2. The spinach plant height under PAW treatment and different GC.

Plant height (cm)								
Sampling date	Measurement 1 4 April 2024		Measurement 2 19 April 2024		Measurement 3 2 May 2024		Measurement 4 16 May 2024	
PAW treatment	GC		GC		GC		GC	
	G	F	G	F	G	F	G	F
PAW 0	7.00	5.67	8.10	7.33	12.67	12.37	18.8 bB	17.23 bB
PAW 1			8.17	7.53	14.57	13.17	19.47 bB	24.57 aA
PAW 2			7.90	9.90	15.57	15.50	19.77 bB	27.37 aA
F_{PAW}			n.s. ($p < 0.05$, $F = 1.32$)		n.s. ($p < 0.05$, $F = 2.61$)		*($p < 0.05$, $F = 7.29$)	
F_{GC}			n.s. ($p < 0.05$, $F = 0.09$)		n.s. ($p < 0.05$, $F = 0.3$)		*($p < 0.05$, $F = 9.19$)	
Interaction								
$F_{\text{PAW}}-F_{\text{GC}}$			n.s. ($p < 0.05$, $F = 1.91$)		n.s. ($p < 0.05$, $F = 0.15$)		*($p < 0.05$, $F = 4.99$)	

PAW 1—treatment 1; PAW 2—treatment 2; PAW 0—control; G—greenhouse; F—field; F_{PAW} —F test PAW treatment; F_{GC} —F test GC; $F_{PAW}-F_{GC}$ —F test interaction.

Values between the same line followed by different lowercase letters are statistically significantly different (LSD) at $p < 0.05$; values within the same columns followed by different capital letters are statistically significantly different (LSD) at $p < 0.05$.

*Statistical significance.

GC, growing conditions; LSD, least significant differences; n.s., no statistical significance; PAW, plasma activated water.

in the greenhouse with and without PAW treatment. The application of PAW 2 increased the plant height compared with the PAW 1 treatment, but without statistical differences. During the study, the plants in the greenhouse were taller, except for the last measurement where the plants in the field were taller, especially after the PAW treatment.

The influence of PAW treatment and GC on plant diameter was determined and is shown in Table 3. A significant influence of the cultivation conditions was only found in the first measurement and the influence of PAW in the last measurement. In the first measurement, a significantly smaller plant diameter was found in

plants without PAW treatment under field conditions. In the last measurement, a significant influence of PAW 2 treatment was only observed in the plants grown under field conditions compared with the control plants. The positive effect of PAW 2 treatment on plant diameter was observed in all the measurement periods and under all GC.

The effects of the experimental factors (Table 4) showed that the GC had no significant effect on the green mass of spinach, but the PAW treatment had a significant impact after the second and third applications. PAW 2 treatment significantly increased the green mass of spinach under greenhouse conditions at measurement

Table 3. The measurement of spinach plant diameter under PAW treatment and different GC.

Plant diameter (cm)								
Sampling date	Measurement 1 4 April 2024		Measurement 2 19 April 2024		Measurement 3 2 May 2024		Measurement 4 16 May 2024	
PAW treatment	GC		GC		GC		GC	
	G	F	G	F	G	F	G	F
PAW 0	4.50	4.50	10.0 a	5.93 b	11.83	11.40	21.03 AB	17.5 7B
PAW 1			9.7 a	9.93 a	14.00	12.30	20.87 AB	20.8 AB
PAW 2			10.8 a	9.0 a	14.33	13.33	25.3 A	23.97 A
F_{PAW}			n.s. ($p < 0.05$, $F = 1.59$)		n.s. ($p < 0.05$, $F = 0.89$)		*($p < 0.05$, $F = 3.66$)	
F_{GC}			*($p < 0.05$, $F = 3.51$)		n.s. ($p < 0.05$, $F = 0.57$)		n.s. ($p < 0.05$, $F = 0.96$)	
Interaction								
$F_{\text{PAW}} - F_{\text{GC}}$			n.s. ($p < 0.05$, $F = 1.54$)		n.s. ($p < 0.05$, $F = 0.07$)		n.s. ($p < 0.05$, $F = 0.36$)	

PAW 1—treatment 1; PAW 2—treatment 2; PAW 0—control; G—greenhouse; F—field; F_{PAW} — F test PAW treatment; F_{GC} — F test GC; $F_{PAW} - F_{GC}$ — F test interaction.

Values between the same line followed by different lowercase letters are statistically significantly different (LSD) at $p < 0.05$; values within the same columns followed by different capital letters are statistically significantly different (LSD) at $p < 0.05$.

*Statistical significance.

GC, growing conditions; LSD, least significant differences; n.s., no statistical significance; PAW, plasma activated water.

Table 4. The plants green mass under PAW treatment and different GC.

Green mass (g)								
Sampling date	Measurement 1 4 April 2024		Measurement 2 19 April 2024		Measurement 3 2 May 2024		Measurement 4 16 May 2024	
PAW treatment	GC		GC		GC		GC	
	G	F	G	F	G	F	G	F
PAW 0	0.43	0.44	1.09	1.18	6.55 B	9.01 B	33.79 B	31.05 B
PAW 1			1.31	0.85	10.7 AB	9.05 B	43.08 AB	49.47 AB
PAW 2			1.10	1.95	17.31 A	12.6 AB	58.98 A	67.87 A
F_{PAW}			n.s. ($p < 0.05$, $F = 0.93$)		*($p < 0.05$, $F = 4.36$)		*($p < 0.05$, $F = 11.6$)	
F_{GC}			n.s. ($p < 0.05$, $F = 0.3$)		n.s. ($p < 0.05$, $F = 0.41$)		n.s. ($p < 0.05$, $F = 0.63$)	
Interaction								
$F_{\text{PAW}} - F_{\text{GC}}$			n.s. ($p < 0.05$, $F = 1.71$)		n.s. ($p < 0.05$, $F = 1.03$)		n.s. ($p < 0.05$, $F = 0.45$)	

PAW 1—treatment 1; PAW 2—treatment 2; PAW 0—control; G—greenhouse; F—field; F_{PAW} — F test PAW treatment; F_{GC} — F test GC; $F_{PAW} - F_{GC}$ — F test interaction.

*Statistical significance.

Values between the same line followed by different lowercase letters are statistically significantly different (LSD) at $p < 0.05$; values within the same columns followed by different capital letters are statistically significantly different (LSD) at $p < 0.05$.

GC, growing conditions; LSD, least significant differences; n.s., no statistical significance; PAW, plasma activated water.

3 (after the second PAW application) compared with the control. In the last measurement, PAW 2 treatment resulted in the highest green mass compared with untreated plants under both GC.

Impact of GC and PAW treatment on the dry matter of spinach are shown in Table 5. Significant differences in dry matter content after PAW treatment were only observed in the last measurement, but the influence of GC was not determined. In the last measurement, the application of PAW 2 led to a significant increase in plant dry matter under field conditions compared with the control. Plants treated with PAW 2 had a higher dry matter in the greenhouse and in the field than plants treated with PAW 1 and control plants. In the

greenhouse, treatment with PAW 1 increased dry matter compared with the control, but in the field the impact was lower, except for the last measurement.

According to the available studies, the application of PAW has a positive influence on the morphological characteristics of spinach, especially after the third application, as it showed a significant influence on plant height, plant diameter, green mass and dry matter. Since green mass and dry matter represent the amount of biomass produced by the plant, it could be assumed that the main component of PAW is RNS, which is responsible for the increase in biomass in PAW – treated plants. Nitrogen ions in the water are absorbed by the roots and increase the growth of the aerial parts of the

Table 5. The plants dry matter under PAW treatment and different GC (g).

Dry matter (g)								
Sampling date	Measurement 1 4 April 2024		Measurement 2 19 April 2024		Measurement 3 2 May 2024		Measurement 4 16 May 2024	
	GC		GC		GC		GC	
PAW treatment	G	F	G	F	G	F	G	F
PAW 0	0.12	0.12	0.10	0.13	0.89	1.03	5.91 B	4.97 B
PAW 1			0.14	0.08	1.32	0.89	6.1 AB	8.08 AB
PAW 2			0.10	0.24	1.99	1.67	7.79 AB	9.28 A
F_{PAW}			n.s. ($p < 0.05$, $F = 0.99$)		n.s. ($p < 0.05$, $F = 3.2$)		*($p < 0.05$, $F = 4.16$)	
F_{GC}			n.s. ($p < 0.05$, $F = 0.96$)		n.s. ($p < 0.05$, $F = 0.47$)		n.s. ($p < 0.05$, $F = 0.92$)	
Interaction								
$F_{\text{PAW}} - F_{\text{GC}}$			n.s. ($p < 0.05$, $F = 2.05$)		n.s. ($p < 0.05$, $F = 0.34$)		n.s. ($p < 0.05$, $F = 1.06$)	

PAW 1—treatment 1; PAW 2—treatment 2; PAW 0—control; G—greenhouse; F—field; F_{PAW} — F test PAW treatment; F_{GC} — F test GC;

$F_{PAW} - F_{GC}$ — F test interaction.

*Statistical significance.

Values between the same line followed by different lowercase letters are statistically significantly different (LSD) at $p < 0.05$; values within the same columns followed by different capital letters are statistically significantly different (LSD) at $p < 0.05$.

GC, growing conditions; LSD, least significant differences; n.s., no statistical significance; PAW, plasma activated water.

plant as they serve as a nutrient (Thirumdas et al., 2018). RNS promotes the formation of active photosynthetic pigments by increasing the amounts of stromal and thylakoid proteins in the leaves and increasing the formation of chloroplasts, which is important for the growth and green mass of the leaves (Abbaszadeh et al., 2021; Than et al., 2022). Positive effects of PAW on plant growth have been observed by other researchers in different vegetable varieties (Thirumdas et al., 2018; Attri et al., 2020). It was observed that PAW treatment had an impact on plant growth and development over a longer measurement period, especially at higher PAW doses. Similar results were obtained by the authors Ji et al. (2016) who increased germination, growth of spinach seedlings and content of chlorophyll and total phenols after treatment with micro Dielectric Barrier Discharge (DBD) plasma. The influence of GC is only significant for plant height at the last measurement for plants grown under field conditions with PAW application. Regardless of the fact that there are no statistically significant differences between GC, PAW appears to be more effective in controlled environments, potentially due to stable microclimatic conditions. The application of PAW 2 increased spinach green mass by 46% (field) and 57% (greenhouse) and dry matter increased by 53% (field) and 75% (greenhouse). Since the limited number of papers on the use of plasma technology in the pre-harvest process of plasma agriculture (Randeniya and De Groot, 2015; Ito et al., 2018; Puac et al., 2018) are still in the initial stages of development, this paper attempted to evaluate the possibility of using PAW under field conditions.

Results of chemical analysis of plant material

The results of the composition of the macro- and microelements of the plant material grown in the

greenhouse and in the field are shown in Table 6. The results of the chemical analyses of micro and macro elements under greenhouse conditions (Table 6) show the influence of PAW on a higher content of dry matter and N, P and K. Only the application of a lower dose of PAW increased the content of Ca and Mg compared with the control. For trace elements, the application of PAW under greenhouse conditions increased the Fe content but decreased the Cu content compared with the plants that were not treated with PAW.

In spinach samples grown outdoors, the amounts of macro- and microelements are lower than in plant samples grown in a protected area. The content of all macroelements is slightly higher in the samples treated with PAW compared with the untreated variants. For the microelements, higher Zn values were found when a larger amount of PAW was applied compared with the control and PAW 1.

Results of measurement of plant bioactive compounds in spinach

In crop production, PAW is a promising sustainable technology to improve seed germination, plant growth and tolerance to biotic and abiotic stress factors (Gao et al., 2022), but its influence on the production of active compounds in growing plants is less known. Some positive effects of PAW application on many bioactive compounds in plants and food have been observed (Brisset and Pawlat, 2016; Ji et al., 2016; Ito et al., 2018; Puac et al., 2018; Vaka et al., 2019; Oliveira et al., 2022; Than et al., 2022; Pipliya et al., 2023), but only under controlled conditions and less in agricultural practice in the field. Therefore, in this study, addition to the evaluation of PAW application on the growth and development of spinach, the content of bioactive substances, which could serve as a protective mechanism

against stress conditions, was also determined. In the work of Bergquist (2006), it is mentioned that increasing the concentration of bioactive compounds in fruits and vegetables may have not only a positive health effect, but may also extend the shelf life and increase stress tolerance. The evaluation of the measurements of the total phenols, phenolic acids, antioxidant activity, flavonoids and proline under field and greenhouse conditions are shown in Table 7.

The results show that no statistically significant difference was found between the PAW treatment and the control in all parameters measured under field conditions in the spinach samples. Although the differences are insignificant, it was observed that the application of a lower amount of PAW (PAW 1) increased the content of total polyphenols, phenolic acids, antioxidant activity and flavonoids compared with the application of PAW 2 and the control. The results of the measurements of phenols and flavonoids in plant samples grown in a protected area show that the measured values are slightly lower compared with those obtained under field conditions, which could be related to the protective role of PAW. As reported in other studies, phenolic compounds play an important role in plants and are widely distributed. They act as components of supporting and protective tissues,

they are defence signals, participate in reproduction, attract pollinators as attractants and protect the plant from ultraviolet radiation, but are also involved in the interaction of the plant with the environment.

There are also no statistically significant differences in the measured values between PAW – treated and untreated plants, but it was found that the treated plants had higher values than the untreated plants. Higher levels of total phenols, phenolic acids and flavonoids were measured in PAW 2 compared with PAW 1 treatment and the control, while a higher proline content was obtained in PAW 1 treatment.

Plant and dietary proteins are considered to be rich sources of bioactive peptides, which are classified based on their biological effects, for example, antithrombotic, antimicrobial, antihypertensive, opioid, immunomodulatory and antioxidant (Meena et al., 2019; Marinaccio et al., 2023). Proline (Pro) is a central and multifunctional amino acid that may play an important role not only in plant developmental processes but also in responses to biotic and abiotic stress factors. In agreement with other reports (Meena et al., 2019), the role of Pro metabolism enzymes under environmental stress conditions in plants was determined. In terms of proline content, no significant differences were found

Table 6. Results of chemical properties of plant material grown in greenhouse and field conditions ($\text{mg} \cdot 100 \text{ g}^{-1}$ plant).

Treatment	d.m.%	N	P ₂ O ₅	P	K ₂ O	K	Ca	Mg	Fe	Zn	Mn	Cu
PAW 0 G	15.50	347	130	57.0	561	465	280	122	2.28	1.50	0.66	0.152
PAW 1 G	17.24	429	221	96.4	719	597	291	131	3.10	2.02	0.70	0.133
PAW 2 G	16.94	406	202	88.0	924	767	264	115	4.08	1.64	0.67	0.124
PAW 0 F	14.37	302	108	41.5	565	496	190	94	3.56	1.39	0.46	0.089
PAW 1 F	15.68	312	187	52.3	628	521	217	116	3.51	1.46	0.72	0.125
PAW 2 F	16.33	286	120	47.1	743	617	232	111	3.36	1.83	0.58	0.109

PAW 0 G—control in greenhouse; PAW 1 G—PAW 1 greenhouse; PAW 2 G—PAW 2 in greenhouse; PAW 0 F—control in field; PAW 1 F—PAW 1 application if field; PAW 2 F—PAW 2 application in field; d.m.—dry matter.
PAW, plasma activated water.

Table 7. Content of total phenols, phenolic acids, antioxidant properties, flavonoids and proline in spinach samples grown in field and greenhouse conditions.

	Total phenols $\text{mg GAE} \cdot \text{g}^{-1} \text{ d.w.}$	Phenolic $\text{mg CAE} \cdot \text{g}^{-1} \text{ d.w.}$	Antioxidant activity $\mu\text{mol TE} \cdot \text{g}^{-1} \text{ d.w.}$	Flavonoids mg $\text{CE} \cdot \text{g}^{-1} \text{ d.w.}$	Prolines μmol proline $\cdot \text{g}^{-1} \text{ d.w.}$
PAW 0 F	2.105 ± 0.175	0.668 ± 0.109	3.681 ± 0.929	0.493 ± 0.023	0.13 ± 0.025
PAW 1 F	2.357 ± 0.49	0.731 ± 0.07	4.649 ± 1.432	0.524 ± 0.129	0.298 ± 0.083
PAW 2 F	2.110 ± 0.476	0.747 ± 0.144	3.984 ± 0.392	0.493 ± 0.11	0.437 ± 0.259
PAW 0 G	1.379 ± 0.165	0.453 ± 0.036	2.228 ± 0.458	0.251 ± 0.036	0.229 ± 0.108
PAW 1 G	1.455 ± 0.33	0.428 ± 0.069	2.668 ± 0.663	0.284 ± 0.03	0.458 ± 0.082
PAW 2 G	1.617 ± 0.391	0.503 ± 0.078	3.237 ± 1.189	0.313 ± 0.074	0.380 ± 0.165

Values of measured parameters are given as means $\pm$ STDEV of three biological replicates.

PAW 0 G—control in greenhouse; PAW 1 G—PAW 1 greenhouse; PAW 2 G—PAW 2 in greenhouse; PAW 0 F—control in field; PAW 1 F—PAW 1 application if field; PAW 2 F—PAW 2 application in field values of measured parameters are given as means $\pm$ STDEV of three biological replicates.

PAW, plasma activated water.

either, but the application of PAW 2 resulted in a higher proline content compared with plants not treated with PAW or with a lower amount (PAW 1). In this study, proline content was higher when a higher dose of PAW was applied under field conditions, while a lower dose of PAW increased proline content in plants grown in the greenhouse. Reports on the stimulation of proline synthesis and accumulation by the application of nitrogen could be partially confirmed in this study, as the application of a higher PAW dose resulted in a lower N content in the plants under field conditions. Lower PAW doses increased the proline content only under greenhouse conditions, while higher PAW doses increased the proline content under field conditions. As observed in other studies, plant species respond differently to the application of PAW (Holubova et al., 2020.) and also to different doses of PAW. The results of this study showed that the application of PAW to spinach plants increased the content of total polyphenols, phenolic acids, antioxidant activity and flavonoids, especially under field conditions. A lower dose of PAW increased the content of phenolic compounds in the field and a higher dose of PAW increased their content in the greenhouse. Although no statistically significant effect was found, a positive impact of PAW treatment was observed. The increase of most bioactive compounds, particularly in open-field conditions, could presume the possibility for enhanced stress tolerance and physiological activity. Since research on the application of PAW in agriculture through irrigation is still in its infancy and therefore there is not enough solid evidence for a direct comparison, it was suggested that PAW could be used in spinach cultivation even under unpredictable field conditions.

CONCLUSIONS

This study suggests that the application of PAW in spinach cultivation is a useful method for plant growth and the accumulation of bioactive compounds that make spinach a healthy food source. PAW treatment improved all measured growth parameters, but future studies should be conducted on different spinach varieties and during several growing seasons. In this study, the higher PAW dose increased all morphological characteristics of spinach under both growth conditions and bioactive compounds (except prolines) under greenhouse conditions. In addition, studies need to be conducted for individual vegetable crops to determine the most appropriate amount and composition of PAW for the production of primary and secondary metabolites for the plants. For future research, it is recommended to test different PAW varieties, dosages and plant cultivars to obtain reliable results on the amount and composition of PAW suitable for vegetable crops. The application of an appropriate amount and concentration of PAW to plants could achieve the effect of fertilisation and activation of bioactive plant compounds to protect plants from biotic and abiotic factors.

FUNDING

This work was supported by the project 'Green Vegetables for Green Deal' ZEPzaZEP. The project was financed by the Fund for Environmental Protection and Energy Efficiency, Republic of Croatia.

AUTHOR CONTRIBUTIONS

T.B.L. and M.B. conceptualisation. M.B., N.R.F., T.B.L. and K.M. methodology. M.B., N.R.F., R.B., L.J.B.O., T.B.L. and D.Z. investigation. M.B. resources. R.B. and I.K. data processing. M.B. writing-original draft preparation. K.M. and S.A. writing-review and editing. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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