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Efficacy of Ozonated Water in Reducing Microbial Contamination and Maintaining Quality of Sunflower Microgreens During Cold Storage

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Abstract

This study aimed to investigate the effect of ozonated water on the microbial contamination and quality of sunflower microgreens. Sunflower microgreens were arranged in a completely randomized design for ozone treatments, with 5 replications. A 1 g/L concentration of ozonated water reduced the number of harmful microbes on the microgreens with increased washing time, from 30 s to 1, 3, and 5 min. The effect of ozone concentrations of 0, 1, 5, and 10 g/L on sunflower quality showed that the 1 g/L ozone concentration increased the vitamin C content from 1.49 to 1.67 mg 100 g/food waste (FW), and reduced weight loss, after storage at 8±1°C for 3 days. Additionally, the 1 g/L ozonated water and ozonated water combined with ice at 5°C for 5 min reduced weight loss and browning scores after 8 days of storage, as well as maintaining the color (a* value) of the sunflower microgreens. Therefore, to promote food safety and product quality, the research recommends applying this washing technique to postharvest sunflower microgreens.

Keywords: Shelf life, Vegetable quality, Ozone concentration, Contamination reduction, Food safety

1. Introduction

Sunflower (*Helianthus annuus* L.) microgreens are a great source of iron, calcium, folate, protein, zinc, and vitamins C, A, and K; moreover, they can be harvested within just 2 weeks [1]. Cultivated sunflower microgreens are often contaminated by various microorganisms from soil that affect seed germination and greatly impact seed quality by producing toxins [2], which can be harmful to the health of humans who consume the microgreens. Therefore, to ensure safe food consumption, the washing of sunflower microgreens before eating or cooking is very important. The efficient washing of vegetables can reduce microbial activity; there are many ways to achieve this, such as washing with vinegar, potassium permanganate, or chlorine. Ozone, a good oxidizer of other biomolecules, represents a green technology for washing vegetables. Ozone has been used in the export of fruits and vegetables and is generally recognized as safe regarding the quality of the food remains; additionally, it can extend the shelf life of fruits and vegetables. Ozonated water at a concentration of 9.0 mg/L reduced the levels of *Escherichia coli* O157 (by 2.89 log CFU/g), *Salmonella typhimurium* (by 2.56 log CFU/g), and *Listeria monocytogenes* (by 3.06 log CFU/g) without destroying vegetable cell structure [3]. Alexopoulos et al. [4] reported that washing cauliflower (*Lactuca sativa*) and *Capsicum annuum* with 0.5 mg/L ozonated water reduced bacterial counts. Moreover, Karaca and Velioglu [5] found that ozonated water at a concentration of 12 mg/L and chlorinated water at 100 mg/L were able to control *Escherichia coli* and *Listeria innocua* in celery. However, the amount of chlorophyll, vitamin C, and total phenolic compounds, as well as the antioxidant activity, decreased. The report of Geransayeh [6] showed that fruits treated with ozone had lower levels of decay and weight loss than controls, and that the combination of ozone and slurry ice allowed good maintenance of quality and promised further extension of product shelf life [7]. Moreover, Wang and Long [8] reported that hydrocooling cherry fruit in appropriate $CaCl_2$ solutions (i.e., 0.2-0.5 %) for 5 min and then passing the fruit through cold flume water for 15 min increased fruit firmness, retarded losses in vitamin C, titratable acidity, and skin color, and reduced splitting and decay following 4 weeks of cold storage. Therefore, the aim of this experiment was to study the effects of ozonated water alone and ozonated water combined with cold water to maintain quality and reduce microbial contamination of sunflower microgreens during cold storage.

2. Materials and methods

2.1 Effect of ozone exposure time on microbial contamination

Sunflower seeds were soaked for 24 h in water and then distributed over the wet surface of vermiculite-filled plastic trays. The trays were kept in a shade house at 25±5°C. The crop was kept moist until harvesting, and no fertilizer was applied. Microgreens were harvested after 7 days of germination, then cut and washed in the ozonated water [9].

The sunflower microgreens were washed with 1 g/L ozonated water at the time points of 30 s and 1, 3, and 5 min. The two controls comprised microgreens washed with tap water, and unwashed microgreens. The experimental design was completely randomized, with 5 replications. After storage at 8°C for 7 days, 10-g samples of sunflower microgreens from each treatment were placed in 90 mL of maximum-recovery diluent (MRD) and were serially diluted (10-fold) until 10^{-5} dilution. A 0.1-mL aliquot of the initial 3 dilutions in each sample was plated and spread on the surface of the potato dextrose agar (PDA) with streptomycin, and then incubated at room temperature for 72 h prior to counting the fungal colonies. The fungal colonies were isolated and purified to identify species of fungi by analyzing their morphological characteristics under a compound microscope (Olympus, Model CX23). Coliform- and total bacteria were counted on the surfaces of plate count agar (PCA) and Chromocult coliform agar (CCA). A 0.1-mL aliquot of the final three dilutions in each sample was plated and spread onto PCA or CCA agars and incubated at room temperature for 48 h to count the total bacterial colonies on PCA or the red colonies of coliform bacteria on CCA. The number of estimated colony forming units (CFU) for each sample was calculated by the formula CFU/mL = (number of colonies × dilution factor (inverse of the dilution)/volume of sample (0.1), and was then taken as a log value (log CFU/mL). The bacterial colonies were isolated and purified for classification using gram staining, endospore staining, and morphological analysis under a compound microscope [10].

2.2 Effect of ozone concentration on sunflower microgreen qualities

Ozonated water at 1, 5, and 10 g/L concentrations was produced using an ozone generator (Ebase, Model OZ-3090A, Thailand). Microgreens were harvested after 7 days of germination, cut, and washed either in the ozonated water or in tap water (control) for 5 min. After washing, 100 g of sunflower microgreens was immediately analyzed for quality. Additionally, 100 g of sunflower microgreens was placed in a plastic bag and stored at 8±1°C for 3 days, after which the qualities of vitamin C content, weight loss, and total soluble solids were determined [9].

The vitamin C content of the sunflower microgreens was determined using the 2, 6-dichloroindophenol titrimetric AOAC method. The titration volumes were compared with 1 g/L vitamin C (Sigma-Aldrich, St. Louis, MO, USA), and the results were expressed as mg of vitamin C per 100 g of fresh weight (g/food waste (FW)).

The weight of individual replications was recorded, and the percentage of weight loss was calculated by the formula [(before weight – after weight) ÷ before weight] × 100.

Total soluble solids (TSS) were measured with a hand refractometer (TLEAD, Model RHB-62ATC, Thailand) and expressed as a percentage.

2.3 Effect of ozonated water and ice combination on sunflower microgreen qualities

The experimental sunflower microgreens were washed with 1 g/L ozonated water alone and ozonated water combined with ice, while the control microgreens were washed with tap water and tap water with ice, for 5 min. The sunflower microgreens were analyzed immediately after washing and every 2 days during storage at 8±1°C for 8 days. The weight loss percentage, color a* value, and firmness were determined [9].

The weight loss percentage (%) was determined by comparing the weight of the vegetables on the sampling day with their initial weight, which had been determined on day 0.

The 1-10-level (hedonic) browning score was determined by 10 people, while the color determination a* values (green) of sunflower sprout leaves were determined using a color analyzer (Hunter Lab, Model Color Quest XE) at 3 datapoints/replication with 5 replications.

The firmness of 20 g of the sunflower microgreens was measured using a digital texture analyzer (Stable Micro System, Model TA. XT Plus, UK) with HDP KS5, and expressed in N.

2.4 Statistical analysis

Statistical analysis of the data obtained in the present study was carried out using a completely randomized design (CRD). Data obtained from the experiments in each replication were subjected to analysis of variance (ANOVA) and were evaluated with a regression analysis using the SPSS software package (Version 20.0, Chicago, III). Means were compared using Duncan's New Multiple Range test, and the differences in means were considered to be significant at $p \leq 0.05$.

3. Results

3.1 Effect of ozone exposure time on microbial contamination

An investigation of the effect of ozonated water on microbial contamination revealed that washing sunflower microgreens with 1 g/L ozonated water for **5** min was most effective in reducing the total fungal count, to a low of 1.10 log CFU/mL. The next most effective treatments involved washing with 1 g/L ozonated water for 3 min, 1 min, and 30 s to obtain fungal amounts of 1.46, 1.63, and 1.75 log CFU/mL, respectively. On the other hand, the control treatments—washing with tap water, and not washing—yielded the highest fungal amounts, 2.09 and 2.16 log CFU/mL (Table 1), which were significantly different from the ozonated treatments ($p \leq 0.05$). In addition, the identification of contaminant fungi using morphological characteristics under the microscope revealed that were *Aspergillus flavus* (Figure 1A), *A. niger* (Figure 1B), *Curvularia* sp.(Figure 1C), and *Fusarium* sp. (Figure 1D).

The determination of coliform and total bacterial counts on sunflower microgreens revealed that washing in 1 g/L ozonated water for 5 min yielded the lowest total bacterial count, of 4.21 log CFU/mL, and the lowest coliform bacterial count, of 4.07 log CFU/mL. This was followed by the 3-min, 1-min, and 30-s ozonated-water treatments, which resulted in total bacterial counts of 4.59, 4.70, and 4.77 log CFU/mL, as well as coliform counts of 5.20, 4.33, and 4.48 log CFU/mL, respectively. The ozonated treatments significantly reduced total-bacterial counts and coliform counts compared to the tap water control, which yielded values of 5.32 and 5.29 log CFU/mL, respectively. On the other hand, the non-washing control resulted in the highest total-bacterial count and coliform count, yielding values of 5.51 and 5.48 log CFU/mL, respectively (Table 1).

Table 1 Effect of washing in 1 g/L ozonated water for different durations on the amount of contaminant fungi, total bacteria, and coliform bacteria on sunflower microgreens.

Treatment	Contaminant fungi (log CFU/mL)	Contaminant total bacteria (log CFU/mL)	Contaminant coliform bacteria (log CFU/mL)
Without washing	2.16 ± 0.08^{a}	5.51 ± 0.14^{a}	5.48 ± 0.15^{a}
Tap water	2.09 ± 0.05^{a}	5.32 ± 0.11^{b}	5.29 ± 0.13^{ab}
O_3 water for 30 s	1.75 ± 0.07^{b}	4.77 ± 0.10^{c}	5.20 ± 0.12^{b}
O_3 water for 1 min	1.63 ± 0.09^{bc}	4.70 ± 0.08^{cd}	4.48 ± 0.09^{c}
O_3 water for 3 min	1.46 ± 0.08^{c}	4.59 ± 0.07^{d}	4.33 ± 0.08^{c}
O_3 water for 5 min	1.10 ± 0.03^{d}	4.21 ± 0.05^{e}	4.07 ± 0.06^{d}
CV (%)	12.32	14.54	13.41

Values (mean±SD) in the same columns with different lowercase letters (a, b, c, d, e) are significantly ($p \leq 0.05$) different.

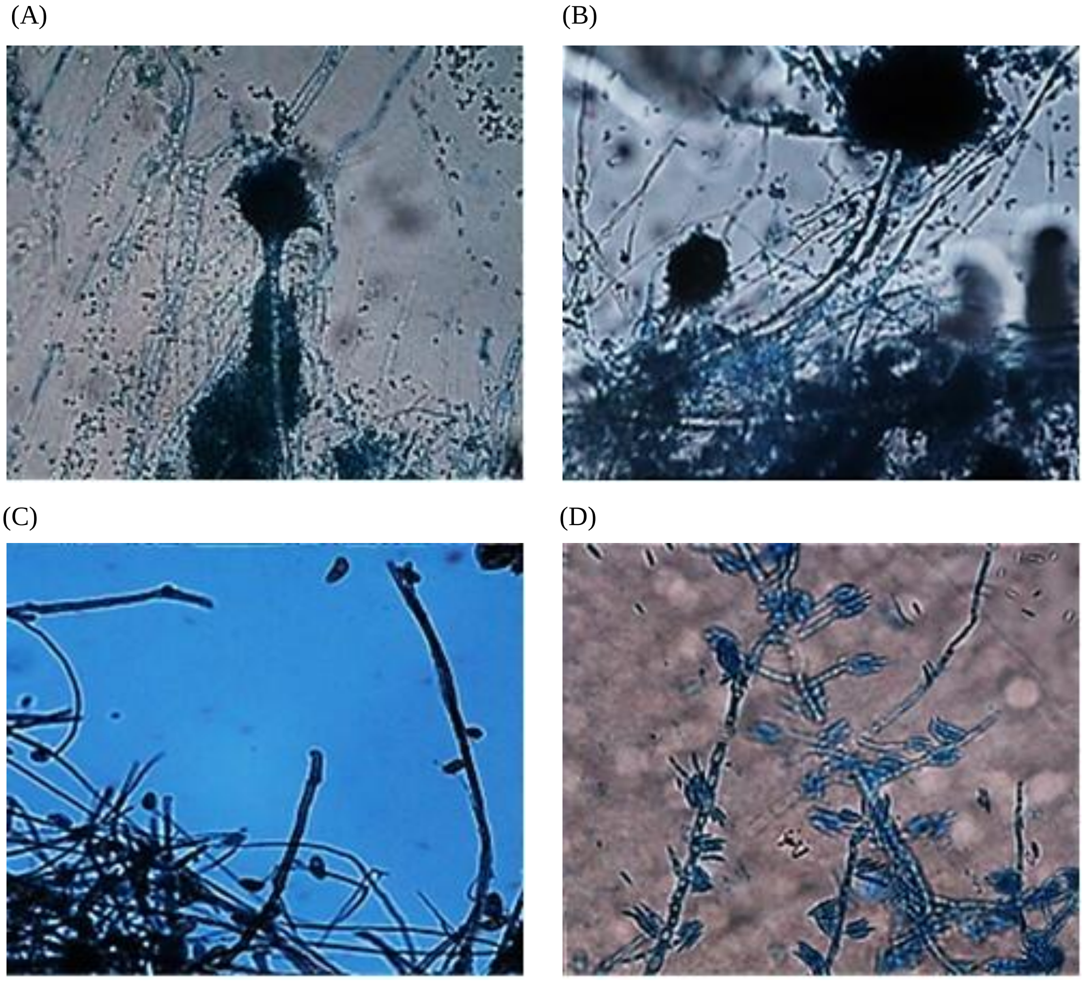

Figure 1 Fungal contaminants of the studied sunflower microgreens. *Aspergillus flavus* (A), *A. niger* (B), *Curvularia* sp. (C), and *Fusarium* sp. (D).

3.2 Effect of ozone concentration on sunflower microgreen qualities

The sunflower microgreens exhibited lower vitamin C contents after ozonated water treatments compared with tap water treatments. In addition to their storage time increasing by 3 days, the sunflower microgreens treated with 1 g/L ozone had significantly increased vitamin C content, rising from 1.49 to 1.67 mg 100 g/FW, while the other treatments resulted in decreased vitamin C content. However, the sunflower microgreens showed a decrease in vitamin C content with increasing ozone concentration.

The sunflower microgreens treated with 1 g/L ozonated water showed the lowest percentage of weight loss after storage at 8°C for 3 days, at 0.04%, which differed significantly from the tap water treatment (with 0.07% weight loss). Meanwhile, the sunflower microgreens treated with 5 and 10 g/L ozonated water had weight losses of 0.06 and 0.08%, respectively.

The TSS percentage of sunflower microgreens in the tap water treatment decreased from 2.95 to 2.55% when stored for 3 days, whereas the treatments with ozonated water at 5 and 10 g/L increased TSS from 2.08 to 2.13% and from 2.48 to 2.60%. In contrast, the treatment with 1 g/L ozonated water did not affect the TSS concentration of sunflower microgreens (Table 2).

Table 2 Effect of different concentrations of ozonated water on the quality of sunflower microgreens after washing and after 3 days in storage at 8±1°C.

Treatment	Storage 8±1°C (d)	Vitamin C (mg 100 g/FW)	Weight loss (%)	Total soluble solids (%)
Tap water	0	2.26±0.19[a]	0.00±0.00[c]	2.95±0.05[a]
	3	1.15±0.07[b]	0.07±0.00[a]	2.55±0.05[a]
O_3 water at 1 g/L	0	1.49±0.17[b]	0.00±0.00[c]	2.05±0.05[c]
	3	1.67±0.00[a]	0.04±0.01[b]	2.05±0.03[c]
O_3 water at 5 g/L	0	1.67±0.15[a]	0.00±0.00[c]	2.08±0.08[c]
	3	1.49±0.15[b]	0.06±0.02[ab]	2.13±0.03[bc]
O_3 water at 10 g/L	0	1.91±0.11[a]	0.00±0.00[c]	2.48±0.18[b]
	3	1.01±0.04[b]	0.08±0.01[a]	2.60±0.16[a]
CV (%)		17.40	19.65	16.23

Values (mean±SD) in the same columns with different lowercase letters (a, b, c) are significantly ($p \leq 0.05$) different.

3.3 Effect of ozonated water and ice combination on sunflower microgreen qualities

The weight loss percentage of all treatments increased with increased storage time at 8±1°C. The sunflower microgreens in the treatment of 1 g/L ozonated water combined with ice for 5 min presented the lowest weight loss percentage, producing values of 0.09**-**5.14%, which was significantly different compared with the other treatments (3.94 to 11.34%), after storage for 8 days (Figure 2). Moreover, the treatments of ozonated water and ozonated water with ice provided significantly reduced browning scores compared with treatment with tap water and tap water with ice after storage at 8±1°C for 6 and 8 days (Figure 3). The ozone treatments also showed a significant tendency toward low a* value (with the values for green to red color ranging from -11.49 to -9.85) in the green color of the sunflower leaves compared with the tap water treatment (with values for green to red color ranging from -10.94 to -8.85)**.** The firmness of sunflower microgreens was not significantly different between treatments during the storage period (Table 3).

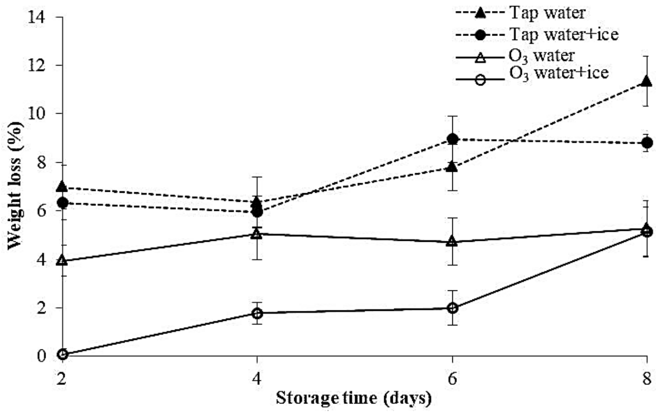

Figure 2 Effect of washing with ozonated water and tap water, with and without ice, on the weight loss of sunflower microgreens during storage at 8±1°C.

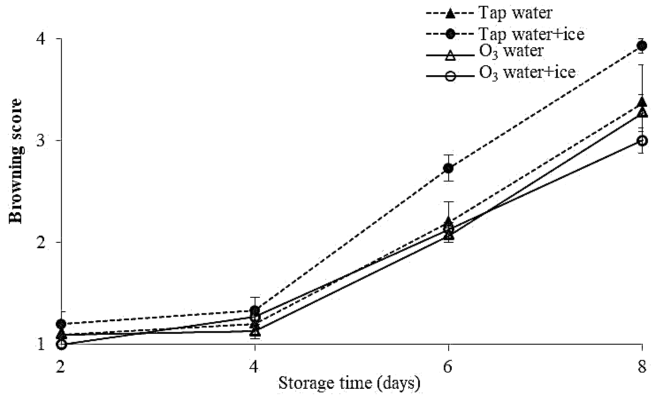

Figure 3 Effect of washing with ozonated water and tap water, with and without ice, on the browning score of sunflower microgreens during storage at 8±1°C.

Table 3 Effect of washing with ozonated water (1 g/L) and tap water, with and without ice, on the a* color value and firmness of sunflower microgreens during storage at 8±1°C.

Parameters	Treatment	Storage time (days)			
		2	4	6	8
a* color value	Tap water	-10.26±0.41^{a}	-10.21±0.29^{ns}	-10.78±0.55^{ns}	-8.85±0.81^{a}
	Tap water+ice	-10.93±0.50^{ab}	-10.94±0.38^{ns}	-10.53±0.49^{ns}	-9.89±0.42^{ab}
	O_3 water	-10.74±0.39^{ab}	-10.13±0.65^{ns}	-10.70±0.31^{ns}	-10.94±0.36^{b}
	O_3 water+ice	-11.49±0.15^{b}	-10.10±0.29^{ns}	-9.85±0.63^{ns}	-10.21±0.37^{ab}
Firmness (N)	Tap water	5.33±1.37^{ns}	4.22±0.74^{ns}	3.00±0.53^{ns}	5.44±1.76^{ns}
	Tap water+ice	5.00±0.60^{ns}	4.00±0.73^{ns}	4.44±0.87^{ns}	6.67±2.03^{ns}
	O_3 water	7.11±1.50^{ns}	3.33±0.58^{ns}	3.22±0.83^{ns}	7.44±1.14^{ns}
	O_3 water+ice	4.44±0.71^{ns}	5.22±0.76^{ns}	4.67±0.80^{ns}	3.11±0.61^{ns}

Values (mean±SD) in a column with different lowercase letters (a, b) are significantly ($p\leq0.05$) different and "ns" refers to non-significant difference ($p>0.05$).

4. Discussion

Ozone effectively kills microorganisms through oxidation of their cell membranes [7]. The present study demonstrated the high efficacy of ozonated water at a 1 g/L concentration in reducing total fungal contamination of sunflower microgreens, especially when used at longer washing times for 5 min. Most of the contaminant fungi in our study were *Aspergillus flavus*, *A. parasiticus*, and *A. niger*, which is a cause of cancer in humans; a few fungal pathogens were also identified as *Curvularia* sp. and *Fusarium* sp. In comparison, Afzal et al. [11] reported 13 phytopathogenic fungal species associated with seven cultivars of sunflower to reduce seed germination by 10-20% and seedling mortality by 10-12%, including *A. flavus*, *A. fumigatus*, *A. niger*, *Curvularia lunata*, *Fusarium solani*, and *F. moniliforme*. Çetinkaya et al. [12] demonstrated that applying ozone to tomato and cucumber seeds inactivated *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radicis-lycopersici*, respectively, with no negative effect on seed germination rate. Similarly, in the present study, a 1 g/L concentration of ozonated water considerably reduced the total- and coliform-bacterial counts on contaminated sunflower microgreens, especially when washing for 5 min, which reduced the total bacterial contamination by 1.3 log CFU/mL compared with the control treatment (no washing). Karaca and Velioglu [5] found that washing lettuce, spinach, and coriander in 12 mg/L ozonated water for 20 min affected the amount of *Escherichia coli* and *Listeria innocua* bacteria. Similarly, Feliziani et al. [13] reported that ozone was toxic to microorganisms when used at high concentrations, with a high concentration (2 g/L) eliminating the various bacteria and fungi studied in a short time [14]. Hernández-Arias et al. [15] found that ozone at a concentration of 1 g/L for 3 min inactivated *E. coli* in water by 83%. Similarly, our study showed that only 3 min of washing microgreens with 1 g/L ozonated water significantly reduced the coliform bacteria (by 1.46 log CFU/mL) compared to the unwashed control.

Vitamin C (ascorbic acid) is an essential nutrient involving in many physiological functions in the human body, such as collagen synthesis, neuromodulation, and immune system maintenance [16]. Vitamin C content in sunflower microgreens washed in 1 g/L ozonated water significantly increased from 1.49 to 1.67 mg 100 g/FW after storage at 8°C for 3 days. It has been suggested that the activities of several enzymes, such as ascorbate peroxidase (APX) and ascorbate oxidase (AO), are stimulated under ozone stress, leading to the biosynthesis of vitamin C in plants. According to Yeoh et al. [17], the vitamin C concentration increased in fresh-cut papaya treated with 9.2 µL/L ozone for 10 min. However, the vitamin C decreased with high concentrations of ozonated water (5 and 10 g/L) in the present study, which may be explained by the fact that the AO enzyme is degraded by high ozone concentrations, as demonstrated through ozone-related vitamin C reduction in carrot sticks [18], fresh-cut parsley [5], and green chili pepper [19].

According to Glowacz and Rees [19], ozone at 0.9 µmol/mol reduced the weight loss of red and green chili peppers during storage at 10°C. Similarly, the application of 1.6 mg/kg ozone-enriched atmosphere 12 h per day led to a decrease in weight loss in Valencia oranges following 14 days of cold storage [20]. The present study found that ozonated water at a concentration of 1 g/L reduced the weight loss percentage of sunflower microgreens compared with tap water (control), whereas the high ozone concentrations of 5 and 10 g/L increased the weight loss of the sunflower microgreens. This may be because the ozonated water at 5 and 10 g/L concentrations was too strongly oxidating, increasing the effects on transpiration and damaging the sunflower microgreens. Similarly, Glowacz and Rees [19] found that the increase in ozone concentration was a cause of increasing weight loss in red and green chili peppers. In line with the above, the increase in TSS detected in sunflower microgreens after treatment with 5 and 10 g/L ozonated water was related to increased weight loss.

Additionally, this study showed that the sunflower microgreens treated with 1 g/L ozonated water combined with ice at 5°C for 5 min had the smallest weight loss after a storage time of 8 days. This result suggests that ozone and precooling treatment can induce the shrinkage of stomata in the epidermis, which reduces water transpiration and delays the decomposition of plant cell walls [21]. After treatment with ozonated water and ozonated water with ice, there was delayed browning when compared with that of tap water treatments after storage at 8±1°C for 6 and 8 days. Moreover, low temperatures can reduce the chemical reactions in sunflower microgreens. In addition, the ozone treatments showed a tendency for significantly lower a* values (green color) for sunflower leaves than the tap water treatment, as the ozone did not damage the chlorophyll in sunflower leaves. Similarly, Karaca and Velioglu [5] reported that 12 mg/L ozone did not affect chlorophyll a or chlorophyll b in lettuce, spinach, or parsley. On the other hand, the sunflower leaves in the tap water treatments had higher a* values (red color) and browning scores than did leaves subjected to the ozone treatments. The ozone treatments did not increase chlorophyll degradation, whereas the tap water treatment showed increased chlorophyll degradation with increased storage time, indicating the senescence of plant metabolism and catabolism. It has been reported that chlorophyll-degradation-related genes affect color during postharvest storage of broccoli [22] and green pepper [23].

In addition to color, ozone treatment had no negative effect on the firmness of sunflower microgreens during storage**.** The ozone oxidized only the surface of the sunflower microgreens but did not damage their internal cell structure. Similarly, Souza et al. [24] reported that carrot exposure to ozonated water did not change the characteristics of firmness, weight loss, or color**.**

Overall, our results indicate that ozone dissolved in low-temperature water has high efficacy in reducing harmful microorganisms and maintaining the quality of sunflower microgreens.

5. Conclusions

Washing sunflower microgreens in 1 g/L ozonated water for 5 min effectively eliminated harmful microorganisms, with no negative effect on vitamin C content and weight loss after storage at 8±1°C for 3 days. Combined treatment of 1 g/L ozonated water and ice at 5°C for 5 min yielded significantly lower weight loss percentages and browning scores throughout the storage period (8 days) than tap water, with no negative effect on firmness and a* color values. Therefore, the 1 g/L ozonated water combined with ice precooling effectively reduced the contamination and quality maintenance of sunflower microgreens.

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7. Conflicts of interest

The authors have no conflicts of interest to declare.

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