

Evaluation of Nonthermal Plasma Treatment by Measurement of Stored Citrus Properties

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Abstract

Decay of fruit is one of the greatest issues in fruit storage. **Purpose:** In this study, citrus sterilization was performed to evaluate a dry sterilization method using an atmospheric-pressure nonthermal plasma treatment based on a dielectric-barrier discharge technique. **Methods:** Citrus samples were stored under four different environmental conditions as follows: group A had cold storage with plasma treatment with a temperature of $6.2 \pm 1.0^\circ\text{C}$ and relative humidity (RH) of $93.4 \pm 8.2\%$, group B had ambient-temperature storage with $22.9 \pm 2.3^\circ\text{C}$ and $82.1 \pm 4.5\%$ RH, group C ambient-temperature storage with plasma treatment with $25.3 \pm 2.2^\circ\text{C}$ and $90.0 \pm 2.8\%$ RH, and group D had cold storage with $5.7 \pm 1.0^\circ\text{C}$ and $93.4 \pm 6.5\%$ RH. **Results:** As a result of citrus surface sterilization by plasma treatment, treatment groups A and C together showed an average of 16.1 CFU/mL of mold colonies, while control groups B and D showed an average of 2.2×10^2 CFU/mL or approximately 13 times greater than the treatment groups. Regarding the mean concentration of aerobic bacteria colonies, the treatment groups (A and C) and control groups (B and D) showed an average of 7.1 CFU/mL and 1.9×10^3 CFU/mL, respectively. This is approximately a 270-fold difference in the concentration of pathogen colonies between treatment and control groups. **Conclusions:** The results showed the potential of nonthermal plasma treatment for citrus storage in enhancing storage duration and quality preservation.

Keywords: Citrus, Storage, Dry sterilization, Microorganism, Nonthermal plasma treatment

Introduction

Citrus is the most popular domestic fruit for Koreans (MAFRA, 2015) and is in particular the young generation's favorite fruit (KREI, 2015). The island of Jeju is the main citrus production location in the Republic of Korea (KR), and a main variety (*Citrus unshiu*) is grown on 86.4% of the citrus farms. Because of recent consumer demand for organic and safe fruits for the sake of health, the quantity of organic and pesticide-free agricultural products is increasing (Kim et al., 2013). The area occupied by organic citrus farms is estimated to be 1,500 ha or 7% of all citrus farms in Jeju (Ku et al., 2015).

The *Citrus unshiu* variety is harvested from November to the following January such that the price drops for a few months during harvest. During the season after January, citrus fruits may be damaged during storage period and the quality may decline. The product sales volume drops from 30% in December to 10% in the following February such that the price of citrus increases from March onward (Ahn et al., 2018). Yang et al. (1997) reported that a storage period of 90 days is reasonable for citrus.

One of the causes of quality degradation during citrus storage is green mold such as *Penicillium italicum* or *Penicillium digitatum* (Holmes and Eckert, 1999). These molds are widely reported to be the two most significant postharvest pathogens in citrus. Infected citrus may become completely covered with the green and bluish spores of *P. italicum* and *P. digitatum*. The citrus infection

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spreads in the box and sound fruits are directly affected regardless of damage. To date several studies have been undertaken to develop sterilization methods against fungi. A customary method of fungal control for agricultural products is washing and waxing; however, Yang et al. (1997) and Kim et al. (2016) developed alternative methods as well as pesticides and fumigation, respectively. Hyun et al. (2001) reported a curing method based on washing with hot water but such washing may cause spoilage and quality degradation. Recently, studies of dry sterilization methods for agricultural products have emerged.

Nonthermal plasma is an emerging dry sterilization technology that involves electrons, ions, and neutral particles produced at room temperature (Brun et al., 2012). The atmospheric-pressure nonthermal plasma treatment is based on dielectric barrier discharge (DBD) technology and uses high-voltage electric discharge to produce gases such as nitrogen dioxide and ozone that can contribute to microbial inactivation. DBD is a common example of an atmospheric-pressure nonthermal plasma-generating method based on two charged electrodes separated by insulated material (Brandenburg, 2017). Other research relates to cold-plasma dry sterilization of microorganisms on onion powder to improve the safety and quality of the powder products (Won et al., 2016), sterilization of food poisoning pathogens on dried peppers (Song et al., 2016), improvement of sterilization with cold plasma and ultraviolet C for pathogens (*E. coli*, *Salmonella typhimurium*, and *Listeria monocytogenes*) spiked on cabbage (Seong et al., 2017), and inactivation of food poisoning bacteria (*Salmonella typhimurium*) on eggs (Mok and Song, 2013). There are also comparisons of sterilization methods for sea water and fresh water using plasma treatment and ozone treatment (Oehmigen et al., 2010).

Although many studies exist for agri-food sterilization using cold plasma, few studies have been undertaken on both fungi and aerobic bacteria sterilization based on ambient and cold-temperature storage with plasma treatment for citrus. Besides, the studies are limited to low concentrations (less than 1 ppm) of gas such as ozone and nitrogen dioxide for sterilization of agricultural products at different storage temperatures. The objectives of this work are to evaluate (1) the efficacy of atmospheric-pressure nonthermal plasma (APNP) treatment for the reduction of spoilage mold and aerobic bacteria on the surface of citrus fruit at ambient temperature and at cold

temperature, and (2) the effect of APNP treatment on weight, color, sugar content, acidity, and firmness of the citrus.

Materials and Methods

Samples and storage condition

Samples of citrus (*Citrus unshiu*) that were harvested in Jeju in July 2018 were purchased at the Seoul Garak market. All samples were analyzed visually for damages such as bruises or scars; damaged and undersized samples were then removed. Collected samples were numbered and the weights measured before storing the samples according to four storage conditions. During the storage period, using the randomly selected 120 samples from four storage conditions, traceable properties of the selected sample such as weight and color were measured. In this study, four storage conditions were applied: cold storage with nonthermal plasma treatment (C+P, group A), ambient-temperature storage (A, group B), ambient-temperature storage with nonthermal plasma treatment (A+P, group C), and cold temperature storage (C, group D). We selected the number of samples according to the space for each storage facility. Ambient-temperature storage (group C) permitted only one box, while cold storage for plasma treatment (group A) had enough space for more samples. The total number of samples n for each storage condition was as follows: $n = 450$ for group A, $n = 150$ for group B, $n = 300$ for group C, and $n = 300$ for group D.

Nonthermal plasma actuator and measurement of gas concentration

The nonthermal plasma actuator (an in-house system by the Plasmapp Co. Ltd., Daejeon, KR) was developed based on DBD for the two storage groups A and C with three and ten discharge cylinders, respectively. The discharge source had two coils inside and outside that were part of a 2 mm thick ceramic cylinder. Group A (C+P) was in a storage container measuring 2.3 m (long) by 3.8 m (wide) by 2.3 m (high) while group C (A+P) used a 1.0 m by 1.0 m by 1.0 m container (Fig. 1). Gas flow was circulated through a Teflon pipe by a diaphragm pump (model 20RNS, G&M Tech Inc., Gunpo, KR).

We measured the concentration of ozone (O_3) and nitrogen dioxide (NO_2) gases using an O_3 detector based on UV absorption at 254 nm (model 106-L, 2B Technologies

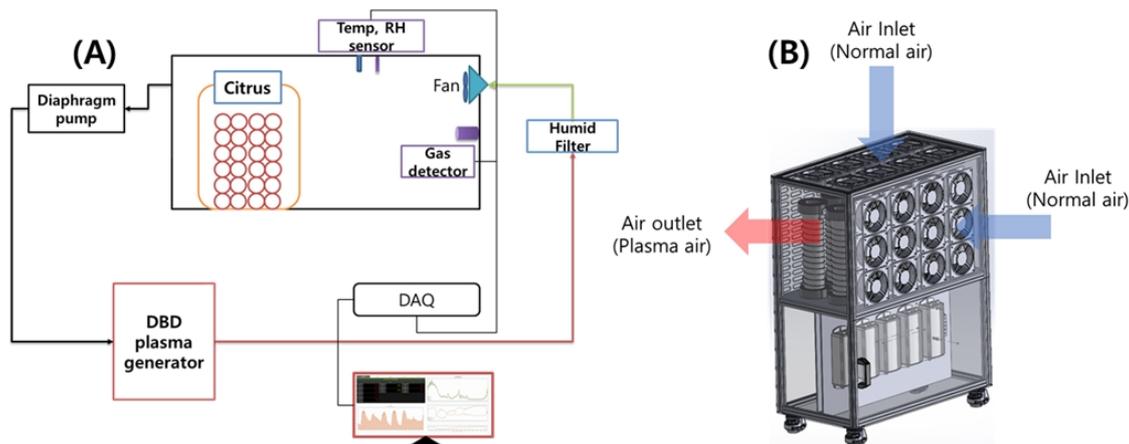


Figure 1. Schematic drawing of plasma generators for (A) ambient temperature with plasma treatment (A+P), and (B) cold storage with plasma treatment (C+P).

Inc., Colorado, USA) in the range of 0-100 ppm with 0.001 ppm resolution and using an NO₂ detector (model ENW, Aeroqual Ltd., Auckland, NZ) in the range of 0-1 ppm with 0.001 ppm resolution, respectively. Measured concentrations of O₃ and NO₂ were < 1 ppm and < 0.9 ppm, respectively. Storage temperature and humidity were measured and averaged using a data logger (model TR-7wf, T&D Corporation Ltd., Hyogo, JP). Initial environmental conditions were as follows: cold storage with plasma (group A) had 6.2 ± 1.0 °C and 93.4 ± 8.2% relative humidity (RH), ambient-temperature storage (group B) had 22.9 ± 2.3 °C and 82.1 ± 4.5% RH, ambient storage with plasma treatment (group C) had 25.3 ± 2.2 °C and 90.0 ± 2.8% RH, and cold storage (group D) had 5.7 ± 1.0 °C and 93.4 ± 6.5% RH.

Measurement of physical properties

During the storage period, physical properties of stored citrus were measured with the randomly selected samples from the each storage conditions. Color difference, weight, sugar content, acidity (pH), and firmness were measured using a colorimeter (model CR-400, Konica Minolta Inc., Seoul, KR), an electronic scale (model MWP, CAS Co., Seoul, KR), a refractometer (model DBX-55, Atago Co. Ltd., Tokyo, JP), a pH meter (model A211, Thermo Fisher Scientific Inc., Massachusetts, USA), and a universal test machine (UTM) (model AG-X 500N, Shimadzu Co., Tokyo, JP), respectively. Weight and color difference were measured of the selected 120 samples to be traceable for the storage period. On the other hand, we randomly collected 40 samples for invasive measurement of sugar contents, acidity and peeled and ground every

sample. To separate the pulp from the juice, we used a fine filter. Using the filtered juice, we measured pH using the pH meter and soluble sugar content (total sugar solids) using the refractometer, replicating the measurements to calculate the mean and the standard deviation (SD). For the firmness of the citrus peel and flesh, we calculated mean values from measurements repeated 180 degrees apart around the circumference of the sample using a 2 mm diameter tip with 10 mm/min speed. Colors were measured without any light interference after calibration with a white Teflon plate (Y: 93.8, x: 0.3131, y: 0.3192) and expressed as Yxy values, which were then converted to CIE L*a*b* color space. The value of color difference (ΔE) was calculated with each color value as follows: $\Delta L^* = (L_1^* - L_0^*)^2$, $\Delta a^* = (a_1^* - a_0^*)^2$, and $\Delta b^* = (b_1^* - b_0^*)^2$ where, L* represents brightness of the material, a* means red (positive) and green (negative), and b* means yellow (positive) and blue (negative) (Gong et al., 2016).

Measurement of microorganisms

To compare sterilization performance for the different storage conditions, we counted the number of colonies of mold and aerobic bacteria. To prepare an aerobic bacteria culture, 9 mL of distilled water was mixed with 1 g of citrus peel, and then homogenized with a stomacher (model 1930, 3M Co. Ltd, Daejeon, KR) for 5 minutes. An aerobic plate (Petri film aerobic count plate, 3M Co. Ltd, Minnesota, USA) was inoculated with 1 mL of homogenized mixture in triplicate and incubated at 35 °C for 24-48 h. The colonies were counted by 3M plate reader (Petri film

plate reader, 3M Co. Ltd, DE). To prepare a mold culture, we followed a procedure similar to that of the aerobic bacteria culture, but a potato dextrose agar (PDA) plate (Difco, PDA, BD Co. Ltd., Maryland, USA) was used instead of a 3M plate for the inoculation. Grown colonies of mold were counted using OpenCFU (Geissmann, 2013), which is an open-source program for counting cells based on an image processing method. Statistical analyses such as analysis of variance (ANOVA), Duncan's multiple range test, and principal component analysis (PCA) were performed using R (ver. 3.3.2, R Core Team, Vienna, AT), which is an open-source statistical program, and its packages.

Results and Discussion

Weight, color, sugar content, pH, and firmness

Results of the weight measurements every week during storage are shown in Figure 2. As shown in the figure, four lines represent the four storage conditions. Weight reduction between the first and the last week measurements with ambient-temperature storage (groups B and C) on average 16.57 g and with cold-temperature storage (groups A and D) on average 3.99 g differed by approximately 4 times. Besides, plasma treatment with ambient-temperature storage showed a 10% difference compared to the

control group, while cold storage plasma treatment showed a 25% difference. The storage period, for example over 3 to 5 weeks of storage, is highly correlated with weight reduction ($P < 0.001$). Weight loss during storage is common and depends somewhat on storage condition because of fruit respiration and evaporation during storage (Lee et al., 2009, Kim et al., 2002).

The visual appearance of the citrus was measured as $L^*a^*b^*$ values and the difference (ΔE) is shown in Figure 3. Although there was little difference between the first and second weeks, ambient-temperature storage with plasma treatment (A+P) showed a color difference as high as 6.1. Over the nine-week period, the A+P value decreased as a log function curve (slope: $-2.36 \ln(X)$, $R^2 = 0.95$). In the last week, the color differences for the groups were 0.79 (C+P), 1.02 (A), 0.89 (A+P), and 1.41 (C), indicating a distribution of ± 0.27 . The rate of reduction of color difference between the last week and the first week was on average 84% probably because the citrus ripened during storage period thereby reducing the color difference. In the last week, the plasma treatment group had $\Delta E = 0.84 \pm 0.07$ and the control group had $\Delta E = 2.14 \pm 0.65$ on average, both of which show a 44% color difference. This result shows a 62% reduction from the first color difference of $\Delta E = 3.49 \pm 3.69$ for the plasma treatment and $\Delta E = 2.14 \pm 0.65$ for the control. Ramazzina et al. (2015) reported that plasma treatment had less effect on kiwi surface hue angle and saturation

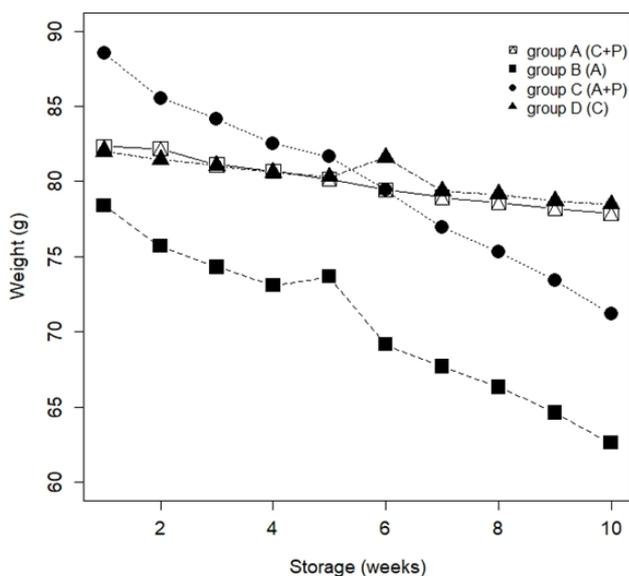


Figure 2. Measurement of weight for four storage conditions during storage period. Results represent the mean ($n = 120$).

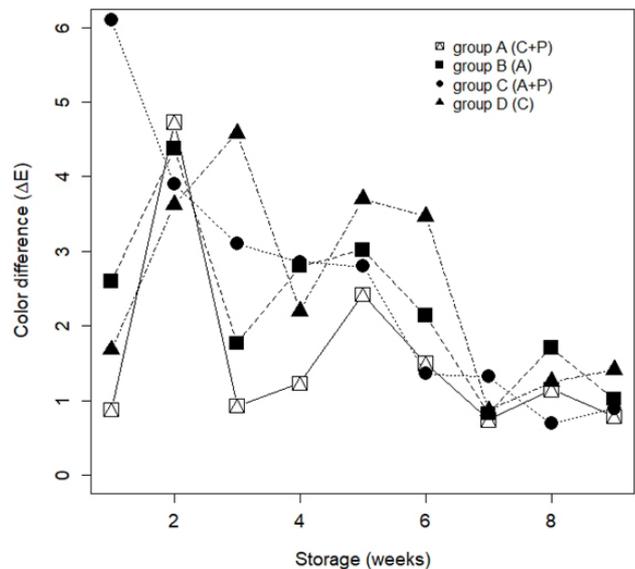


Figure 3. Measurement of color difference (ΔE) for four storage conditions during storage period. Results represent the mean ($n = 120$).

value; however, after storage both parameters showed a significant decrease.

We measured destructive properties such as sugar content ($^{\circ}$ Brix), acidity (pH), and firmness (kilogram force or kgf) with 40 samples randomly selected from four storage conditions. The sugar content over the storage period is presented in Figure 4. Initial sugar content values were 10.0 to 10.8 $^{\circ}$ Brix, and after 8 weeks the values were 10.8 to 12.4 $^{\circ}$ Brix. The mean value increased from 10.4 ± 0.41 $^{\circ}$ Brix initially to 11.4 ± 0.41

$^{\circ}$ Brix after 8 weeks, indicating a 9.5% increase. To evaluate sugar content according to storage conditions, an analysis of variance (ANOVA) was performed for storage conditions and changing sugar content but this analysis was inconclusive. The result showed that sugar content had little relationship with storage conditions. Sugar content changes little during storage because of evaporation from flesh to peel, promoting weight loss and concentration of inner materials (Kim et al., 2002).

The acidity of the citrus was measured with a pH meter,

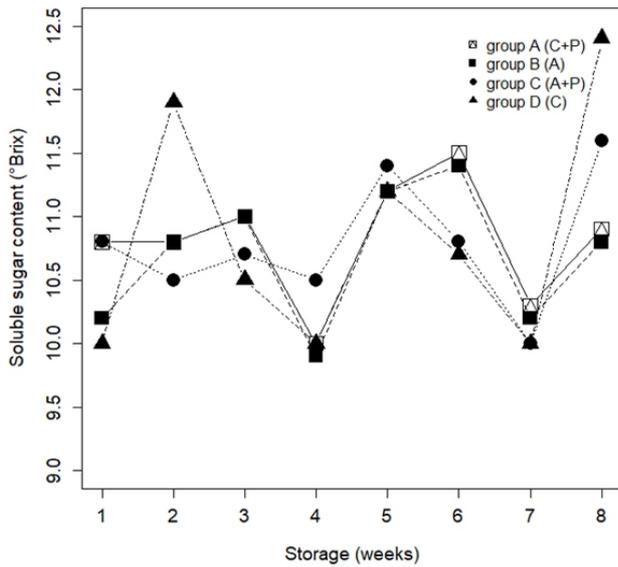


Figure 4. Measurement of soluble sugar content ($^{\circ}$ Brix) for four storage conditions during storage period. Results represent the mean (n = 40).

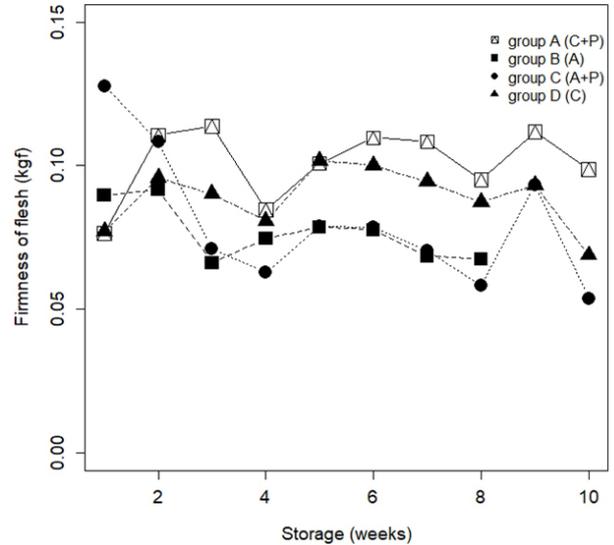


Figure 6. Measurement of firmness of flesh for four storage conditions during storage period. Results represent the mean (n = 40).

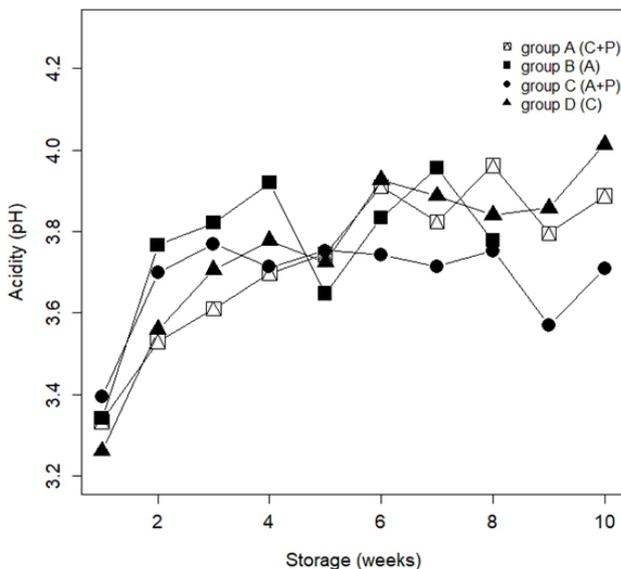


Figure 5. Measurement of acidity (pH) for four storage conditions during storage period. Results represent the mean (n = 40).

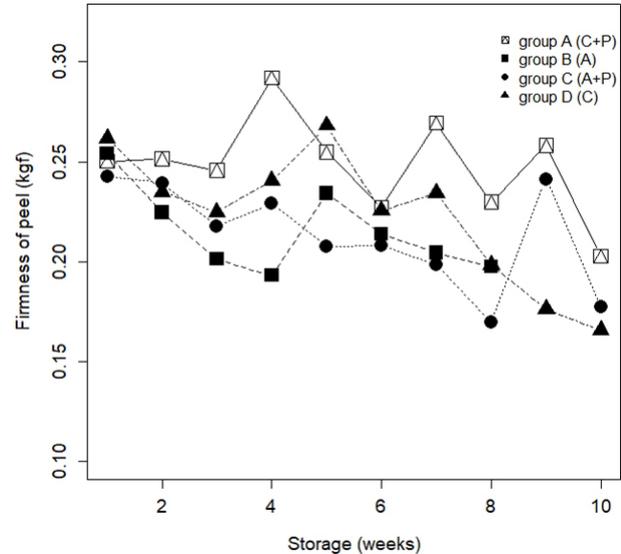


Figure 7. Measurement of firmness of peel for four storage conditions during storage period. Results represent the mean (n = 40).

and the results are shown in Figure 5. In the case of A+P (group C), pH was somewhat reduced from 3.77 to 3.71 over 4 weeks. Ambient-temperature storage (group B) results showed a reduction from 3.92 to 3.65 or as much as 6.9% in 6 weeks. Other studies have shown that, with ambient temperature the concentration of ascorbic acid is reduced (Burdurlu et al., 2006), whereas with cold storage at 5 °C the concentration is increased (Roig et al., 1999).

Firmness was reduced as storage time increased according to the results for peel and flesh illustrated in Figures 6 and 7. The ambient-temperature storage (groups B and C) datasets had similar firmness of flesh results (Fig. 6) of 0.07 to 0.08 kgf in 5 weeks and similar firmness of peel results (Fig. 7) of 0.21 to 0.24 kgf, whereas the corresponding cold storage with plasma treatment (group A) dataset showed 0.09 to 0.15 kgf for flesh and 0.25 to 0.28 kgf for peel.

Fungal control and aerobic bacteria

Table 1 shows the results of fungal sterilization with nonthermal plasma treatment under the cold and ambient-temperature conditions. The plasma treatment datasets (groups A and C) had mean concentrations of 10.62 and 21.7 colony-forming units per milliliter (CFU/mL), whereas the control datasets (groups B and D) had higher mean values of 332.65 and 113.17 CFU/mL.

This indicates that the plasma treatments sterilized mold on the surface of citrus more effectively by a factor of 31 compared to the control groups. Group A (C+P) showed the lowest at 0.6 CFU/mL in the third week; however, this rose to 23.46 CFU/mL in the seventh week. The control groups also showed some low and high values through the entire storage period from 53 to 863 CFU/mL (group B) and from 19 to 302 CFU/mL (group D) even though these fruits were stored without any treatments. The reason for the control group having different mold counts is that different samples were selected every week to inoculate the rinsed samples on the PDA plates. Each individual sample may have its own condition such that it is difficult to homogenize the sterilization condition. Nonetheless, the plasma treatment groups differed significantly ($P < 0.001$) from the control groups. Post-hoc testing with the Duncan test was performed to define the relationship between mean values (Table 1). The control groups are shown in Table 1 with superscript "a" and the plasma treatment groups are shown with superscript "b". The Duncan test reveals that the difference between the mean values for plasma treatment and the mean values for control group is statistically significant. The PCA method was applied to show the relationship of every group in 3D space (Fig. 8). In the figure, black and green spheres illustrate groups A and C, red ones represent group B, and blue ones are for

Table 1. Concentration of mold colonies (in CFU/mL) for four storage conditions during storage period

Storage condition Storage period (week)	group A (C+P)	group B (A)	group C (A+P)	group D (C)
1	55.88 ± 15.82	53.11 ± 49.84	33.0 ± 16.74	19.11 ± 13.96
2	3.60 ± 2.84	471.6 ± 338.52	126.33 ± 29.96	27.86 ± 12.64
3	0.60 ± 0.82	149.66 ± 98.4	40.66 ± 73.33	148.33 ± 110.5
4	2.73 ± 2.54	296.26 ± 365.98	9.4 ± 4.92	50.93 ± 31.12
5	1.93 ± 1.98	59.46 ± 23.06	0.4 ± 0.63	67.4 ± 87.32
6	3.20 ± 2.59	418.2 ± 321.39	2.33 ± 1.29	141 ± 116.44
7	23.46 ± 47.06	170.33 ± 95.55	0.26 ± 0.45	134.66 ± 99.7
8	15.26 ± 9.12	863.26 ± 283.28	0.2 ± 0.56	29.33 ± 27.29
9	0.86 ± 1.55	501.13 ± 315.87	0.2 ± 0.56	208.53 ± 238.72
10	1.06 ± 1.75	X	0.26 ± 0.59	301.8 ± 442.64
Mean ± SD	10.62 ± 16.83 ^b	332.65 ± 249.05 ^a	21.7 ± 39.94 ^b	113.17 ± 91.74 ^a

group A (C+P) : cold storage and plasma treatment, group B (A) : ambient storage
group C (A+P) : ambient storage and plasma treatment, group D (C) : cold storage
mean: average, SD: standard deviation, X: missing data
a, b : Duncan's test to compare means within 95% confidence level

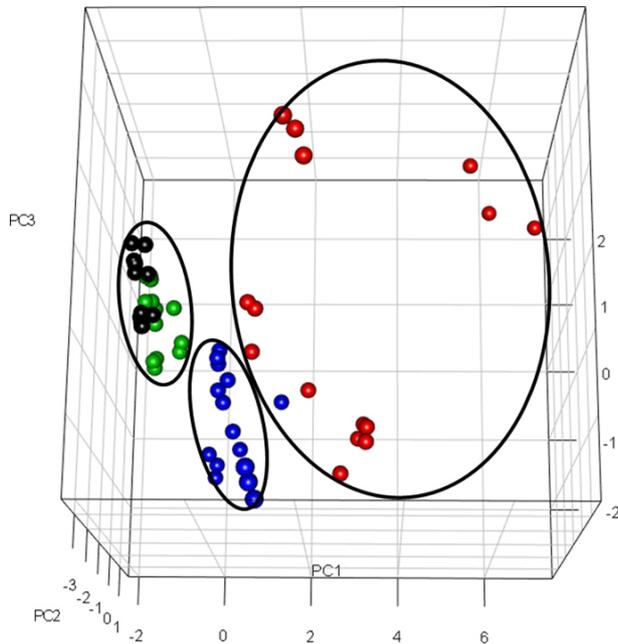


Figure 8. Mold inactivation result based on PC1 and PC3 space. Black-filled circle is group A and green-filled circle is group C (both are plasma treatment samples). Red-filled circle is group B and blue-filled circle is group D (both are control group samples).

group D. The PC1 and PC3 domains illustrate the obvious discriminant result.

Aerobic bacteria sterilization results are shown in Table 2 for 7 weeks. The plasma treatment datasets showed mean concentrations of 2.0 and 12.3 CFU/mL, whereas the control group datasets showed 2.0×10^3 and 1.9×10^3 CFU/mL. The difference is a factor of more than 998 between the two groups. Over 5 weeks, the four groups showed somewhat of a reduction in the number of

bacteria; however, in the last week the control groups showed 4.8×10^3 and 1.9×10^3 CFU/mL. An interesting point is that the cold storage with plasma treatment dataset (group C) had a lower mean value (2.0 CFU/mL) than the ambient storage with plasma treatment dataset (group A) at 12.3 CFU/mL. Table 2 shows a significant difference ($P < 0.024$) between control groups (superscript “a”) and plasma treatment groups (superscript “b”) according to the Duncan test. Other studies have shown that gas plasma treatment at atmospheric pressure and ambient temperatures could offer a potential foodborne pathogen sterilization method for poultry products, especially *Listeria innocua* strain (Noriega et al., 2011).

Conclusions

In Jeju, citrus fruits are usually stored in containers with windows at ambient temperature before they are sold at the market. Atmospheric-pressure nonthermal plasma treatment was applied to sterilize citrus fruits under different storage conditions. The plasma treatment had a significant effect on fungal and aerobic bacterial sterilization. In the case of aerobic bacteria sterilization, the difference between the plasma treatment group and the control group was at least a factor of 151. Physical properties were measured during the storage period, and weight reduction was found to occur at different rates between ambient and cold storage. Further study is necessary to minimize weight loss during storage at

Table 2. Concentration of aerobic bacteria (in CFU/mL) for four storage conditions during storage period

Storage period (week)	Storage condition			
	group A (C+P)	group B (A)	group C (A+P)	group D (C)
1	14.6	58.6	0.6	3224.0
2	46.6	326.6	2.6	5600.0
3	10.0	497.3	2.6	1936.0
4	2.6	3479.9	1.3	144.6
5	8.0	42.0	1.3	25.3
6	2.0	4798.5	2.0	158.6
7	2.6	4789.5	3.3	1924.6
Mean ± SD	12.3 ± 15.8 ^b	1997.6 ± 2251.4 ^a	2.0 ± 0.9 ^b	1859.0 ± 2043.7 ^a

group A (C+P) : cold storage and plasma treatment, group B (A) : ambient storage
 group C (A+P) : ambient storage and plasma treatment, group D (C) : cold storage
 mean: average, SD: standard deviation
 a, b : Duncan's test to compare means within 95% confidence level

ambient temperature, and to evaluate the effect of plasma treatment on important citrus nutrients such as vitamins, fiber, nitrogen, and potassium.

Conflict of Interest

The authors have no conflicting financial or other interests.

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