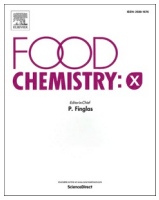
Food Chemistry: X 22 (2024) 101345

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



The pH acidity and nitrate accumulation by plasma discharge enhanced the growth and phytochemicals of soybean sprouts grown in reused water

Jong-Seok Song [a,b,*], Sunkyung Jung [a]

[a] Institute of Plasma Technology, Korea Institute of Fusion Energy, 37 Dongjangsan-ro, Gunsan, 54004, Republic of Korea
[b] Department of Plasma and Nuclear Fusion, UST-KFE School, 169-148 Gwahak-ro, Yuseong-gu, Daejeon 34133, Republic of Korea

ARTICLE INFO

Keywords:
Acidity
Nitrogen
Plasma
Reuse
Sprout

ABSTRACT

This study investigated the effect of plasma treatment on reused water and evaluated the interactions of the plasma-treated water (PTW) with plants or microbes to determine the optimal PTW for reuse. The repeated treatment gradually accumulated nitrate (NO_3^-) in the PTW and lowered its pH; afterward, it led to the sprouted soybeans accumulating other inorganic ions in the PTW. The biomass of soybean sprouts was enhanced by the accumulated NO_3^- but decreased due to the pH effect. Meanwhile, the acidic pH reduced the microbial counts, but they increased after sprinkling the PTW over the sprouts. The optimal PTW in our study, which had a gradual increase of NO_3^- ($\leq$321.8 mg·L^{-1}) with an acceptable pH ($\geq$pH 3), significantly enhanced the biomass by 4.2% compared to the untreated control. Additionally, it increased the total content of amino acids and isoflavones by 9% and 18% in the growing part, respectively.

1. Introduction

Sprouting, the germination stage of a seed, is a simple and economical strategy for producers to produce phytonutrient-rich plant foods. In particular, soybean is popularly consumed as a sprout product because of its nutritional benefits, such as a higher content of amino acids and isoflavones. Soybean sprout ranked first among 16 common sprout foods in terms of total contents of amino acids and isoflavones (Moreno, Pérez-Balibrea, & García-Viguera, 2006). Generally, soybean sprouts are produced on trays by sprinkling seeds with fresh water for 3 to 7 days under controlled environments. Many factors, such as biological (e.g., microbes in water) and environmental factors (e.g., water quality) are well known to affect the sprouting process (Wang, Zhang, Jiang, Cao, & Jiang, 2022a). For example, the water for sprouting is the most critical factor for healthy soybean sprouts (Choi, 2019; Yun, 2003) and their phytochemical products (Hwang, 2012; Kim, Yoon, Kim, Dhungana, & Shin, 2020). Thus, new methods based on the water are worth investigating for better sprout production.

Plasma-treated water (PTW) could be used as a disinfectant or fertilizer for food and agricultural products, as addressed by research groups in Korea (Choi et al., 2022; Yoo, 2015), Japan (Attri, Ishikawa, Okumura, Koga, & Shiratani, 2020; Ito, Oh, Ohta, & Shiratani, 2018), Germany (Weltmann et al., 2019), and the United States (Fridman, 2008). It is simple to produce by exposing water to a high electric discharge in air for a given treatment time; this technique enables gaseous active species formed in the discharge to dissolve in the water (Brisset & Pawlat, 2016; Song, Kim, Ryu, Oh & Kim, 2020a). Therefore, PTW chemically has different compositions under different treatment conditions, depending on the device types and treatment time (Ranieri et al., 2021; Tachibana & Nakamura, 2019; Zhou et al., 2020). For example, dielectric barrier discharge (DBD) is commonly used for broad applications due to its simple structure and ease of use. Hydrogen peroxide, as a disinfectant, in PTW increases slowly over treatment time when the DBD is operated in direct contact with water (Hong, Ma, Kim, & Shin, 2019; Tian et al., 2015). On the other hand, nitrate fertilizer is mainly formed in PTW after applying DBD on water; however, it spontaneously contributes to its low pH (Ka et al., 2021; Lee et al., 2023; Lee, Lim, Hong, & Kim, 2020; Ryu & Park, 2009; Song, Lee, et al., 2020b; Song et al., 2021). Given that PTW has different uses, finding the optimal PTW needs to be investigated under practical user conditions. There are many operating factors affecting the PTW, but in particular, the constituents and pH of the PTW can be easily adjusted by controlling the length of water treatment time.

Many studies have focused on the positive impact of PTW on the growth and quality of sprouted soybeans in terms of controlling the microbes (Lee, Khan, Shim, & Kim, 2018; Mošovská et al., 2022; Wang,

* Corresponding author at: Institute of Plasma Technology, Korea Institute of Fusion Energy, 37 Dongjangsan-ro, Gunsan, 54004, Republic of Korea
E-mail address: jongseoksong@kfe.re.kr (J.-S. Song).

https://doi.org/10.1016/j.fochx.2024.101345
Received 16 August 2023; Received in revised form 26 March 2024; Accepted 31 March 2024
Available online 2 April 2024

Zhang, Jiang, et al., 2022a) or supplying a nitrogen source (Guragain et al., 2021; Ji et al., 2022; Porto et al., 2018). The PTW reduces the amount of microbes affecting food safety by up to 6 log(CFU·mL^{-1}) and has various effects on growing sprouts (Wang, Zhang, Jiang, et al., 2022a). However, the optimal PTW for cultivating sprouted soybeans in practice has not been reported to the best of our knowledge. As the main factor of PTW, nitrate enhances the growth and phytochemicals of sprouted soybeans. Conversely, the acidity of the PTW reduces them due to cellular damage to the sprouts. The various interactions of PTW with plants and microbes should be investigated to determine the optimal PTW for the practical reuse of water. Therefore, this study investigated the effect of plasma treatment on reused water and determined the relationships between PTW and microbes and the plant growth and phytochemical composition of soybean sprouts.

2. Materials and methods

2.1. Plant materials

The seeds from Korean soybeans (*Glycine max* cv. Haepum) were harvested in October 2018 from an experimental field at the National Institute of Crop Science, Rural Development Administration, Korea. The harvested seeds were stored in a temperature-controlled chamber maintained at 4 °C. Before the treatment, they were uniformly placed as a single layer into a plastic tray (800 seeds per tray according to the manufacturer's recommendations).

2.2. Water treatment and growing condition

Growth experiments were performed with six commercial seed sprouters (Chungsiru SC-9000TS, Shinchang INC, Osan, Korea), each equipped with a 9-L plasma treatment chamber (**Fig. S1**). Two liters of deionized water inside the chambers were either untreated as the control or treated with four different treatment times from 5 to 30 min at a distance of about 10 cm from 2 surface DBD electrodes connected to a power supply unit at the top of the lid. The DBD electrodes generated a high electric discharge at an average power of 60 W with a driving frequency of 18 kHz and a peak-to-peak voltage of 6 kVp-p, as previously described by Song, Lee, et al. (2020b) and Song et al. (2021).

The untreated deionized water or PTW was transferred into a water container of the sprouter from the chamber and sprinkled onto the soybean seeds for 30 s at intervals of 30 min. After sprinkling for 24 h, the used water was transferred back to the chamber and either was not treated or treated again with the same plasma treatment time. The PTW inside the chambers was transferred again into the sprouter and sprinkled onto the soybean. The untreated water transferred back into the container was either reused as the negative control or replaced daily with fresh deionized water as the positive control. Up to 4 days after sowing, the soybean sprouts were grown at room temperature in the dark under the different water treatments.

2.3. Physiochemical analysis of the water

The water was collected from the sprouter before and after the plasma treatment at different times daily and analyzed for its physicochemical characteristics, including the temperature, pH, and inorganic ions present. In this study, hydrogen peroxide was not detected, probably because of its short lifetime, as previously reported by Lim, Byeon, Hong, Ryu, and Kim (2021). The water temperature and pH were measured using a benchtop meter equipped with a ROSS Ultra pH/ATC Triode (Orion 8302BNUMD, Thermo Scientific, USA). Then, 2 mL of the water were analyzed for the concentration of anions (e.g., NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, and Cl^-) with ion chromatography (Dionex ICS-2100, Thermo, USA) using an IonPac AG25 column (4 × 50 mm) and ASRS-300 (4 mm) suppressor (Song, Lee, et al., 2020b). Another 2 mL of the water were analyzed for the concentration of the cations (e.g., Mg^{2+}, K^+, Ca^{2+}, Na^+, and NH_4^+) with ion chromatography (Dionex ICS-1600, Thermo, USA) using an IonPac CG12A column (4 × 50 mm) and CSRS-300 (4 mm) suppressor (Song et al., 2021).

2.4. Analysis of the microbial counts

The water was also analyzed for the concentration of bacteria and fungi before and after the plasma treatment at different times daily. The concentration of bacteria and fungi was measured by plating on plate count agar and potato dextrose agar, respectively. Two milliliters of the water were sampled from each of the six sprouters. One milliliter of the water was serially diluted 10-fold and poured onto each agar plate. The plate for bacteria was grown for 48 h at 35 °C, and the fungal culture was grown for 96 h at 25 °C.

2.5. Analysis of the growth characteristics

Soybean sprouts from each sprouter were harvested on day 4 and weighed for total biomass. The total biomass was defined as the weight of 800 sprouts. Then, their dry weight was determined after drying at 90 °C in an oven for 3 days.

2.6. Analysis of the free amino acids

Soybean sprouts harvested from each sprouter were lyophilized at a temperature below −70 °C and divided into cotyledon and hypocotyl plus root parts. Each part was pooled and hydrolyzed with 6.0 M hydrochloric acid (HCl) at 110 °C for 22 h and dried at 50 °C under vacuum in a rotary evaporator. After drying, the powder was dissolved in 0.02 M HCl and filtered through a 0.45-μm syringe filter (Whatman) before analysis. The analysis of free amino acids was performed using an amino acid analyzer (Hitachi L-8900, Hitachi High-Technologies Co., Japan). The other procedures used were previously described by Song et al. (2021).

2.7. Analysis of isoflavones

The whole plants of the sprouted soybeans were lyophilized at a temperature below −70 °C and ground to a powder. For each sample, a 1-g aliquot was dissolved into 5 mL of 70% aqueous ethanol (v/v) containing 0.1% acetic acid (v/v) and then extracted using an orbital shaker for 6 h at room temperature. The extract was centrifuged at 8000 rpm for 10 min, and the supernatant was filtered through a 0.45-μm syringe filter before analysis. Isoflavone analysis was performed by HPLC (Agilent Technologies 1260, USA) equipped with a diode array detector. The used column was an Inertsil ODS-SP column (4.6 × 250 mm, 5-μm, GL Sciences), and the column temperature was 35 °C. The mobile phase was a gradient of 0.1% acetic acid in deionized water (A) and 0.1% acetic acid in acetonitrile (B): 15% B (0–8 min), 35% B (8–42 min), and 35% B (42–47 min). The flow rate of the mobile phase was 1.5 mL·min^{-1}, and the absorbance of the isoflavones was measured at 254 nm. A calibration curve method was used to quantify the 12 isoflavones in the sample. The isoflavone standards (Sigma-Aldrich, USA) were prepared in DMSO (dimethyl sulfoxide). They included malonylglucosides (malonyldaidzin, malonylgenistin, and malonylglycitin), acetylglucosides (acetyldaidzin, acetylgenistin, and acetylglycitin), β-glucosides (daidzin, genistin, and glycitin), and aglycones (daidzein, genistein, and glycitein). The calibration curve of the isoflavones showed good linearity ($R^2 > 0.999$).

2.8. Statistical analyses

The growth experiments were performed three times in a temperature-controlled laboratory maintained at 25 °C for over 2 years. All the data were initially subjected to analysis of variance, and a mean comparison was made by Tukey's HSD (honestly significant difference)

test at $P = 0.05$. For the used water, physicochemical and microbial data were analyzed with sampling dates in each treatment as the main factor and the replicate as a random factor. For soybean sprouts, growth and phytochemical data were analyzed with water treatments at different times as the main factor and the replicate as a random factor. All the statistical analyses were done with the 'agricolae' package in the R statistical program (Version 3.6.2, R core team, 2019).

3. Results and discussion

3.1. Effect of the plasma treatment on the reused water

The water inside the chamber became warmer and more acidic immediately after the air discharge within 5 to 30 min compared to the untreated control. The temperature and pH value of the PTW were 23.7 °C and pH 3.8, 24.7 °C and pH 3.2, 27.3 °C and pH 2.9, and 30.9 °C and pH 2.6 for the discharge of 5, 10, 20, and 30 min, respectively, while that of the untreated water was 23.1 °C and pH 6.5 (Fig. 1 **and Fig. S2**). In addition, the NO_3^- was abundant in the PTW due to the first discharge to the water. The initial NO_3^- concentration was 14.8 mg·L^{-1}, 45.1 mg·L^{-1}, 97.5 mg·L^{-1}, and 165.9 mg·L^{-1} for the discharge of 5, 10, 20, and 30 min, respectively (Fig. 2). After sprinkling for 24 h, the PTW was cooled down to room temperature at 23.5 °C (**Fig. S2**) and returned to a near neutral pH compared with the acidic water measured after the first discharge (Fig. 1). The pH value of the PTW was pH 7.2, pH 7.2, pH 6.7, and pH 5.4 within 24 h after the discharge for 5, 10, 20, and 30 min, respectively, while that of the untreated water was pH 7.2 (Fig. 1). The pH recovery of the PTW was accompanied by NO_3^- reduction and other inorganic accumulations in the water. The NO_3^- concentration was 0.1, 14.8, 78.0, and 169.9 mg·L^{-1} within 24 h after the discharge for 5, 10, 20, and 30 min, respectively (Fig. 2). The concentrations of other anions such as NO_2^-, SO_4^{2-}, PO_4^{3-}, and Cl^- in the PTW were <10 mg·L^{-1} (**Fig. S3**), while the total cation concentration was 55.8, 60.7, 89.2, and 116.4 mg·L^{-1} within 24 h after the discharge for 5, 10, 20, and 30 min, respectively (**Fig. S4**). In the PTW, the K^+ accounted for approximately 89% of the total cation concentration and other cations such as Na^+, Mg^{2+}, Ca^{2+}, and NH_4^+ for <11%.

The PTW was again heated up and acidified after the discharge for the same range of treatment times each day and compared with the water measured before the discharge. The temperature of the PTW was 24.6 to 31.3 °C, 25.3 to 33.2 °C, 25.7 to 34.2 °C, and 26.3 to 35.3 °C for 5 to 30 min of the repeated discharge on day 1, day 2, day 3, and day 4, respectively; thereafter, all the PTW was cooled down to room temperature between 23.9 and 24.5 °C within 24 h after the discharge. The pH value of the PTW was pH 7.0 to pH 2.6, pH 6.5 to pH 2.5, pH 4.7 to pH 2.4, and pH 4.3 to pH 2.4 for 5 to 30 min of the discharge on day 1, day 2, day 3, and day 4, respectively; however, it increased again within 24 h after the discharge (Fig. 1). The repeated discharge finally resulted

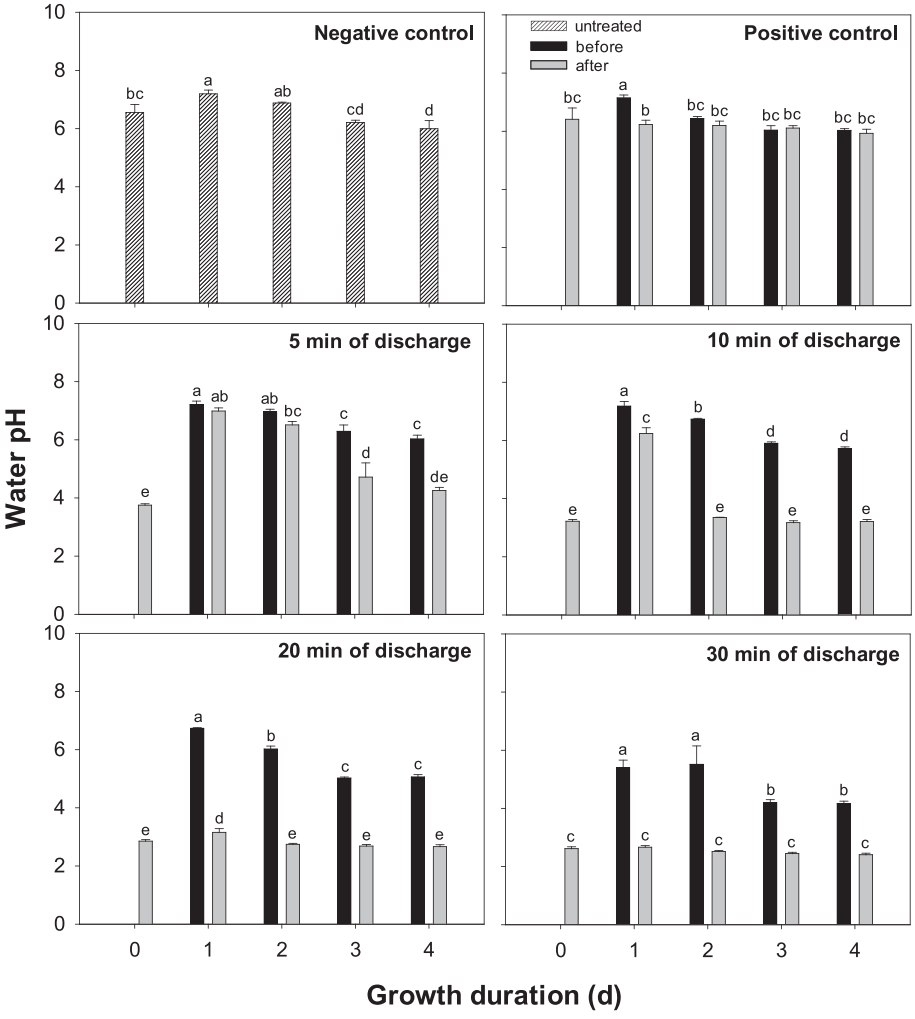


Fig. 1. pH changes in the used water, each treated daily with the plasma discharge for 0 to 30 min and afterward sprinkled for up to 4 days. The untreated water was either reused as the negative control or replaced daily with fresh deionized water as the positive control. For the used water, the pH data were analyzed with the sampling dates in each treatment. Each bar represents the mean of three replicates. Means with the same letter are not significantly different by Tukey's HSD (honestly significant difference) test at $P = 0.05$.

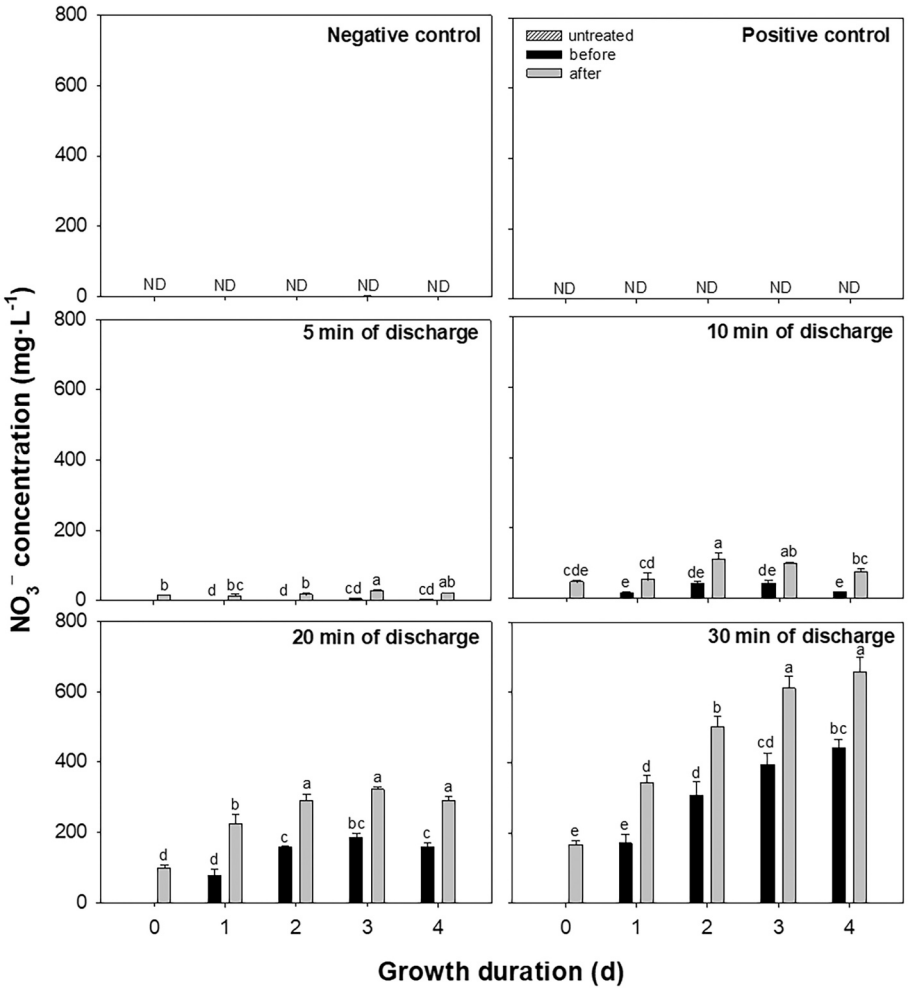


Fig. 2. The changes of nitrate (NO_3^-) concentration in the used water, each treated daily with the plasma discharge for 0 to 30 min and afterward sprinkled for up to 4 days. The untreated water was either reused as the negative control or replaced daily with fresh deionized water as the positive control. For the used water, nitrate data were analyzed with the sampling dates in each treatment. Each bar represents the mean of three replicates. Means with the same letter are not significantly different by Tukey's HSD (honestly significant difference) test at $P = 0.05$. ND indicates that the nitrate was not detected.

in the accumulation of NO_3^- in the PTW; additionally, for the sprouted soybeans, it caused the accumulation of other inorganic ions in the PTW against the acidic pH environment. The NO_3^- concentration of the PTW was 15.3 to 342.9 mg·L^{-1}, 16.8 to 501.4 mg·L^{-1}, 26.9 to 610.9 mg·L^{-1}, and 19.7 to 656.8 mg·L^{-1} for 5 to 30 min of the repeated discharge on day 1, day 2, day 3, and day 4, respectively (Fig. 2). The total cation concentration was 55.8 to 116.4 mg·L^{-1}, 35.6 to 151.2 mg·L^{-1}, 19.7 to 161.5 mg·L^{-1}, and 12.5 to 162.0 mg·L^{-1} in the PTW treated daily with the discharge for 5 to 30 min over 4 days, respectively (**Fig. S4**).

As previously reported by our works (Ka et al., 2021; Lee et al., 2020; Lee et al., 2023; Ryu & Park, 2009; Song et al., 2021; Song, Lee, et al., 2020b), pH acidity and NO_3^- accumulation in the PTW were the main effects of applying DBD to the water. These effects subsequently led to a large accumulation of cations in the PTW (**Fig. S4**); the reason was that the sprinkling of the acidic PTW caused oxidative damage to the growing sprouts. Still, the sprouts would grow again because of the accumulated NO_3^-, while microbes in the PTW would be decreased due to the pH effect. Thus, the optimal PTW should be determined considering sprout safety and microbial control under NO_3^- abundant but acidic conditions.

3.2. Effect of the plasma-treated water on the microbial counts

The aerobic bacterial counts present in the PTW were immediately reduced after the first discharge for 5 to 30 min compared to the untreated control due to the acidity of the water. The concentration of total aerobic bacteria in the PTW was 2.6, 2.6, 1.5, and 1.2 log(CFU·mL^{-1}) when the pH of the PTW was 3.8, 3.2, 2.9, and 2.6 after discharge for 5, 10, 20, and 30 min, respectively (Fig. 1 **and Fig. S5**). In comparison, the concentration in the untreated water was 3.6 log(CFU·mL^{-1}). After sprinkling for 24 h, the bacteria in the PTW grew again to a level of >6 log(CFU·mL^{-1}) except for the water treatment for 30 min (**Fig. S5**). The pH of the PTW was 7.2, 7.2, 6.7, and 5.4 within 24 h after the first discharge for 5, 10, 20, and 30 min, respectively, while the pH value of the untreated water was pH 7.2 (Fig. 1). The bacterial counts in the PTW were again reduced due to the acidic pH immediately after the repeated discharge for the same range of treatment times each day compared with the bacteria measured before the discharge. The reduction of total aerobic bacteria was 48%, 56%, 58%, and 63% at pH 2.7, pH 2.5, pH 2.4, and pH 2.4 in the PTW treated with the discharge for 30 min on day 1, day 2, day 3, and day 4, respectively; in the PTW treated with the discharge for 20 min, it was 37%, 48%, 64%, and 73% at pH 3.2, pH 2.7, pH 2.7, and pH 2.7 on day 1, day 2, day 3, and day 4 (Fig. 1 **and Fig. S5**). Thus, total aerobic bacteria were significantly affected by the change of the water pH after the discharge for 20 to 30 min (**Fig. S5**; $P < 0.05$). The bacterial counts in the PTW were decreased more due to a lower acidic pH immediately after the discharge each day. However, under neutral or slightly acidic conditions, the bacterial counts in the PTW were not

reduced despite the repeated discharge. The reduction of total aerobic bacteria was not observed for the first two days in the PTW treated daily with the discharge for 5 or 10 min. The counts thereafter were decreased by approximately 25% and 12% at nearly pH 4.5 after the discharge for 5 min on day 3 and day 4, respectively; on the same days, they were decreased by 28% and 31% at pH 3.2 after the discharge for 10 min (Fig. 1 **and Fig. S5**). Still, they increased again to >6 log(CFU·mL^{-1}) as the PTW returned to a near neutral pH due to a cation leakage from growing sprouts into the acidic PTW (**Fig. S5**). Previous studies on antimicrobial activity of PTW revealed that plasma-induced acidification to below pH 4 could effectively inactivate the bacteria when they were suspended in a non-buffered solution (Ikawa, Kitano, & Hamaguchi, 2010; Kawakami, Aihara, Izumi, Shirai, & Mukai, 2022). The critical pH for bacterial inactivation could be related to pH homeostasis because bacteria have a defense system to maintain a stable intracellular pH (Oehmigen et al., 2010).

The counts for the yeast and mold in the PTW were only slightly decreased due to the acidic pH caused by the first discharge for 5 to 30 min compared to the untreated control. The concentration of yeast and mold in the PTW was 1.6, 1.3, 1.2, and 0.3 log(CFU·mL^{-1}) when the pH value of the PTW was pH 3.8, pH 3.2, pH 2.9, and pH 2.6 after discharge for 5, 10, 20, and 30 min, respectively, while the concentration in the untreated water was 2.2 log(CFU·mL^{-1}) (Fig. 1 **and Fig. S6**). However, after a gradual increase to >4 log(CFU·mL^{-1}) within 24 h, the yeast and mold in the PTW appeared not significantly affected despite the pH change after repeated discharge. There was little or no significant difference between their counts measured before and after the discharge in the PTW (**Fig. S6**; $P > 0.05$). The low or little efficacy of the PTW against yeast and mold can be attributed to the thick wall of fungal cells, in addition to the organic materials which resulted from the sprouted soybeans after sprinkling with the PTW. A literature review indicated that organic materials react with the PTW, which provides a physical barrier to microbial cells (Zhao, Patange, Sun, & Tiwari, 2020). The greater resistance exhibited by fungi against the PTW is probably related to the fact that the fungal cell has a nuclear membrane surrounding the nucleus, which offers a further barrier against DNA damage compared to the bacterial cell (Klämpfl et al., 2012).

3.3. Effect of the plasma-treated water on the growth of soybean sprouts

Soybean sprouts sprinkled with the PTW were more enhanced due to the NO_3^- accumulation in the water during the discharge except for the 30 min treatment compared to the untreated control. The biomass of the soybean sprouts sprinkled with the PTW was 535.3, 547.9, 543.4, and 475.6 g when the PTW was used for the sprinkling and after that treated daily with the discharge for 5, 10, 20, and 30 min, respectively (Fig. 3). The biomass of the sprouts sprinkled with the untreated water was 525.9 or 506.5 g when the untreated water was either reused as the negative control or daily replaced with fresh deionized water as the positive control, respectively (Fig. 3). The biomass difference in the untreated controls was attributed to the loss of nutrients by the water replacement, so the effect of the PTW on the sprouts was compared with the reused water as the negative control. The biomass of the soybean sprouts sprinkled with the PTW was enhanced as follows: equivalent to approximately a 1.8%, 4.2%, and 3.3% increase at a NO_3^- accumulation up to 26.9, 110.4, and 321.8 mg·L^{-1} in the PTW treated daily with the discharge for 5, 10, and 20 min, respectively, compared with the biomass of the negative control (Figs. 2, 3, and 4). However, the biomass of the soybean sprouts sprinkled with the PTW was reduced to 90.4% of the negative control, although the NO_3^- accumulation increased up to 656.8 mg·L^{-1} in the PTW treated daily with the discharge for 30 min (Fig. 4). The reduced biomass might be mainly attributed to a strong acidity accompanied by the excessive accumulation of NO_3^- in the PTW (Figs. 1 and 2). The acidity below pH 3 in the PTW, together with excess NO_3^-, significantly inhibited the seedling growth, showing severe damage on the root (Figs. 1 and 4; $P < 0.05$). There was a significant leakage of cations into the PTW due to the cellular damage in the root ($P < 0.05$), while anions except for NO_3^- were not significantly affected (**Fig. S3 and Fig. S4**). After the soybean sprouts were dried thoroughly, their dry weights were as follows: in the PTW treated daily with the discharge for 5, 10, 20, and 30 min, respectively, equivalent to approximately 1.1%, 3.0%, 3.0%, and 1.9% increase compared with the dry weight in the untreated control, 63.4 g (Figs. 2 and 3). In the case of PTW discharged for 30 min daily, the dry weight of sprouts was high, but their biomass was lower compared to the untreated control, which might be due to a sudden water loss from damaged cells despite the greater rate of nitrogen assimilation in the sprouts.

Our results demonstrated that NO_3^- accumulation in the PTW, although maintained at acidic pH, can positively affect the growth of soybean sprouts. The biomass of the soybean sprouts increased by 4.2% at a NO_3^- accumulation of up to 110.4 mg·L^{-1} in the acidic water and after that, gradually decreased due to a further acidic pH (Figs. 1, 2, and 3). Similar results were previously reported in a literature review for the optimal PTW (Priatama, Pervitasari, Park, Park, & Lee, 2022; Song, Kim et al., 2020a). Thus, the optimal PTW required acceptable levels for pH acidity and NO_3^- accumulation to show positive effects on the sprouts compared with the untreated water, and should be treated with plasma discharge for the appropriate amount of time. That is, 10 to 20 min of the discharge was the critical time for the reused water for the soybean sprouts to increase the NO_3^- accumulation by 110.4 to 321.8 mg·L^{-1} with a pH of 3 or higher in the PTW in 4 days.

3.4. Effect of the plasma-treated water on the amino acids and isoflavones of the soybean sprouts

Total amino acids were enhanced in the growing part of the sprouts due to the NO_3^- consumption in the PTW. Sprinkling with the PTW significantly increased the total amino acid contents in the hypocotyl

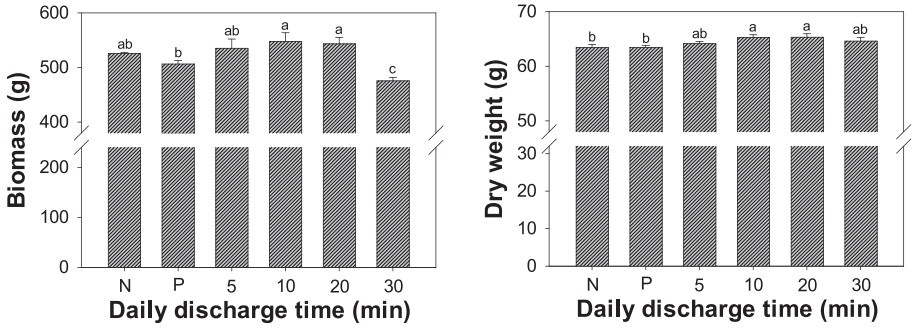


Fig. 3. Biomass and dry weight of the soybean sprouts, each sprinkled for up to 4 days with the water treated daily with the plasma discharge for 0 to 30 min. The untreated water was either reused as the negative control (N) or replaced daily with fresh deionized water as the positive control (P). Each bar represents the mean of three replicates. Means with the same letter are not significantly different by Tukey's HSD (honestly significant difference) test at $P = 0.05$.

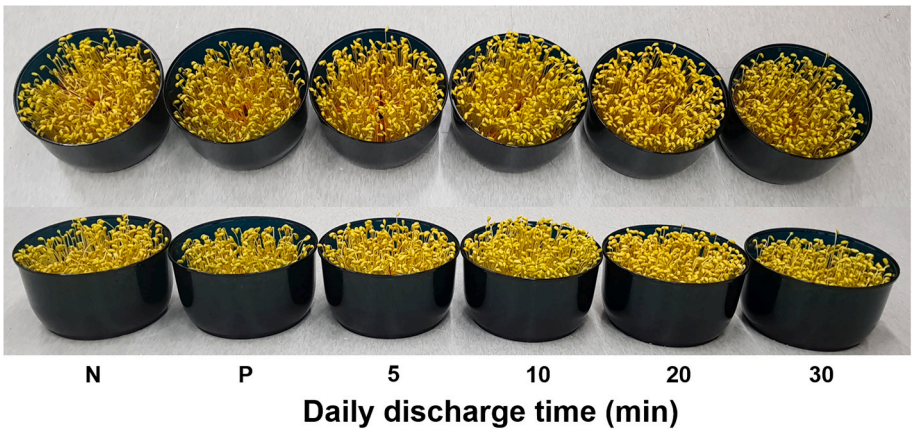


Fig. 4. A photograph of the soybean sprouts, each sprinkled for up to 4 days with the water treated daily with the plasma discharge for 0 to 30 min. The untreated water was either reused as the negative control (N) or replaced daily with fresh deionized water as the positive control (P).

and root by 1.6%, 9.6%, 9.1%, and 17. 2% compared to the untreated control (approximately 747 mg per 100 g) when the NO_3^- concentration was decreased to 11.9–23.3, 10.3–81.3, 19.5–163.9, and 37.1–169.5 $mg{\cdot}L^{-1}$ in the PTW collected daily after the discharge for 5, 10, 20, and 30 min, respectively (Figs. 2 and 5; $P < 0.05$). Regardless of the PTW treatment, aspartic acid accounted for approximately 40% of the total amino acids in the hypocotyl and root; glutamic acid and valine accounted for about 11%, and the other 28 amino acids for <49% (**Table S1**). In particular, sprinkling with the PTW increased the aspartic acid content in the hypocotyl and root by 16.5% compared to the untreated control (approximately 294.8 mg per 100 g) when the PTW was treated daily with the discharge for 30 min. In the case of the cotyledon, it had little effect on the total amino acid contents (Fig. 5 **and Fig. S7**; $P > 0.05$). Aspartic acid and glutamic acid accounted for approximately 32% of the total amino acids in the cotyledon; leucine, valine, lysine, and arginine accounted for about 27%, and the other 25 amino acids for <41% (**Table S2**).

The isoflavones in the soybean sprouts increased with their increasing biomass in relation to the NO_3^- accumulation and pH acidity in the PTW. However, the PTW effect on the isoflavones was not significant (**Table S3**; $P > 0.05$). Sprinkling with the PTW increased the total isoflavone contents in the sprouts by 7.0%, 18.0%, and 15.9% compared to the untreated control when the biomass at day 4 was enhanced by 1.8%, 4.2%, and 3.3% in the PTW treated daily with the discharge for 5, 10, and 20 min, respectively (Fig. 3 **and Fig. S8**). In addition, it changed the composition of the individual isoflavones in the sprouts by sprinkling with the PTW treated daily with the discharge for 5 to 20 min. With a discharge of 10 min, malonylglucosides, acetylglucosides, and β-glucosides increased by 30.8%, 50%, and 30.7% in the sprouts compared to the untreated control, respectively, while aglycones decreased by 8.1% (**Table S3**; $P > 0.05$). There was little difference in the total isoflavone content between the sprouts sprinkled with the PTW for the discharge of 30 min and untreated control. The reason may be that the sprinkling of the strongly acidic PTW caused severe damage to the growing sprouts and the resultant loss of the isoflavones.

Sprinkling with the PTW can affect metabolic processes, including primary or secondary metabolites in the sprouted soybeans (Fig. 6). As expected, it increased the total contents of the amino acids and isoflavones in the growing part of the sprouts. Similarly, our previous works reported that the total content of amino acids was enhanced in other sprouted crops, including barley (Song, Lee, et al., 2020b), ginseng (Song et al., 2021), and oat (Lee et al., 2023) and when treated with the PTW. It is suggested that the enhanced content of the amino acids can be mainly related to the accumulated NO_3^- in the PTW because there was no nitrogen source except for the seed tissues, such as the cotyledons. The total content of amino acids in our study clearly showed a gradual increase in the hypocotyl and root with the increasing discharge time for the PTW, even though the total content in the cotyledon was not statistically different among the treatments (Fig. 5). The highly accumulated NO_3^- in the PTW would be absorbed by the root and consequently reduced to amino acids through nitrogen metabolism in the sprouts. In the case of the isoflavones, the PTW treated with the 10 and 20 min of the discharge increased the total content of the isoflavones in the sprouts by 18.0% and 15.9% compared to the untreated control, respectively, showing a similar response as their biomass increased (**Fig. S8**). Although it was not significant due to higher variation, the PTW effect on the isoflavones could be assumed. An acceptable NO_3^- accumulation in the PTW, together with an acidic pH, could be the main factor for the higher isoflavone content, based on a review (Wang, Zhang, Jiang, et al., 2022a; Wang, Zhang, Zhu, et al., 2022b) regarding factors affecting

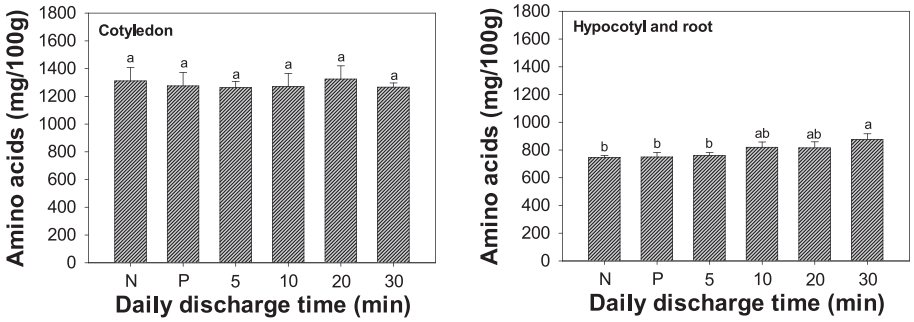


Fig. 5. Total contents of the 38 free amino acids of the soybean sprouts, each sprinkled up to 4 days with the water treated daily with the plasma discharge for 0 to 30 min. The untreated water was either reused as the negative control (N) or replaced daily with fresh deionized water as the positive control (P). Each bar represents the mean of three replicates. Means with the same letter are not significantly different by Tukey's HSD (honestly significant difference) test at $P = 0.05$.

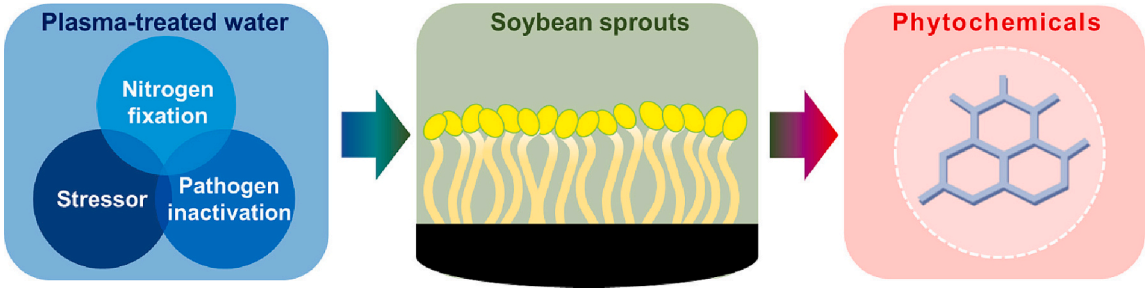


Fig. 6. The proposed factors of the plasma-treated water that affect the growth and phytochemicals of soybean sprouts.

isoflavones in soybean sprouts. In response to oxidative stress, nitric oxide (NO) acts as a signaling molecule for the biosynthesis of isoflavones (Wang, Zhang, Jiang, et al., 2022a; Wang, Zhang, Zhu, et al., 2022b). Nitric oxide is synthesized by the chemical reduction of NO_2^- and NO_3^-, and the NO synthesis requires an acidic pH (Wendehenne, Durner, & Klessig, 2004).

4. Conclusion

The pH acidity and NO_3^- accumulation in the PTW interactively affected the plants or microbes, thus indicating the optimal PTW for cultivating soybean sprouts in practice. That is, 10 to 20 min of the discharge was the appropriate time daily for the reused water to reach the NO_3^- requirement for the soybean sprouts with an acceptable pH. The optimal PTW, which had an accumulation of NO_3^- up to 321.8 $mg{\cdot}L^{-1}$ and a pH of 3 or higher in 4 days, significantly enhanced the growth of the soybean sprouts by 4.2% compared to the untreated control. It increased the total content of the amino acids and isoflavones by 9% and 18% in the sprouts, respectively. In addition, the pH acidity might act as a stressor or by inactivating pathogens. Therefore, the optimal PTW used for sprinkling in our study can be applicable enough to enhance the growth and phytochemicals of sprouted soybeans in commercial sprouters (Fig. 6).

Funding

This research was supported by the R&D Program of "Plasma Advanced Technology for Agriculture and Food (Plasma Farming, Project No. 1711124797)" through the Korea Institute of Fusion Energy (KFE) funded by Government funds, Republic of Korea.

CRediT authorship contribution statement

Jong-Seok Song: Data curation, Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Sunkyung Jung:** Data curation, Investigation.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgements

We thank Dr. Mi Ja Lee at the National Institute of Crop Science (NICS), Rural Development Administration (RDA) for providing the seed samples.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101345.

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