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Ozonated water soaking improves the flower growth, antioxidant activity, and bioactive compound accumulation in *Agastache rugosa*

Vu Phong Lam^{1,2†}, Dao Nhan Loi^{2,3†}, Sunwoo Kim³, Juhyung Shin³ and Jongseok Park^{1,3*}

Abstract

Ozonated water (OW) is now being recognized as an innovative and eco-friendly solution for managing plant growth while also promoting the production of bioactive compounds and essential plant metabolites. This study aimed to identify the most effective duration of OW treatment to promote plant growth and accumulation of antioxidant activity and bioactive compounds in *Agastache rugosa* in a plant factory. Whole plants were subjected to OW soaking treatments for varying durations (0, 1, 10, 20, 40, and 80 s) at a concentration of 1 $\mu\text{mol}\cdot\text{mol}^{-1}$, once per week, at 0, 1, 2, 3, and 4 weeks after transplantation. Five weeks after transplantation, plant samples were collected for the analysis of their plant growth parameters, photosynthetic pigments and parameters, total flavonoid, antioxidant activity, and bioactive compounds. Stem length was decreased in all OW treatments, while the number of flower branches, the flower fresh and dry weights were significantly increased under 20, 40, and 80 s OW treatments than in the control group. The net photosynthetic rate decreased significantly in 20, 40, and 80 s OW treatments compared with the control. Chlorophyll a concentration was the highest in the 20-s OW treatment, and chlorophyll b concentration was the highest in the 10-s OW treatment. Total flavonoid levels in plants increased significantly under 20-, 40-, and 80-s OW treatments, and their antioxidant activity (superoxide dismutase, catalase, and peroxidase) were significantly higher under 40- and 80-s OW treatments than in the control. Rosmarinic acid content increased significantly under the 10- and 40-s OW treatments, whereas the tilianin and acacetin contents increased significantly under the 20-, 40-, and 80-s OW treatments compared to those in the control. Our results suggest that soaking whole plants in OW for 20–80 s enhances the flower growth and bioactive compounds in *A. rugosa* for medicinal use.

Keywords Antioxidant activity, Bioactive compound, Chlorophyll, Plant factory, Flavonoid

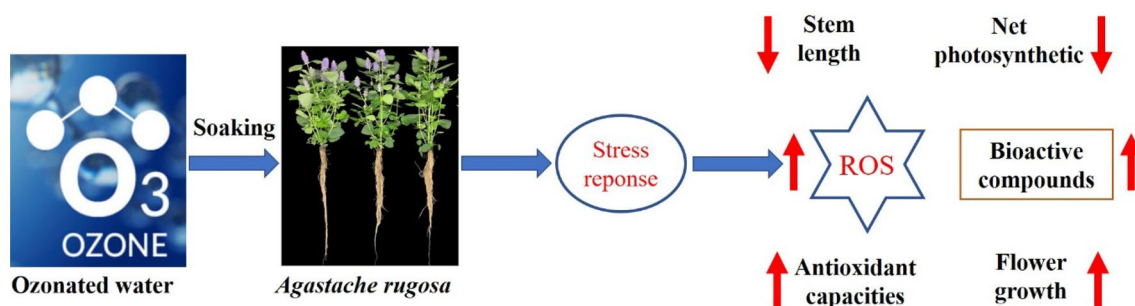
[†]Vu Phong Lam and Dao Nhan Loi contributed equally to this work.

*Correspondence:

Jongseok Park
jongseok@cnu.ac.kr

Full list of author information is available at the end of the article

Graphical Abstract



Background

Agastache rugosa, commonly known as “Korean mint”, is a perennial herbaceous plant belonging to the *Lamiaceae* family that is widely distributed in Asian countries, such as Japan, China, and Korea. It is used as a traditional medicine and culinary seasoning in these regions [1, 2]. *A. rugosa* possesses antioxidant [3], anticancer [4], antimicrobial [5], anti-inflammatory [6], analgesic [7], and cardiovascular properties [8]. It also exerts immunomodulatory effects by promoting human immune responses [9]. Tilianin, acacetin, and rosmarinic acid (RA) are the primary bioactive compounds in *A. rugosa* [10]. RA exerts diverse pharmacological effects, including antitumorigenic, antioxidative, anti-inflammatory, and anti-apoptotic effects [11]. Tilianin is associated with various beneficial properties, such as antidiabetic, cardioprotective, antihypertensive, neuroprotective, antiatherogenic, antidepressant, anti-inflammatory, and antioxidant properties [12]. Acacetin, a flavone found in *A. rugosa*, exerts anticancer, anti-inflammatory, and anti-metabolic disorder effects [13]. Recently, there have been growing concerns regarding the bioactive quality and nutritional value of food as well as the economic and environmental sustainability of crop management practices. Therefore, it is imperative to explore sustainable, economically viable, and ecologically beneficial approaches to enhance the quality, particularly the nutritional value and ecological impact of *A. rugosa*.

Ozonated water (OW) treatment is a cutting-edge and eco-friendly approach to plant growth and management [14, 15]. Since 2003, the US Food and Drug Administration has approved the use of ozone in food products, with the permissible residual dissolved ozone concentration of 0.4 mg L⁻¹ in bottled water [16]. Ozone can penetrate plant tissues via lenticels, stomata, and physical openings in the cuticle. Upon entering the

plant tissues, ozone initiates reactions with molecules present in the cell wall, apoplastic fluid, and plasma membrane, resulting in the generation of reactive oxygen species (ROS), such as hydroxyl, hydrogen peroxide radicals, and superoxide [17]. Plants activate defense mechanisms at the genetic, biochemical, and transcriptional levels in response to oxidative stress triggered by ozone and its byproducts [18, 19]. These defense mechanisms include the activation of antioxidants, such as glutathione and ascorbate, and enzymes, such as superoxide peroxidases, dismutases, and catalases [18, 20]. When exposed to ground-level ozone concentrations, plants exhibit an elevation in the activity of antioxidant-related enzymes as a response to oxidative stress [21]. Plants exposed to ozone have been documented to experience an augmentation in phenylpropanoid metabolites, leading to the synthesis of diverse phenolic compounds. This phenomenon, in turn, activates plant cell metabolism, aiding in the restoration and upkeep of cellular structure [22, 23].

Additionally, plants produce various metabolites, such as phenolics, terpenoids, and carotenoids, as part of their defense responses against ozone-induced damage [24, 25]. Ozone is a highly reactive oxidizing agent that readily dissolves in water and exerts potent antibacterial and antifungal effects [26, 27]. Ozone serves as a potent oxidizing agent that interacts with various biomacromolecules, such as proteins, nucleic acids, fatty acids, and carbohydrates. Unlike radicals and ROS, ozone itself is not a radical but can readily react with radicals [17]. Plants have an inherent defense mechanism to cope with ozone stress that involves the production of various antioxidant compounds capable of scavenging free radicals [28]. Therefore, ozone has the potential to serve as a valuable resource for enhancing plant tolerance to diverse abiotic stresses without posing any significant environmental concern [29]. Ozone, a representative abiotic ROS

inducer, triggers early differential expression of miRNAs in *Arabidopsis thaliana* ecotype Col-0, a tolerant strain. These miRNAs, also responsive to UV-B stress, negatively regulate target genes associated with development, implying a potential role in resource allocation during oxidative stress [30]. Ozone triggered enhanced levels of superoxide dismutase, peroxidases, glutathione reductase, and ascorbate peroxidase in *Arabidopsis thaliana* [31]. The pericarp of O₃-treated plants exhibited a notable 52.8% increase in total carotenoid content and a 17% rise in total phenolic compound content. However, in the seeds of O₃-treated plants, there was a substantial 87% reduction in total antioxidant potential [32]. O₃ serves as a robust stimulant, significantly influencing the levels of secondary metabolites and antioxidants within antidiabetic *Costus pictus* plants. This alteration has the potential to impact the plant's medicinal properties [33]. Exposure to O₃ led to a general augmentation in phenolic compounds. Furthermore, the antioxidant capacity of all examined extracts showed an enhancement due to O₃ exposure. This suggests that the controlled application of ozone for a defined duration could serve as a promising biotechnological method to enhance the quality of *Salvia officinalis* leaf extracts [34]. In a plant factory setting, elevated ozone concentrations were found to enhance the accumulation of bioactive compounds like total phenolics, antioxidant capacity, and total flavonoids in red leaf

lettuce (*Lactuca sativa* L.) [23]. Previous studies have shown that OW stimulates the moderate production of ROS, which trigger the plant defense mechanisms and promote the synthesis of bioactive compounds [15, 17]. These findings indicate that ozone control is an effective method for boosting the accumulation of antioxidant capacities and bioactive compounds in plants.

Application of OW in hydroponic nutrient solutions with whole-plant soaking intervals is very rare. In this study aimed to investigate the effects of whole-plant soaking duration in OW on the growth and quality of *A. rugosa*, which has not been evaluated in previous studies. This study hypothesized that varying plant soaking durations in OW could trigger different physiological and biochemical responses, leading to increased biosynthesis of secondary metabolites without negatively affecting the plant growth in *A. rugosa*. To verify these hypotheses, this study investigated the effects of different soaking durations in OW on the growth, photosynthetic parameters, photosynthetic pigments, antioxidant capacities, and bioactive compound accumulation in *A. rugosa*.

Methods

Seedling growth conditions

A. rugosa seeds from Danong Seed Co., Ltd. in Seoul, Republic of Korea were sown in a germination seed tray measuring 40×60 cm and containing 240 holes.

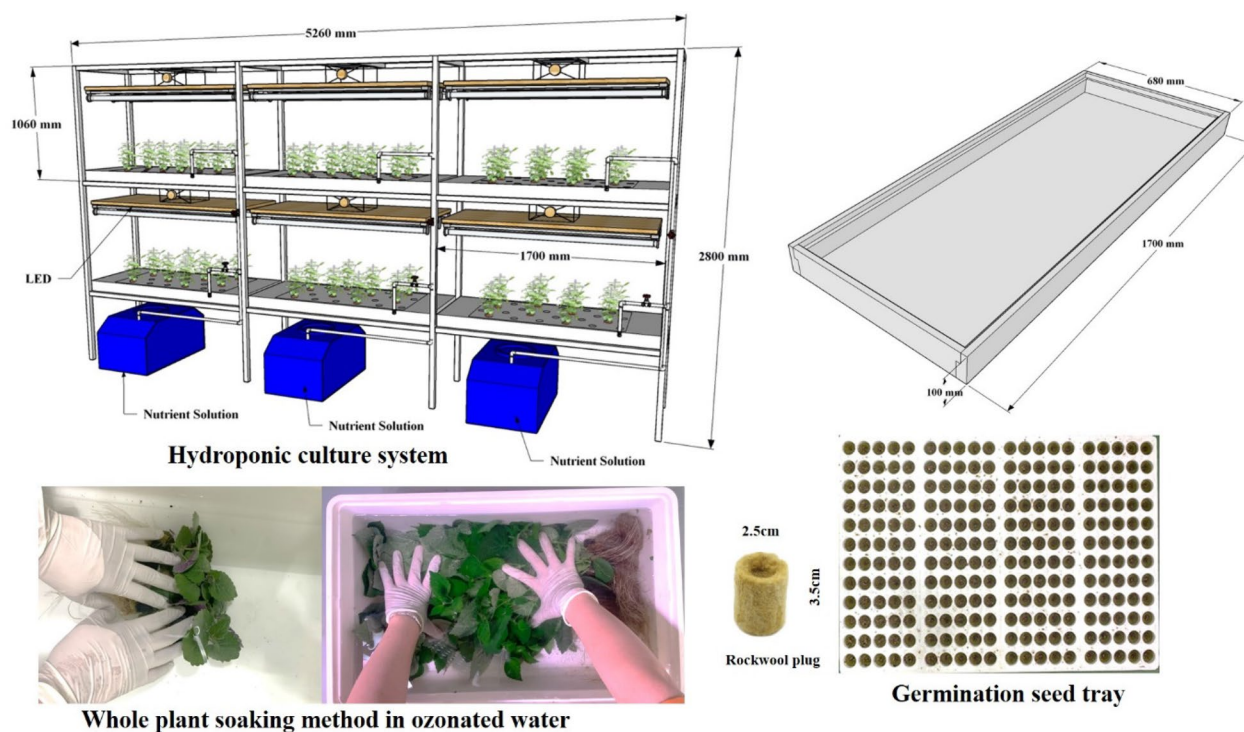


Fig. 1 Germination seed tray, and a deep flow technique system in a plant factory

The tray was used with UR Rockwool from Suwon, Korea (as shown in Fig. 1). Each hole, measuring 2.6×3.6 cm (width $\times$ height), was seeded with a single *A. rugosa* seed. Germination was facilitated using rockwool plugs sized at 2.5×3.5 cm (width $\times$ height) (Fig. 1). The seedlings were grown in an enclosed room at a temperature of $21.8/18$ °C, relative humidity of $70 \pm 5\%$, and photosynthetic photon flux density (PPFD) of 220 ± 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$, provided by LED lights (TL5 14W/865 Philips, Amsterdam, Netherlands) under a 16 h/8 h light/dark cycle. Hoagland solution, which had an electrical conductivity (EC) of 1.2 dS m^{-1} and pH of 6.0, was provided to the seedlings from 2 weeks after sowing.

Treatments

On the 39th day after sowing, the seedlings were transplanted to a deep flow technique system (Fig. 1) situated in a plant factory. The cultivation conditions were identical to those used for seedling growth. The plants were grown for 35 days in Hoagland solution with a pH of approximately 6.5 and an EC of 2.0 dS m^{-1} . Whole plants were subjected to OW soaking treatments for 0, 1, 10, 20, 40, and 80 s at a concentration of $1 \mu\text{mol mol}^{-1}$ (Fig. 1). OW soaking treatments were conducted once per week, at 0, 1, 2, 3, and 4 weeks after transplantation. Subject the entire plant to OW solution for varying durations as specified in the experiment's treatment conditions. Plant samples were collected 35 days after transplantation (DAT) for further analysis. The experiment was conducted in an enclosed room with artificial lighting.

Ozonated water supply

For OW generation, ozone gas was supplied by the ozone generator from an oxygen gas cylinder at a rate of 1.5 L/min. The ozone generator used power to discharge oxygen (O_2) and convert it into ozone (O_3). Next, the tap water was directed into a water tank, where it was exposed to an OW contactor. This contactor enabled dissolved the ozone generated inside the ozone generator in water, resulting in the production of OW within the tank. A ventilation system was installed in the water tank to minimize ozone gas generation.

Measurements of plant growth parameters

Thirty-five DAT, all samples were harvested and various growth parameters, including leaf length, leaf width, root length, leaf number, number of flower branches, stem length, leaf area, and fresh weights of leaves, stems, flowers, shoots, and roots, were measured. Moreover, the dry weights (DW) of flowers, leaves, stems, and roots were evaluated after 7 days of oven drying at 70 °C. Flower or leaf DW ratio was calculated as the ratio of the flower,

leaf area ratio, or leaf DW to the total DW of the plant. To determine the specific leaf area, the leaf area of the plant was divided by the DW of the leaves.

Evaluation of photosynthetic characteristics

Photosynthetic characteristics, namely the net photosynthetic rate (P_n ; $\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$), stomatal conductance (g_s ; $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$), intercellular CO_2 concentration (C_i ; $\mu\text{mol CO}_2 \text{ mol}^{-1}$), and transpiration rate (T_r ; $\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$) were determined using a portable photosynthesis system (LICOR 6400; Licor. Inc. Nebraska, NE, USA). The leaf chamber was configured with a CO_2 concentration of $400 \mu\text{mol mol}^{-1}$, PPFD of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$, leaf temperature of 25 °C, and an air-flow rate of $500 \text{ cm}^3 \text{s}^{-1}$ before taking the measurements. Photosynthetic characteristics were assessed between 9:00 AM and 12:00 AM on the third intact leaf from the apex of the plant. The data collection process was automated, and within each OW treatment, three measurements were taken for every replication ($n=3$).

Evaluation of photosynthetic pigments and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The soil plant analysis development (SPAD) value was measured at 39 days after transplanting on the third intact leaf from the top of the plant by a portable chlorophyll meter (502, Minolta Camera Co., Ltd., Osaka, Japan). Six plants ($n=6$) per replicate were used to determine SPAD value. Upon harvesting, the leaves, stems, and flowers of each *A. rugosa* plant replicate were promptly immersed in liquid nitrogen and stored in a deep freezer at -70 °C; subsequently, they were transferred to a dry freezer at -50 °C (TFD5503, IL Shinbiobase Co. Ltd, Gyeonggi-do, Korea) within 4 d. A porcelain mortar and pestle was employed to ground each sample, and the dry powder was filtered through mesh sieves. Chlorophyll (Chl) a, Chl b, and total carotenoid (Car) concentrations and DPPH radical scavenging activity of plant samples were analyzed using an Epoch microplate spectrophotometer (EPOCH-SN; Agilent Technologies, Inc., Santa Clara, CA 95051, USA). Powdered shoot (20 mg DW) was extracted with 2 mL of 90% MeOH and centrifuged for 20 min at $1308 \times g$. Chl a, Car, and Chl b were detected at 652.4, 470, and 665.2 nm, respectively, following the methods described by Lichtenthaler [35]. DPPH radical scavenging assay was performed at 517 nm according to the method described by Rahman et al. [36]. Chl a, Chl b, Car, and total Chl a + b concentrations, DPPH activity, Chl a/b ratio were calculated as follows:

$$\text{Chl a (mg g}^{-1}\text{)} = (16.82 \times A_{665.2} - 9.28 \times A_{652.4})/10$$

$$\text{Chl b (mg g}^{-1}\text{)} = (36.92 \times A_{652.4} - 16.54 \times A_{665.2})/10$$

$$\begin{aligned} \text{Total carotenoid (mg g}^{-1}\text{)} \\ = ([1000 \times A_{470} - 1.91 \times \text{Chl a} - 95.15 \text{ Chl b}]/ \\ 225)/10 \end{aligned}$$

$$\text{DPPH (\%)} = (A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}} \times 100$$

$$\text{Chl a : b ratio (Chl a/b)} = \text{Chl a/Chl b}$$

$$\text{Total Chl a + b (mg g}^{-1}\text{)} = \text{Chl a} + \text{Chl b},$$

where A is the absorbance at wavelength.

Determination of total flavonoid content and antioxidant activity

The Lin and Tang [37] method, employing aluminum chloride (AlCl_3), was utilized for the colorimetric determination of the total flavonoid content in *A. rugosa* plants. Briefly, A mixture of 20 mg of dry samples and 2 mL of 90% methanol was prepared, followed by sonication at 20 °C for 20 min. After mixing, centrifugation was performed on the solution at a speed of $1308 \times g$ for a duration of 20 min, while maintaining a temperature of 4 °C.

Then, 100 μL of sample was taken and mixed with 300 μL of 95% ethanol, 20 μL of 10% (w/v) AlCl_3 , 20 μL (w/v) potassium acetate (CH_3COOK), and 600 μL of tertiary distilled water. Subsequent to a 40-min incubation at ambient temperature, the absorbance of the reaction mixture was assessed at 415 nm using an Epoch microplate spectrophotometer (EPOCH-SN; Agilent Technologies, Inc.). Quercetin served as the standard, and a calibration curve was established by preparing quercetin solutions with concentrations of 0, 50, 75, 125, 250, and 500 $\mu\text{g/mL}$ in methanol. The total flavonoid content of *A. rugosa* was assessed in triplicate ($n=3$), and the findings are presented as milligrams of quercetin equivalents (QE) per gram of dry weight (DW) of the extracted powder.

Next, this study used a modified version of the nitro blue tetrazolium (NBT) method described by Kiani et al. [38] to measure the superoxide dismutase (SOD, EC 1.15.1.1), and peroxidase (POD, EC 1.11.1.7). In brief, 20 mg of dry sample was mixed with 2 mL of 50 mM phosphate-buffered saline (PBS) at pH 7.0 and sonicated thrice for 10 min each in liquid nitrogen. The mixture was subjected to centrifugation at a speed of $1308 \times g$ for a duration of 20 min at a temperature of 4 °C.

Superoxide dismutase (SOD, EC 1.15.1.1) activity

To determine the SOD activity, a reaction mixture containing 20 μL of the sample, 24.5 μL of NBT, 52 μL

methionine, 2 μL EDTA, 93.5 μL PBS at pH 7.0, and 8 μL of riboflavin was prepared and added to a 96-well plate. The plate was then exposed to LED light ($200 \mu\text{mol m}^{-2} \text{ s}^{-1}$) for 8 min. The absorbance was measured at 560 nm using an Epoch microplate spectrophotometer (EPOCH-SN; Agilent Technologies, Inc.). One unit of SOD enzyme was defined as "the amount of enzyme that can inhibit the photoreduction of NBT by 50% under experimental conditions. SOD activity was quantified in $\text{U mg}^{-1} \text{ DW}$ and determined using the following formula:

$$\begin{aligned} \text{SOD (Unit mg}^{-1} \text{ DW)} \\ = \frac{((\text{control} - \text{sample}) \times 100\% \times 200 \mu\text{L})}{(\text{control} \times 50\% \times 20 \mu\text{L} \times 0.2 \text{ mg})}. \end{aligned}$$

Peroxidase (POD, EC 1.11.1.7) activity

A solution containing 66.6 μL of PBS with a pH of 6.1, 33.3 μL of hydrogen peroxide (H_2O_2), and 80 μL of guaiacol was used for the assay of the enzyme. At 25 °C, the reaction was initiated by adding 20 μL of the sample extract. Enzyme activity was defined as the increase in absorbance of 1 unit of enzyme at 470 nm per min at a temperature of 25 °C. The specific activity of POD was reported in $\text{U mg}^{-1} \text{ DW min}^{-1}$:

$$\begin{aligned} \text{POD (U mg}^{-1} \text{ DW min}^{-1}\text{)} \\ = \frac{\left[\left(a^{\text{initial}} - a^{\text{1min}} \right) \times \text{enzyme liquid total volume (200 } \mu\text{L)} \right]}{[1 (\text{min}) \times 20 \mu\text{L} \times 0.2 \text{ mg sample quality}]}. \end{aligned}$$

Catalase (CAT, EC 1.11.1.6) activity

CAT activity was assessed following the procedure outlined by Aebi, 1984 [39], as depicted in the equations below. To conduct the enzyme assay, a solution comprising 193.6 μL of PBS with a pH of 7.0 and 3.4 μL of 3% H_2O_2 was prepared. The enzymatic reaction was initiated by adding 3 μL of the sample extract. A reference mixture did not contain any enzyme extract, referred to as the 'blank' was placed in a spectrophotometer for 4 to 5 min to attain temperature equilibrium. The absorbance was measured at a wavelength of 240 nm in the spectrophotometer for 3 min. The extinction coefficient was $43.6 \text{ M}^{-1} \text{ cm}^{-1}$. CAT activity was quantified by determining the quantity of enzyme that decomposed 1 μM of H_2O_2 and expressed as $\mu\text{mol per milligram of DW per minute (}\mu\text{mol mg}^{-1} \text{ DW min}^{-1}\text{)}$:

$$\begin{aligned} \text{CAT (}\mu\text{ mol ml}^{-1} \text{ min}^{-1}\text{)} \\ = \frac{(\text{A}_{240}/\text{min}) \times \text{total volume} \times 1000}{43.6 \times \text{enzyme volume}}, \end{aligned}$$

$$CAT \left(\mu\text{mol mg}^{-1} \text{DW min}^{-1} \right) = \frac{\mu\text{mol min}^{-1} \text{ml}^{-1}}{\text{enzyme}(\text{mg ml}^{-1})}.$$

Determination of the concentrations and contents of acacetin, tilianin, three acacetin glycosides, and RA

The roots, stems, leaves, and flowers of *A. rugosa* from each replicate were promptly immersed in liquid nitrogen and stored in a deep freezer at -70°C ; subsequently, they were transferred to a dry freezer at -50°C (TFD5503, IL Shin-biobase Co. Ltd, Gyeonggi-do, Korea) within 4 d. Subsequently, a porcelain mortar and pestle were used to grind each sample, and the dry powder was filtered through a mesh sieve. To determine the concentrations of RA, tilianin, acacetin, and the three acacetin glycosides, acacetin 7-O-(2"-O-acetyl)- β -D-glucopyranoside (acacetin 1), acacetin 7-O-(6"-O-malonyl)- β -D-glucopyranoside (acacetin 2), and acacetin 7-O-(2"-O-acetyl-6"-malonyl)- β -D-glucopyranoside (acacetin 3), 10 mL of 100% methanol was used to dissolve 200 mg of dry powder from flowers, leaves, roots, and stems. The mixture was sonicated for 30 min before analysis. The mixed extract was centrifuged at $1358 \times g$ for 20 min. Extract solution (1 mL) was passed through a $0.45\text{-}\mu\text{m}$ filter and analyzed via high-performance liquid chromatography (HPLC; 1260 Infinity; Agilent Technologies Inc.). The mobile phase was acetonitrile (solvent B) and 0.1% formic acid in water (solvent A). The gradient program was as follows: 0–5 min: 20% B, 5–10 min: 50% B, 10–20 min: 50% B, and 20–22 min: 100% B. The injection volume and flow rate were 10 μL and 0.8 mL min^{-1} , respectively [40]. HPLC chromatogram was obtained at a wavelength of 330 nm. Calibration curves were constructed using standard compounds obtained from Sigma-Aldrich (St. Louis, MO, USA).

Retention times of RA, tilianin, and acacetin were 11.655, 12.542, and 19.659 min, respectively. Moreover, acacetins 1, 2, and 3 were detected at 13.131, 14.485, and 15.351 min, respectively. Concentrations of these bioactive compounds, expressed as mg g^{-1} DW, were measured in the flowers, leaves, roots, and stems of plants. The total concentration of each compound in the whole plant (mg g^{-1} DW) was calculated using Eq. (1):

$$\begin{aligned} \text{BCx in the whole plant (mg g}^{-1}\text{DW)} \\ = \Sigma(\text{amount of BCx in each part} \\ \times \% \text{ DW of each plant organ per DW of the entire plant}), \quad (1) \end{aligned}$$

where BC: bioactive compound, x: tilianin, acacetin 1, acacetin 2, acacetin 3, or RA.

BC content of the whole plant (mg/plant DW) was expressed as the BC concentration (mg.g^{-1} plant DW) multiplied by the entire plant DW (g).

Statistical analysis

Growth parameters and SPAD value were measured in six plants ($n=6$), whereas photosynthetic characteristics, photosynthetic pigments, DPPH radical scavenging activity, total flavonoid content and antioxidant activity, and BC were determined in three plants ($n=3$) for each replicate. The OW experiment was performed using a completely randomized design and included two replicates. The statistical analysis was conducted utilizing SPSS version 20.0 software (SPSS 20, IBM Corp., Armonk, N.Y., USA). The data underwent analysis using one-way analysis of variance, followed by Tukey's multiple range test.

Results

Plant growth parameters

Leaf length, leaf number, leaf width, leaf area, leaf fresh weight, stem fresh weight, root fresh weight, root length, specific leaf area, leaf area ratio, leaf dry weight (DW), and stem and root DW of *A. rugosa* were not significantly affected by OW treatments (Fig. 2; Tables 1 and 2). However, all OW treatments significantly reduced the stem length of *A. rugosa*, in comparison to the untreated plants. In contrast, number of flower branches, the flower fresh weight, DW, and flower weight ratio were significantly increased in response to OW treatments for 20, 40, and 80 s, respectively, in comparison to the untreated plants (Tables 1 and 2). Shoot and whole plant DW were both significantly increased by 15.61 and 15.57%, respectively, in response to the 40-s OW treatment when compared to the untreated plants (Table 2).

Photosynthetic parameters

Photosynthetic parameters were affected by soaking time in the OW treatments (Fig. 3). Specifically, the net photosynthetic rate (P_n) was significantly lower in the 20-, 40-, and 80-s OW treatments than in the control (Fig. 3A). However, the intercellular CO_2 concentration (C_i) was significantly higher in the 40- and 80-s OW treatments than in the control (Fig. 3C), whereas no significant difference was observed in stomatal conductance (g_s) and transpiration rate (T_r) between the OW treatments and the control group (Fig. 3B and D).

Chlorophyll and total carotenoid levels

The highest concentration of chlorophyll a (2.91 mg g^{-1}) was found in the 20-s OW treatment, while the control treatment had the lowest concentration (1.79 mg g^{-1}). Additionally, the results found that the concentration of chlorophyll a was significantly higher (1.63 times) in the 20-s OW treatment group than in the group without OW treatment (Fig. 4A). The concentration of chlorophyll b showed a significant increase in response to the 10-s OW

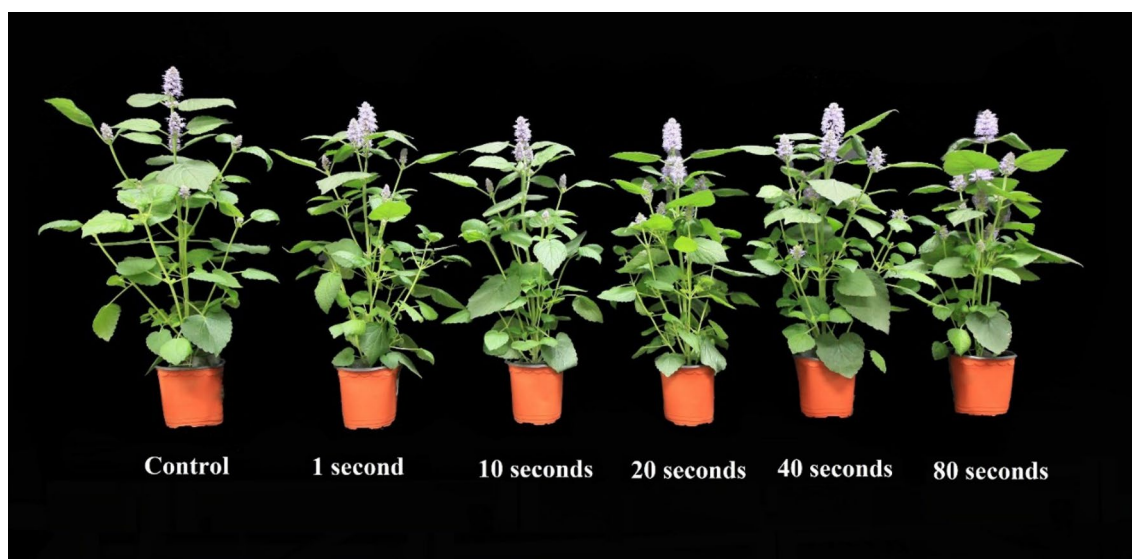


Fig. 2 *Agastache rugosa* plant growth under different ozonated water soaking treatments (0, 1, 10, 20, 40, and 80 s at $1 \mu\text{mol mol}^{-1}$) after five weeks of transplantation

treatment, with levels 1.23 times higher than those of the control group, representing the highest values observed (Fig. 4B). The highest values for the chlorophyll a/b ratio and total chlorophyll were observed in the 20-s OW treatment group (Fig. 4C and D). Based on our findings, it appears that the most effective OW treatment duration for increasing chlorophyll levels in *A. rugosa* was in the low range, specifically the 10- and 20-s OW treatments. In contrast, exposure to longer durations of OW (40- and 80-s treatments) led to decreased chlorophyll levels. Furthermore, the 80-s OW treatment group had the highest total carotenoid content, which was significantly greater (2.16 times) than that of the control group (Fig. 4E). There were no significant differences in the soil plant analysis development (SPAD) values between the OW treatments and control groups (Fig. 4F).

Total flavonoids and antioxidant activity

In comparison with the control, the levels of total flavonoids were significantly increased by 52.49, 49.32, and 49.32% following OW treatment for 20, 40, and 80 s, respectively (Fig. 5A). SOD enzyme activity was significantly higher (38.65 and 36.34%) when treated with OW for 40 and 80 s, respectively, compared to that of the control (Fig. 5B). Similarly, when compared to the control group, exposing the samples to OW for 40 and 80 s led to a notable rise in POD enzyme activity by 73.91% and 70.35%, respectively (Fig. 5C). Similarly, the application of OW for 40 and 80 s, when compared with the control, resulted in a significant increase in CAT enzyme activity by a factor of 2.43 and 2.36, respectively (Fig. 5D).

The DPPH radical scavenging activity of plants was significantly increased in response to 20-, 40-, and 80-s OW treatments, exhibiting 2.33-, 4.02-, and 3.30-fold increases, respectively, compared to untreated plants (Fig. 5E).

Concentrations and contents of acacetin, tilianin, and RA

Concentrations of the bioactive compounds, RA, tilianin, and acacetin, varied significantly among the various organs of *A. rugosa*. The roots had the highest concentration of RA, whereas the flowers had the highest concentrations of tilianin and acacetin. The flowers treated for 1 s exhibited the highest RA accumulation, with the RA concentration being 14.56% higher than that observed in untreated plants (Table 3). The concentration of RA in the stems was significantly elevated following the 1- and 10-s treatments compared to that in the control. Under 10-s treatments, the concentration of RA in the leaves was the highest among all treatments. Compared with the control, the concentration of RA in the roots significantly increased following 10-, 20-, and 40-s treatments. The highest concentrations of tilianin were observed in the flowers and leaves following 80- and 40-s treatments, respectively. In all OW treatments, the concentration of tilianin in the stems was lower than that in the control group (except for 10 s treatment). The higher tilianin concentrations in roots were found under 10 and 40 s treatments than those of the control. A significant elevation in the concentration of acacetin in flowers was observed after 20-, 40-, and 80-s treatments compared with that in the control and other OW treatments. OW treatments

Table 1 Growth characteristics of *Agastache rugosa* under different ozonated water treatments (0, 1, 10, 20, 40, and 80 s at 1 μmol mol⁻¹)

^w Ozone soaking time (s)	Leaf length (cm)	Leaf width (cm)	Number of leaves (leaves)	Leaf area (cm ²)	Stem length (cm)	Flower fresh weight (g)	Leaf fresh weight (g)	Stem fresh weight (g)	Shoot fresh weight (g)	Root fresh weight (g)	Root length (cm)
0	9.65	8.20	98.17ab	1176.22	44.38a	4.02b	16.47	15.42	35.90ab	13.03	55.08ab
1	9.70	7.73	94.00b	1226.62	41.32b	3.90b	15.18	14.47	33.55b	11.87	47.07b
10	9.28	7.42	102.50ab	1227.74	41.60b	4.12b	14.72	13.28	32.12b	11.82	48.33b
20	9.33	7.83	110.00a	1310.26	41.33b	7.25a	17.17	15.07	39.49a	12.53	50.68ab
40	9.48	8.08	102.17ab	1281.32	41.63b	7.18a	16.73	15.17	39.08a	12.82	52.05ab
80	9.85	8.20	100.50ab	1237.53	40.88b	6.05a	16.78	13.65	36.48ab	14.67	60.80a
Significance ^z	NS	NS	NS	NS	**	***	NS	NS	***	NS	*
L ^y	NS	*	NS	NS	NS	***	*	NS	*	**	***
Q ^x	NS	NS	*	NS	NS	***	*	NS	**	*	***

^w Ozone soaking time; NS not significant ($p > 0.05$); ^z significant at * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$; ^y L linear, ^x Q quadratic in the regression analysis. Values represent the means of six samples ($n = 6$). Different letters indicate the significant differences among treatments at $p < 0.05$, as determined using the Tukey's test

Table 2 Growth characteristics of *A. rugosa* under different ozonated water treatments (0, 1, 10, 20, 40, and 80 s at 1 μmol mol⁻¹)

^w Ozone soaking time (s)	Number of flower branches	Flower dry weight (g)	Leaf dry weight (g)	Stem dry weight (g)	Root dry weight (g)	Shoot dry weight (g)	Specific leaf area (SLA) (cm ² /g)	Whole plant dry weight (g)	Leaf area ratio (Cm ² /g)	Leaf weight ratio	Flower weight ratio
0	14.67c	0.72c	2.88	2.09	0.98ab	5.70b	411.48	6.68b	176.51	0.43ab	0.11c
1	15.83bc	0.85c	2.88	2.01	0.93b	5.74b	426.68	6.67b	183.85	0.43ab	0.13bc
10	16.17abc	0.88c	3.07	1.85	1.03ab	5.79b	405.54	6.82b	181.51	0.45a	0.13bc
20	20.33ab	1.37ab	2.80	2.04	1.14ab	6.21ab	469.86	7.36ab	178.12	0.38c	0.19a
40	20.83a	1.38a	3.05	2.16	1.13ab	6.59a	426.43	7.72a	166.17	0.39bc	0.18a
80	20.17ab	1.15b	2.83	2.23	1.18a	6.21ab	437.45	7.40ab	167.56	0.38c	0.15ab
Significance ^z	***	***	NS	NS	**	**	NS	**	NS	***	***
L ^y	**	**	NS	*	***	**	NS	**	*	***	**
Q ^x	***	***	NS	NS	***	***	NS	***	*	***	***

^w Ozone soaking time; NS not significant ($p > 0.05$); *significant at $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$; ^yL linear, ^xQ: quadratic in the regression analysis. Values represent the means of six samples ($n = 6$). Different letters indicate the significant differences among treatments at $p < 0.05$, as determined using the Tukey's test

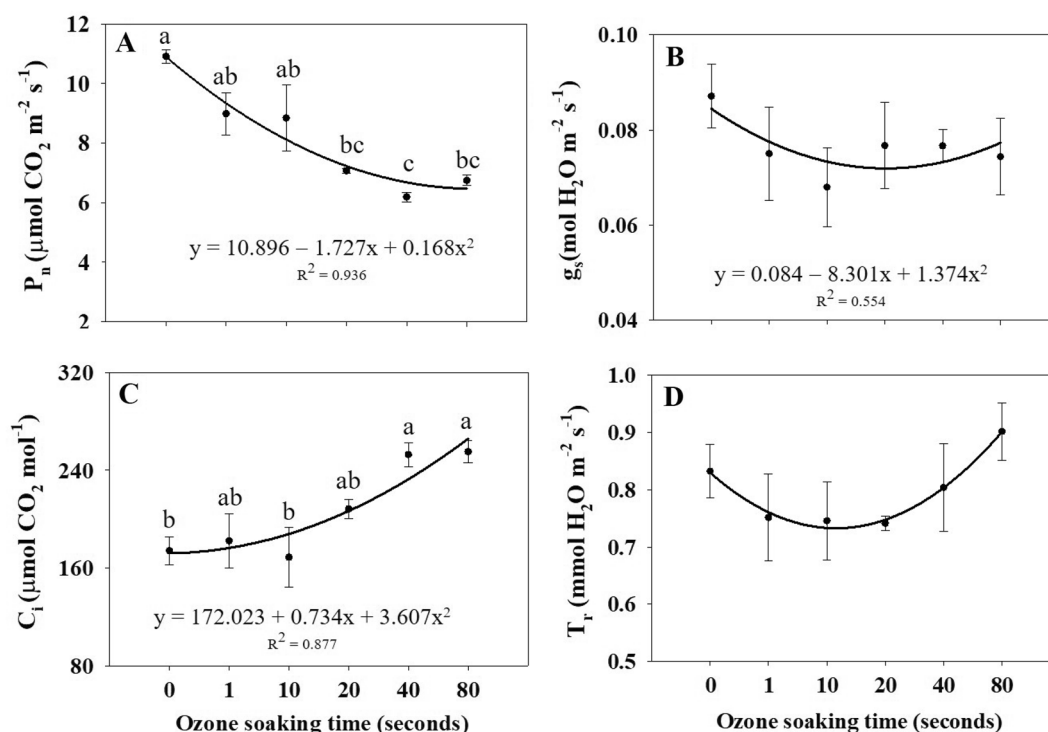


Fig. 3 Net photosynthetic rate (P_n ; **A**), stomatal conductance (g_s ; **B**), intercellular CO_2 concentration (C_i ; **C**), and transpiration rate (T_r ; **D**) of *A. rugosa* under different ozonated water treatments (0, 1, 10, 20, 40, and 80 s at $1 \mu\text{mol mol}^{-1}$). Each value indicates the mean $\pm$ standard error (SE) of three samples ($n=3$). Different letters represent the significant differences at $p \leq 0.05$, as assessed using the analysis of variance (ANOVA), followed by Tukey's multiple range test

significantly reduced acacetin concentration in stems compared with the control (Table 3).

The application of the 20-, 40-, and 80-s treatments significantly increased the RA content in the flowers compared to the untreated plants (Table 4). The stems of *A. rugosa* showed a significant increase in RA contents after treatment for 1 and 10 s compared to the untreated plants. The leaves exhibited the highest RA content when exposed to a 10-s treatment. The RA content in the roots was significantly higher in the OW treatments lasting for 10 and 40 s than in the control and other OW treatments. Compared with the control, flowers treated with OW for 20, 40, and 80 s exhibited a significant increase in tilianin content. The tilianin content in the stems was reduced after a 20-s treatment compared to the control, but no significant difference in tilianin content was found among other OW treatments and the control. The 40-s treatment showed the highest tilianin content in the leaves. Compared with the control and other OW treatments, both the 10- and 40-s treatments resulted in a significant increase in tilianin content in the roots. Acacetin content in flowers was significantly higher in 20-, 40-, and 80-s OW treatments than in the control and other OW treatments. All OW treatments led to a significant

decrease in acacetin content in the stems compared to the control group. However, the highest acacetin content in leaves was observed in the 40-s treatment (Table 4).

The concentration of RA in whole plants that underwent a 10-s OW treatment was significantly higher (by 43.77%) than that in the untreated control group (Fig. 6A). However, the RA content of whole plants that underwent both the 10- and 40-s OW treatments was significantly higher than that of the control group, with increases of 50.37% and 32.61%, respectively, because of the DW of the whole plant (Fig. 6D). There was a significant increase in tilianin concentration in plants treated with 20-, 40-, and 80-s OW treatments, with increases of 19.19, 16.60, and 20.09%, respectively, compared to the untreated controls (Fig. 6B). Similarly, tilianin contents in plants subjected to 20-, 40-, and 80-s OW treatments significantly increased by 42.13, 49.42, and 41.79%, respectively, compared to that in the untreated control group (Fig. 6E). A significant increase in acacetin concentration was observed in plants treated for 20- and 80-s, exhibiting an increase of 18.39 and 14.53%, respectively, compared to the untreated controls (Fig. 6C). Nevertheless, plants exposed to 20-, 40-, and 80-s OW treatments showed a significant increase in acacetin content, with

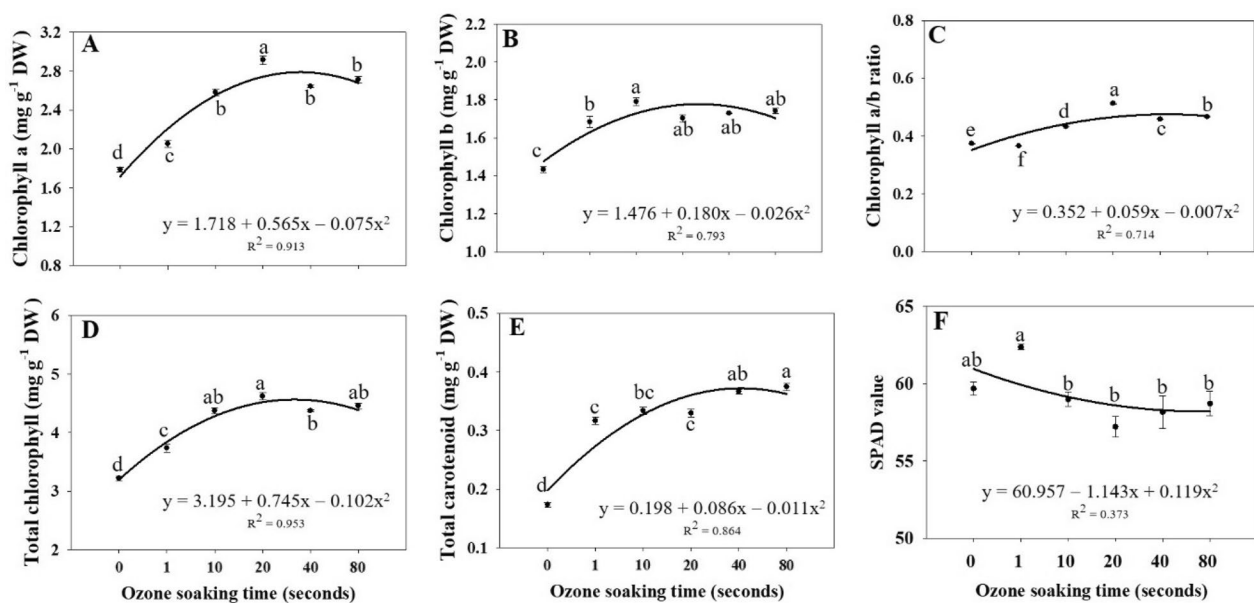


Fig. 4 Chlorophyll a levels **A**, chlorophyll b levels **B**, chlorophyll a/b ratio **C**, total chlorophyll levels **D**, total carotenoid levels **E**, and Soil Plant Analysis Development (SPAD) values (**F**) in *A. rugosa* under different ozonated water treatments (0, 1, 10, 20, 40, and 80 s at 1 $\mu\text{mol mol}^{-1}$). Each value indicates the mean $\pm$ SE of three samples ($n = 3$). Different letters represent the significant differences at $p \leq 0.05$, as assessed using ANOVA, followed by Tukey's multiple range test

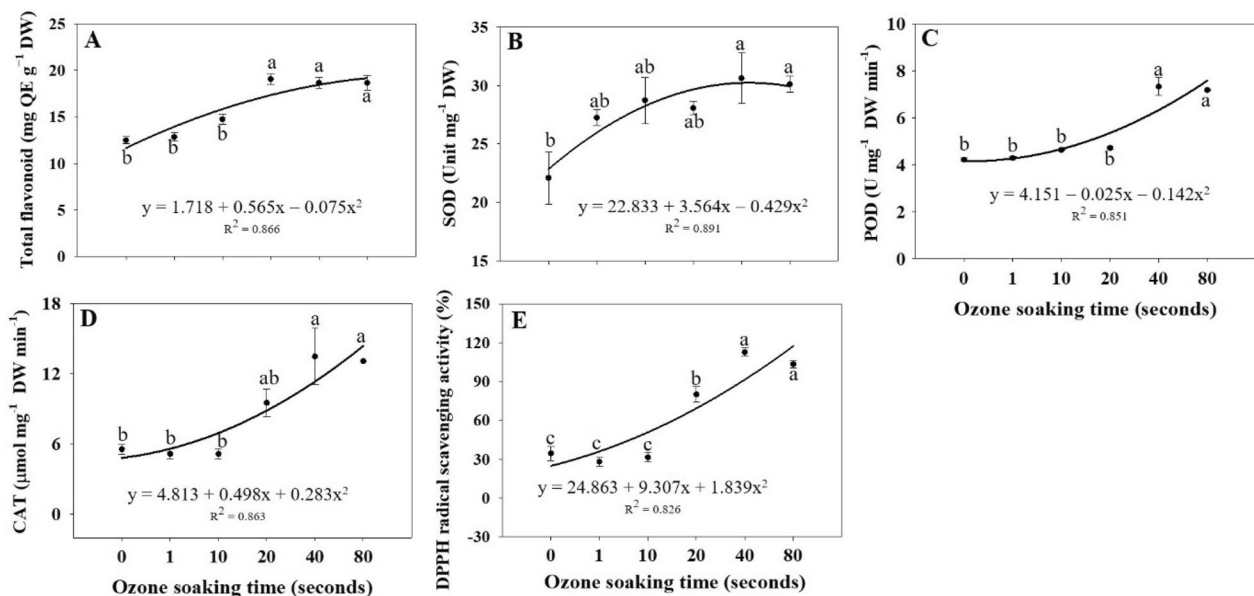


Fig. 5 Total flavonoid levels **A**, superoxide dismutase (SOD) levels **B**, peroxidase (POD) levels **C**, catalase (CAT) levels **D**, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity **E** of *A. rugosa* under different ozonated water treatments (0, 1, 10, 20, 40, and 80 s at 1 $\mu\text{mol mol}^{-1}$). Each value indicates the mean $\pm$ SE of three samples ($n = 3$). Different letters represent the significant differences at $p \leq 0.05$, as assessed using ANOVA, followed by Tukey's multiple range test

Table 3 Rosmarinic acid (RA), tilianin, and acacetin concentrations (mg g^{-1} DW) in the flowers, stems, leaves, and roots of *A. rugosa* under different ozonated water treatments (0, 1, 10, 20, 40, and 80 s at $1 \mu\text{mol mol}^{-1}$)

^w Ozone soaking time (s)	RA concentration in plant organs (mg g^{-1} DW)				Tilianin concentration in plant organs (mg g^{-1} DW)				Acacetin concentration in plant organs (mg g^{-1} DW)			
	Flowers	Stems	Leaves	Roots	Flowers	Stems	Leaves	Roots	Flowers	Stems	Leaves	Roots
0	3.64b	0.64b	5.24bc	10.45c	6.16bc	1.43a	2.77c	0.15b	1.24c	0.73a	0.57a	ND
1	4.17a	0.82a	5.51b	11.28c	6.15bc	1.29b	3.18ab	0.167b	1.14d	0.51c	0.55a	ND
10	3.61b	0.91a	7.30a	15.84a	5.81c	1.44a	2.79bc	0.35a	1.09d	0.55b	0.29b	ND
20	3.46b	0.54b	2.84e	12.89b	6.73b	1.05c	3.06abc	0.20b	1.92a	0.43d	0.56a	ND
40	3.42b	0.65b	4.16d	15.95a	6.03bc	1.13c	3.30a	0.33a	1.51b	0.45d	0.60a	ND
80	3.79ab	0.63b	4.54 cd	11.14c	7.77a	1.29b	3.15abc	0.21b	2.01a	0.54bc	0.54a	ND
Significance ^z	***	***	***	***	***	***	**	***	***	***	***	ND
L ^y	NS	NS	NS	NS	**	NS	NS	NS	***	NS	NS	ND
Q ^x	**	NS	NS	***	***	**	NS	*	***	**	NS	ND

^w Ozone soaking time; NS: not significant ($p > 0.05$); ^zsignificant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; ^yL linear, ^xQ quadratic in the regression analysis. Values represent the means of three assays ($n = 3$). Different letters indicate the significant differences among treatments at $p < 0.05$, as determined using the Tukey's test. ND not detected, RA rosmarinic acid, DW dry weight

Table 4 RA, tilianin, and acacetin contents ($\text{mg/plant organs DW}$) in the flowers, stems, leaves, and roots of *A. rugosa* under different ozonated water treatments (0, 1, 10, 20, 40, and 80 s at $1 \mu\text{mol mol}^{-1}$)

^w Ozone soaking time (s)	RA content in plant organs ($\text{mg/plant organs DW}$)				Tilianin content in plant organs ($\text{mg/plant organs DW}$)				Acacetin content in plant organs ($\text{mg/plant organs DW}$)			
	Flowers	Stems	Leaves	Roots	Flowers	Stems	Leaves	Roots	Flowers	Stems	Leaves	Roots
0	2.53b	1.28b	14.87b	10.00c	4.28b	2.84a	7.87b	0.14c	0.86c	1.46a	1.61b	ND
1	3.32b	1.61a	15.56b	10.26c	4.90b	2.53ab	8.99b	0.15c	0.91c	1.01c	1.57b	ND
10	3.09b	1.59a	21.59a	16.85a	4.97b	2.52ab	8.24b	0.38a	0.93c	0.96c	0.85c	ND
20	4.71a	1.12b	8.12c	14.81b	9.14a	2.17b	8.75b	0.23bc	2.61a	0.88c	1.61b	ND
40	5.18a	1.42ab	13.40b	18.03a	9.12a	2.47ab	10.65a	0.37a	2.29b	0.98c	1.94a	ND
80	4.60a	1.43ab	12.75b	13.33b	9.43a	2.92a	8.86b	0.25b	2.44ab	1.23b	1.53b	ND
Significance ^z	***	**	***	***	***	**	***	***	***	***	***	ND
L ^y	**	NS	NS	NS	***	NS	NS	NS	***	NS	NS	ND
Q ^x	***	NS	NS	***	***	**	*	**	***	*	NS	ND

^w Ozone soaking time; NS not significant ($p > 0.05$); ^zsignificant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; ^yL linear; ^xQ quadratic in the regression analysis. Values represent the means of three assays ($n = 3$). Different letters indicate the significant differences among treatments at $p < 0.05$, as determined using the Tukey's test. ND not detected, RA rosmarinic acid, DW dry weight

increases of 35.64, 32.46, and 32.25%, respectively, compared to the untreated control group (Fig. 6F).

Concentrations and contents of acacetins 1, 2, and 3

The concentration of acacetin 1 in flowers was significantly lower in plants treated with 10-, 20-, and 40-s OW treatments compared to the control group. However, no significant differences were observed between the 1- and 80-s OW treatments and the control group (Table 5). Compared to the control group, plants treated with 1-, 20-, 40-, and 80-s OW treatments had significantly lower concentrations of acacetin 1 in the stems; however, no significant difference was observed between the 10-s

OW treatment and the control group. No significant differences were observed in the acacetin 1 concentration in the leaves between the OW soaking treatments and untreated plants. Acacetin 1 concentration in the roots was the highest value under the 1-s OW treatment (Table 5). The highest concentration of acacetin 2 was observed in flowers under the 80-s treatment, stems under the 10-s treatment, leaves under the 40-s treatment, and roots under the 1-s treatment (Table 5). The highest concentration of acacetin 3 was observed in flowers under the 80-s treatment, stems under the 10-s treatment, and leaves under the 40-s treatment. However, no significant differences were observed in the concentration

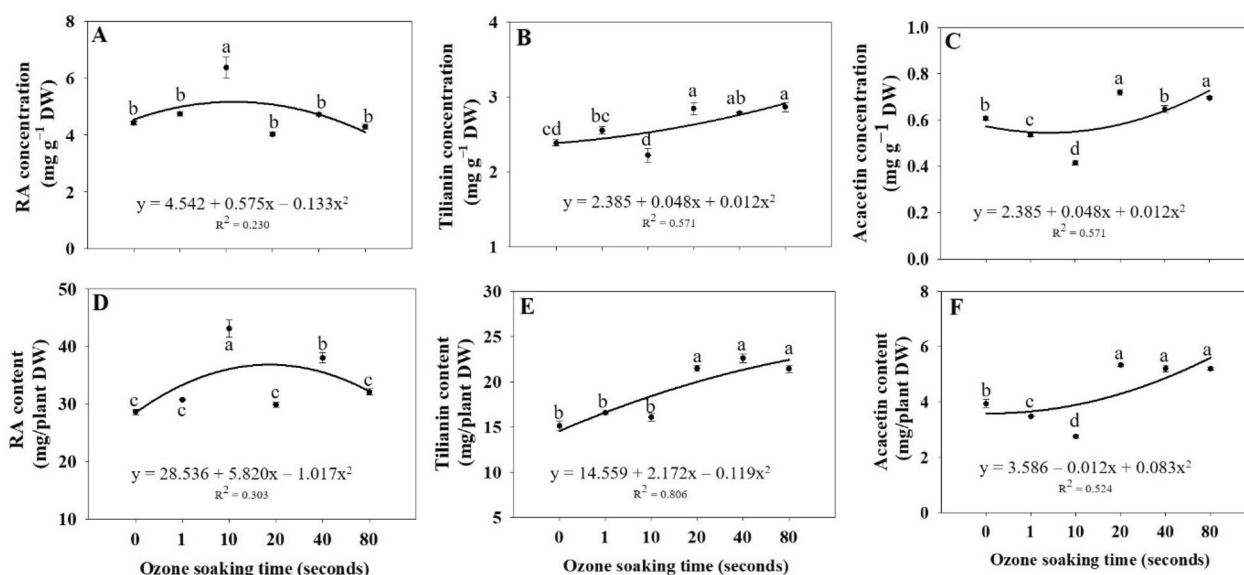


Fig. 6 Rosmarinic acid (RA) concentration (A) and content (D), tiliacin concentration (B) and content (E), and acacetin concentration C and content F in *A. rugosa* whole plant under different ozonated water treatments (0, 1, 10, 20, 40, and 80 s at 1 µmol mol⁻¹). Each value indicates the mean ± SE of three samples ($n = 3$). Different letters represent the significant differences at $p \leq 0.05$, as assessed using ANOVA, followed by Tukey's multiple range test

Table 5 Acacetin 7-O-(2"-O-acetyl)β-D-glucopyranoside (acacetin 1), acacetin 7-O-(6"-O-malonyl)β-D-glucopyranoside (acacetin 2), and acacetin 7-O-(2"-O-acetyl-6"-malonyl)β-D-glucopyranoside (acacetin 3) concentrations (mg.g⁻¹ DW) in the flowers, stems, leaves, and roots of *A. rugosa* under different ozonated water treatments (0, 1, 10, 20, 40, and 80 s at 1 µmol.mol⁻¹)

Ozone soaking time (s)	Acacetin 1 concentration in plant organs (mg.g ⁻¹ DW)				Acacetin 2 concentration in plant organs (mg.g ⁻¹ DW)				Acacetin 3 concentration in plant organs (mg.g ⁻¹ DW)			
	Flowers	Stems	Leaves	Roots	Flowers	Stems	Leaves	Roots	Flowers	Stems	Leaves	Roots
0	8.48a	2.13a	2.68	0.04c	6.10bc	0.82bc	1.34c	0.04c	15.44ab	2.03bc	2.23c	0.09ab
1	8.25a	1.73c	2.98	0.09a	6.58abc	0.72d	1.85b	0.06a	15.01ab	1.69d	2.80ab	0.16a
10	7.21b	2.15a	2.45	0.07b	5.70c	0.95a	1.25c	0.05bc	13.17bc	2.40a	2.29bc	0.12ab
20	6.91b	1.55d	2.71	0.07b	6.88ab	0.73 cd	1.85b	0.05bc	12.64bc	1.85 cd	2.38bc	0.12ab
40	6.21b	1.65 cd	2.93	0.07b	5.98bc	0.81bcd	2.33a	0.05b	11.55c	1.99bc	3.31a	0.14ab
80	8.91a	1.90b	2.75	0.04c	7.65a	0.84b	1.97ab	0.03d	16.03a	2.10b	2.78ab	0.08b
Significance ^z	***	***	NS	***	***	***	***	***	***	***	***	NS
L ^y	NS	NS	NS	NS	**	NS	*	***	NS	NS	NS	NS
Q ^x	***	*	NS	NS	**	NS	**	***	***	NS	NS	NS

^w Ozone soaking time; NS not significant ($p > 0.05$); ^z significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; ^y L linear, ^x Q quadratic in the regression analysis. Values represent the means of three assays ($n = 3$). Different letters indicate the significant differences among treatments at $p < 0.05$, as determined using the Tukey's test. DW dry weight

of acacetin 3 in the roots of OW and untreated plants (Table 5).

Acacetins 1, 2, and 3 contents in flowers were significantly increased by OW treatment for 20, 40, and 80 s, respectively, compared to those in the control group (Table 6). Acacetin 1 content in stems exhibited a significant decrease when treated with OW for 1 and 20 s compared to that in the control group.

Acacetin 1 content in leaves was not significantly different between the OW treatment and the control group. Compared with the control, all OW treatments resulted in a significant increase in acacetin 1 content in the roots. No significant difference in acacetin 2 and 3 content in stems was found between the OW treatment and the control group. OW treatment lasting for 40 s resulted in the highest content of acacetin 2 and 3

Table 6 Acacetins 1, 2, and 3 contents (mg/plant organs DW) in the flowers, stems, leaves, and roots of *A. rugosa* under different ozonated water treatments (0, 1, 10, 20, 40, and 80 s at $1 \mu\text{mol mol}^{-1}$)

^w Ozone soaking time (s)	Acaceten 1 content in plant organs (mg/plant organs DW)				Acaceten 2 content in plant organs (mg/plant organs DW)				Acaceten 3 content in plant organs (mg/plant organs DW)			
	Flowers	Stems	Leaves	Roots	Flowers	Stems	Leaves	Roots	Flowers	Stems	Leaves	Roots
0	5.88b	4.24a	7.63ab	0.04c	4.22b	1.62abc	3.80 cd	0.04c	10.68b	4.04abc	6.35b	0.09
1	6.58b	3.39b	8.44ab	0.08a	5.24b	1.41c	5.24bc	0.05ab	11.96b	3.32c	7.92b	0.14
10	6.16b	3.77ab	7.25b	0.08a	4.88b	1.67abc	3.70d	0.05ab	11.26b	4.20ab	6.78b	0.12
20	9.41a	3.19b	7.74ab	0.07a	9.36a	1.51bc	5.29b	0.06a	17.19a	3.82bc	6.80b	0.14
40	9.40a	3.62ab	9.43a	0.08a	9.06a	1.77ab	7.50a	0.06a	17.49a	4.37ab	10.67a	0.16
80	10.82a	4.30a	7.72ab	0.05b	9.28a	1.91a	5.55b	0.04c	19.46a	4.77a	7.82b	0.09
Significance ^z	***	**	***	***	***	**	***	***	***	**	***	NS
L ^y	***	NS	NS	NS	***	***	*	NS	***	***	NS	NS
Q ^x	***	*	NS	*	***	**	**	***	***	**	*	*

^w Ozone soaking time; NS not significant ($p > 0.05$); ^zsignificant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; ^yL: linear; ^xQ quadratic in the regression analysis. Values represent the means of three assays ($n = 3$). Different letters indicate the significant differences among treatments at $p < 0.05$, as determined using the Tukey's test. DW dry weight

in the leaves. Acaceten 2 content in the roots showed a significant increase after treatment with OW for 1, 10, 20, and 40 s compared to the control group. No significant difference was observed in the acaceten 3 content in the roots between the OW treatments and the control group.

Although there was no significant difference in the whole plant acaceten 1 concentrations between the OW treatments and control group, acaceten 1 contents

were significantly higher in the 20-, 40-, and 80-s OW-treated groups by 20.79, 26.65, and 28.70%, respectively, than in the control group owing to the DW of the plant (Fig. 7A and D). Acaceten 2 concentrations in the whole plant were significantly increased by 23.26, 52.73, 52.70, and 50.04% under 1-, 20-, 40-, and 80-s OW treatments, respectively, compared with those in the control group. Similarly, acaceten 2 contents in the whole plant were significantly increased by 10.08, 36.43, 54.36, and 52.08%

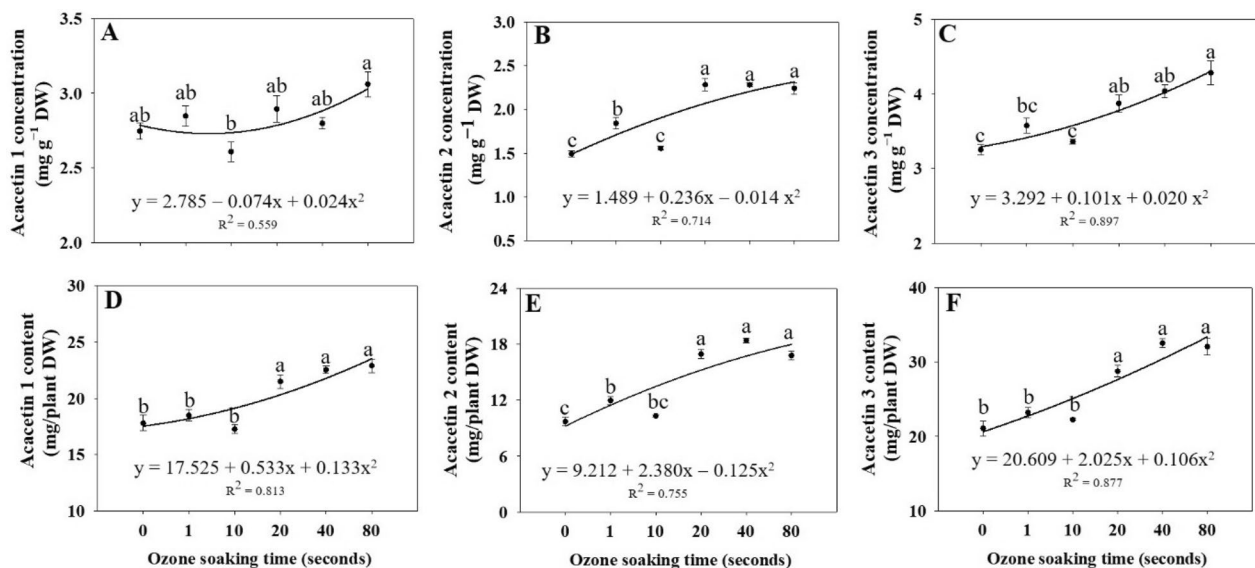


Fig. 7 Acaceten 7-O-2"-O-acetyl)- β -D-glucopyranoside (acaceten 1; **A** and **D**), acaceten 7-O-(6"-O-malonyl)- β -D-glucopyranoside (acaceten 2; **B** and **E**), and acaceten 7-O-(2"-O-acetyl-6"-malonyl)- β -D-glucopyranoside (acaceten 3; **C** and **F**), concentrations and contents in *A. rugosa* whole plant under different ozonated water treatments (0, 1, 10, 20, 40, and 80 s at $1 \mu\text{mol mol}^{-1}$). Each value indicates the mean $\pm$ SE of three samples ($n = 3$). Different letters represent the significant differences at $p \leq 0.05$, as assessed using ANOVA, followed by Tukey's multiple range test

under 1-, 20-, 40-, and 80-s OW treatments, respectively, compared with those in the control group (Fig. 7B and E). OW treatments for 20, 40, and 80 s significantly increased acacetin 3 concentrations and contents in the whole plant when compared to the untreated plants (Fig. 7C and F).

Discussion

Plant growth parameters

The results of this study indicate that OW treatment has varying effects on the growth parameters of *A. rugosa*. Although leaf length, width, number, root length, area, and fresh and DW of leaves, root, and stems were not significantly affected by the OW treatment, significant changes were observed in other parameters. Stem length was reduced in all OW treatments compared to that in the control group, indicating a negative effect of OW treatment on stem growth. This is in agreement with previous studies that reported the inhibitory effects of ozone on stem elongation in other plant species, such as Pak Choi [41], *Phoebe bournei* and *Phoebe zhennan* [42]. In contrast, the OW treatment had a positive effect on flower growth, with a significant increase in number of flower branches, flower fresh and DW and the flower weight ratio observed in response to OW treatments for 20, 40, and 80 s compared with the control group. This suggests that OW treatment can potentially enhance flower development in *A. rugosa* plants. Ozone-induced oxidative stress triggers a cascade of physiological and biochemical responses in plants. These responses ultimately led to accelerated flower development. One possible mechanism is the modulation of hormone signaling pathways. Ozone exposure has been shown to affect the levels and activity of plant hormones, such as ethylene, auxins, and cytokinins, which play crucial roles in flower development. Changes in hormone levels can influence the timing and rate of flower initiation, bud development, and flowering [43]. Several studies have provided compelling evidence of ozone-induced ethylene biosynthesis in various plant species such as tomato leaves and *Leontodon hispidus* [44, 45]. For instance, Nakajima et al. [44] demonstrated a substantial increase in ethylene generation rates in tomato leaves plants exposed to elevated ozone concentrations. *Leontodon hispidus* leaves exposed to elevated ozone concentrations exhibited an increased production of ethylene, with no discernible impact on leaf tissue ABA concentrations [45]. Their findings highlighted the role of ethylene in mediating ozone stress responses through the activation of antioxidant defense pathways. Furthermore, the molecular underpinnings of this phenomenon have been extensively investigated. It has been established that ozone stress can lead to the enhanced activity of enzymes within the ethylene

biosynthesis pathway. In a study by Moeder et al. [46], it was observed that ozone exposure led to the upregulation of 1-aminocyclopropane-1-carboxylic (ACC) synthase (ACS) and ACC oxidase (ACO) gene expression in tomato. Ethylene is known to influence the transition from vegetative growth to flowering [47]. Furthermore, oxidative stress caused by ozone can affect the expression of genes involved in flower development [48]. Studies have shown that exposure to ozone can alter the expression patterns of genes related to floral meristem identity, flower organ development, and flowering time. These changes in gene expression can directly affect developmental processes associated with flower formation [43, 47, 48]. Therefore, flowers were increased by more extended exposition of OW.

Shoot and whole plant DW were significantly increased by the 40-s OW treatment compared to the control group, indicating a positive effect of OW treatment on overall plant growth. The observed effects of the OW treatment on *A. rugosa* growth parameters could be attributed to its oxidative stress-inducing properties. Ozone has been reported to stimulate the production of ROS in plant cells, which can lead to the activation of stress response pathways and ultimately influences plant growth and development [14, 49]. The impact of ozone on plants varies depending on various factors, such as environmental conditions, plant types, and ozone regimes [50]. Short-term ozone exposure triggered compensatory responses, including an increase in photosynthetic pigments and leaf number. However, prolonged exposure to ozone may alter photosynthetic rate, RuBisCO activity, and soluble protein concentrations [51]. At a concentration of 3.0 mg L⁻¹, OW treatment significantly enhanced shoot dry matter, stem thickness, and leaf area in tomato [52]. Under ozone concentrations of 0.5 and 1 ppm, there was an observed increase in fruit yield, leaf area, as well as fresh and DW of cucumber shoots, in comparison to the control group [53]. Japanese mustard spinach plants treated with OW exhibited significantly higher fresh and DW of leaves, leaf length, number of leaves, leaf width, and ratio of shoot and root DW than the control [54]. The study reveals a mixed impact of OW treatment on growth parameters of *A. rugosa*. While leaf characteristics like length, width, and area were not significantly affected, stem length was reduced compared with the control. This indicates that OW treatment negatively affected stem growth, aligning with previous research on ozone's inhibitory effects on stem elongation [41, 42]. On the other hand, flower growth exhibited positive effects under OW treatment, with increased fresh and dry weights, as well as the flower weight ratio. This suggests that OW treatment has the potential to enhance flower development. The underlying mechanisms could involve

hormone signaling pathways and altered gene expression patterns related to flower development.

Photosynthetic characteristics

The reduction in net photosynthetic rate (P_n) observed in the 20-, 40-, and 80-s OW treatments compared to the control group may be attributed to the oxidative stress caused by the ozone treatment, which can damage the photosynthetic machinery of plants and inhibit photosynthesis [42, 55]. Additionally, the significantly higher intercellular CO_2 concentration (C_i) observed in the 40- and 80-s treatments may indicate that the plants were undergoing photorespiration, which is a protective mechanism employed by plants to dissipate excess energy and protect the photosynthetic machinery under stressful conditions [56]. Moreover, OW treatment significantly impacted the photosynthetic activity of *A. rugosa*. Net photosynthetic rate (P_n) was reduced under longer OW treatment, likely due to the oxidative stress induced by ozone, which can damage the photosynthetic machinery. Moreover, higher intercellular CO_2 concentration (C_i) in the longer OW treatments may indicate that plants were undergoing photorespiration as a protective mechanism against stress. The increase in C_i could be linked to a decrease in the carboxylase activity of RubisCO due to ozone exposure [57]. This may have led to a compensatory increase in the C_i .

Chlorophyll and total carotenoid levels

Chlorophyll (Chl) a and b levels in *A. rugosa* significantly increased in response to all OW treatments compared with the control group, with the most effective durations being in the low range (10- and 20-s treatments). Furthermore, the highest concentrations of Chl a, total Chl, and Chl a/b ratio were observed in the 20-s OW treatment, whereas the highest concentration of Chl b was observed in the 10-s OW treatment. The findings of our study are in agreement with previous research that has demonstrated an increase in both Chl b and Chl a + b in pepper plants upon exposure to O_3 at the concentration from 0.18 to 0.36 mg L⁻¹ [58]. This is also consistent with the results reported by Sloan and Engelke [59], who observed a significant increase in the chlorophyll concentration in *Agrostis stolonifera* in response to OW exposure. When tomato plants were subjected to OW irrigation with 3.0 mg/L, the relative expression levels of genes associated with Chl synthesis, namely SIGLK1, SIGLK2, and SIDCL in tomato leaves, exhibited a substantial increase. This increase reached its peak 12 h after treatment, with expression levels soaring to 59-fold, 23-fold, and 23-fold higher than those of the control, respectively [14]. Elevating the concentration of OW induced an increase in Chl levels in *Brassica* crops compared to the control. The

Chl content exhibited the following increases: 2 mg/L resulted in an 8.3% rise, 4 mg/L in a 14.9% increase, 6 mg/L in a 17.7% elevation, and 8 mg/L in an 18.2% enhancement [60]. The application of OW treatments led to an increase in Chl levels in tomatoes when compared to the control. Notably, the treatment with 6 mg/L OW showed a 20.7% increase over the control [61]. OW treatment resulted in a substantial elevation of Chl a, Chl b, and total Chl levels in tomatoes when compared to the control group [62]. When plants are exposed to environmental stressors, they often produce more Chl as a protective response. OW can be perceived as a stressor by the plant, prompting it to ramp up Chl production to better cope with potential damage from oxidative stress. Moreover, ozone exposure can generate oxidative stress in plants. This oxidative stress triggers various responses, including an increase in the production of reactive oxygen species (ROS). In response, plants may increase their Chl content as a defense mechanism. Chl a and Chl b are involved in the plant's antioxidant system and can help neutralize ROS [62, 63]. However, longer durations of OW treatment (40- and 80-s treatments) led to a decrease in chlorophyll levels, suggesting that excessive OW treatment may have adverse effects on plant physiology. These findings are in line with the results reported in previous studies showing that excessive OW treatment can cause oxidative stress and damage to plant tissues [55, 64]. While moderate OW exposure can stimulate Chl production, excessive OW can lead to oxidative stress in plant cells. This stress can damage cellular components, including Chl molecules, leading to a reduction in Chl content. OW stress can disrupt cellular membranes and organelles, including chloroplasts where Chl is located. This disruption can impair the integrity of chloroplasts and reduce their ability to synthesize Chl. Longer durations of OW exposure can inhibit photosynthesis by interfering with the light-dependent reactions in the chloroplasts. Reduced photosynthesis results in less Chl being produced and can lead to a decrease in Chl content. Excessive OW exposure can lead to the production of ROS within plant cells. These ROS can directly damage Chl molecules and other cellular components, leading to a decline in chlorophyll content [14, 65]. Furthermore, our results showed that the 80-s OW treatment group had the highest total carotenoid value, indicating that longer soaking durations in OW may be beneficial for the accumulation of total carotenoids in *A. rugosa*. This finding is consistent with that of a previous study that showed that OW treatment increased the total carotenoid content in pepper fruit by 52.8% compared with the control group [32]. When plants are exposed to OW over an extended period, the stress response mechanisms are prolonged. Initially, the plant perceives OW as a stressor

and activates pathways that lead to the synthesis of total carotenoids. As the exposure continues, these stress responses persist, leading to a continuous increase in total carotenoid production. Over time, the production of ROS due to OW exposure can continue. ROS can cause oxidative damage to plant cells, and the plant responds by increasing total carotenoid levels to mitigate this damage. Total carotenoids act as antioxidants, helping to neutralize ROS, thereby protecting cellular components [32, 66]. Our results suggest that the duration of the OW treatment is a critical factor in determining its effects on plant pigments. Chl levels responded differently to OW treatment durations. Initially, Chl a and b levels increased in response to OW treatment, with the most effective durations being in the low range (10- and 20-s treatments). However, longer durations (40- and 80-s treatments) led to decreased chlorophyll levels, suggesting excessive OW treatment's adverse effects. Additionally, total carotenoid content increased under longer durations of OW treatment, indicating that prolonged soaking in OW might enhance total carotenoid accumulation.

Total flavonoids and antioxidant capacity

The results showed that longer durations of OW treatment significantly increased the levels of total flavonoids and antioxidant activity of *A. rugosa*. These findings suggest that OW treatment enhances the antioxidant activity of *A. rugosa*, which may have implications for its use in various applications, including pharmaceutical and functional foods. Several studies have reported similar results, showing that OW treatment can increase the levels of total flavonoids and antioxidant enzymes in plants [32, 67]. Total flavonoids are known for their antioxidant properties. They can scavenge ROS generated during prolonged exposure to OW. As the oxidative stress persists, the plant increases the production of total flavonoids to neutralize ROS and protect cellular components from oxidative damage. Prolonged exposure to OW can trigger adaptive mechanisms in plants, leading to an increase in the production of total flavonoids. Flavonoids play a crucial role in plant adaptation by providing protection against environmental stresses, including ozone-induced oxidative stress. longer durations of OW exposure lead to an increase in total flavonoid content as a result of enhanced stress responses, oxidative stress mitigation, and activation of adaptive mechanisms [15, 68, 69]. The defense mechanisms involving SOD, CAT,

POD enzymes, and DPPH radical scavenging activity play crucial roles in countering ROS and safeguarding plants against oxidative harm [70]. SOD, CAT, and POD enzymes, along with DPPH radical scavenging activity, collectively form a comprehensive defense system in plants against ROS [71]. SOD's role is to neutralize

superoxide radicals, CAT catalyzes the decomposition of H_2O_2 into water and molecular oxygen, while POD enzymes are responsible for detoxifying hydrogen peroxide. DPPH radical scavenging activity serves as a tool to measure the overall antioxidant activity of plant compounds, reflecting their ability to protect plants from oxidative damage. Together, these mechanisms ensure that plants can thrive and maintain their health even in the presence of oxidative stressors in their environment [70, 71]. The observed increase in these antioxidant enzymes following OW treatment may be attributed to the enhanced production of ROS owing to ozone exposure, which in turn activates the antioxidant defense system of plants [72]. The results of this study suggest that OW treatment can effectively enhance total flavonoid levels, as well as the activity of antioxidant enzymes SOD, CAT, and POD, and DPPH radical scavenging activity in *A. rugosa*. This enhanced antioxidant activity suggests that OW treatment triggers antioxidant defense mechanisms in *A. rugosa*. In line with the earlier study, it was evident that the application of OW treatments significantly elevated the activity of antioxidant enzymes, including SOD, POD, and CAT in tomato leaves when compared to control plants [14]. The antioxidant activity of the fourth leaf of red leaf lettuce exposed to 100 ppb of ozone demonstrated an immediate increase right after the ozone exposure (at 0.25 h). Furthermore, the antioxidant activity continued to exhibit an upward trend over time, extending up to 48 h, in comparison to the control group [23]. Total flavonoids, SOD, CAT, and POD play crucial roles in scavenging ROS and protecting plants from oxidative damage induced by ozone exposure.

Concentrations and contents of acacetin, tilianin, RA, and three acacetin glycosides

The results of the present study indicate that OW treatment significantly increases the contents of certain phytochemicals, namely RA, tilianin, and acacetin, in *A. rugosa* plants (Figs. 6 and 7). RA content was the highest in plants after 10-s OW treatment, while tilianin and acacetin contents were increased after 20, 40, and 80 s of OW treatment. These findings are consistent with previous reports that OW treatment induces the biosynthesis of secondary metabolites in plants [33, 34]. Numerous studies have investigated the impact of prolonged exposure to ozone on secondary metabolites, which play crucial roles in plant defense mechanisms against various environmental stressors owing to their antioxidant and barrier properties [34, 73, 74]. RA biosynthesis in *Melissa officinalis* is stimulated by exposure to 80 ppb ozone for 5 h [75]. OW treatment triggers the stress response, leading to the activation of various biosynthetic pathways and production of secondary metabolites as a defense

mechanism in plants [76, 77]. Exposure to ozone typically triggers heightened transcription and activity of enzymes involved in the phenylpropanoid, lignin, and flavonoid pathways in plants, which serve as important barriers and antioxidant functions [15]. Therefore, OW treatment can be used to enhance the concentrations and contents of RA, tilianin, and acacetin in *A. rugosa*, suggesting its potential applications in pharmaceutical and nutraceutical industries. OW treatment positively influenced the concentrations and contents of certain phytochemicals, including RA, tilianin, and acacetin. This aligns with previous studies indicating that OW treatment induces the biosynthesis of secondary metabolites as part of the plant's stress response [34, 78]. These secondary metabolites have antioxidant and defense properties, making them valuable for pharmaceutical and nutraceutical industries.

This comprehensive study illuminates the intricate responses of *A. rugosa* to varying durations of OW treatments. While some aspects of plant growth remained relatively stable, the research underscores the activity of OW treatments to influence critical factors including photosynthetic parameters, chlorophyll and total carotenoid concentrations, antioxidant activity, and the levels of bioactive compounds. The findings suggest that briefer OW treatments, particularly those lasting around 10 to 20 s, may exert favorable effects on specific aspects of plant physiology, such as the elevation of chlorophyll levels and the accumulation of bioactive compounds like RA. However, soaking entire plants in OW for durations spanning from 20 to 80 s emerges as the most effective strategy for enhancing flower growth, total flavonoid, and augmenting the content of bioactive compounds in *A. rugosa*, rendering it more suitable for commercial and medicinal purposes. These outcomes offer invaluable insights into the optimization of OW treatments within the realm of *A. rugosa* cultivation, with significant potential implications for applications across agriculture and the pharmaceutical industry.

Conclusions

This study demonstrated that soaking in OW for 1–80 s did not have any adverse effects on the plant growth parameters or cause any visible damage to the leaves of *A. rugosa*. Moreover, soaking in OW for 20–80 s significantly increased the flower weight in *A. rugosa*. Ozone treatment under controlled conditions triggered a metabolic response that increased the levels of bioactive compounds in this plant. Additionally, OW treatment enhanced the antioxidant potential and bioactive compound content in *A. rugosa* plants. Our findings suggest that OW soaking is a valuable technique for producing high-quality *A. rugosa*

plants. However, further research is needed to elucidate the underlying molecular mechanisms driving these responses.

Abbreviations

DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
EC	Electrical conductivity
NBT	Nitro blue tetrazolium
OW	Ozonated water
PBS	Phosphate-buffered saline
POD	Peroxidase
PPFD	Response: photon flux density
RA	Rosmarinic acid
ROS	Reactive oxygen species
SOD	Superoxide dismutase

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Author contributions

VPL: experimental design, experimental conducting, investigation, data collection and analysis, writing—original manuscript, and writing—review and editing. DNL: experimental conducting, investigation and preparation of the manuscript, and writing—review and editing. SWK: experimental conducting, writing—review and editing. JHS: experimental conducting, data collection and analysis, and preparation of the manuscript. JSP: project administration, supervision, experimental design, data analysis, writing—original manuscript, and writing—review and editing.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

This research has been confirmed for publication in the journal.

Competing interests

The authors have no competing interest.

Author details

¹Department of Horticultural Science, Chungnam National University, Daejeon 34134, South Korea. ²Department of Agronomy, Tay Bac University, Son La 360000, Vietnam. ³Department of Bio-AI Convergence, Chungnam National University, Daejeon 34134, South Korea.

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