Control of degreening in postharvest green sour citrus fruit by electrostatic atomized water particles

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ABSTRACT

The effect of electrostatic atomized water particles (EAWP) on degreening of green sour citrus fruit during storage was determined. Superoxide anion and hydroxyl radicals included in EAWP were present on the surface of the fruit peel after the treatment. Hydrogen peroxide was formed from EAWP in an aqueous solution, which could indicate that a hydroxyl radical of EAWP turns to hydrogen peroxide in the fruit flavedo as well as in the aqueous solution. EAWP treatment effectively suppressed the degreening of green yuzu and Nagato-yuzukichi fruits during storage at 20℃. The enhancement in K+ ion leakage of both EAWP-treated fruits reduced in comparison with the control. In spite of EAWP treatment, total peroxide level in both fruits showed almost no changes during storage, suggesting that hydrogen peroxide formed by EAWP treatment could stimulate the activation of hydrogen peroxide scavenging system and control degreening of these fruits during storage.

1. Introduction

Green yuzu (*Citrus junos* Siebold ex. Tanaka) and Nagato-yuzukichi (*Citrus nagato-yuzukichi* hort. ex. Y. Tanaka), which are in the yuzu group, are a highly flavored, acidic, green citrus fruit like a lime (*Citrus latifolia* Tan.). These fruits are harvested in summertime in Japan when the rind shows a green color and the fruits are immature. Peel degreening, in which

chlorophyll (Chl) is degraded in the flavedo tissue, is one of main factors of quality deterioration in these fruits when stored. It is necessary to retain the green peel as long as possible to preserve the quality.

Stress treatments, heat and UV treatments, after harvest have been reported to maintain fruit quality during storage. Heat treatments such as hot air, hot water, and vapor heat indicated physiological effects on the control of ripening and senescence and the tolerance of chilling injury in postharvest horticultural produce. We found that hot-air treatment for 2 hours at 50℃ effectively suppressed Chl degradation during storage in broccoli florets (Funamoto, Yamauchi, Shigenaga, & Shigyo, 2002; Kaewsukusaeng et al., 2007). UV treatment after harvest is also known to maintain quality during storage. UV-C treatment seems to control the postharvest yellowing of broccoli florets (Büchert, Civello, & Martínez, 2010; Costa, Vicente, Civello, Chaves, & Martínez, 2006). Similarly, we reported that UV-B treatment efficiently inhibited Chl degradation in stored broccoli florets (Aiamla-or, Yamauchi, Takino, & Shigyo, 2009). However, UV-A was not effective to suppress floret yellowing. From these observations, we infer that active oxygen species, especially hydrogen peroxide, which is produced by the treatments, could induce activation of the ascorbate-glutathione (AsA-GSH) cycle, and enhancement of the cycle might be involved in the suppression of floret yellowing.

Electrostatic atomized water particles (EAWP) were found to be produced by electrostatic atomization from condensed moisture by applying high voltage to the discharge electrode, and the EAWP included active oxygen species such as the superoxide anion and hydroxyl radical (Yamauchi, Suda, & Matsui, 2007) . Ma et al. (2012) noted that treatment with EAWP delayed yellowing and suppressed the reduction of ascorbic acid in broccoli florets during storage. Thus, it is likely that EAWP treatment could be used to maintain the quality of horticultural produce.

In this paper, we deal with the effect of EAWP on the degreening of green sour citrus fruit during storage.

2. Materials and methods

2.1 Plant materials and treatment of electrostatic atomized water particles

Green yuzu fruit grown in a plastic-film house were harvested in early summer in Kochi Prefecture, and green Nagato-yuzukichi fruit were grown in open culture in late summer in Yamaguchi Prefecture. Lime (*Citrus latifolia* Tan.) fruit imported from Mexico were purchased from Tokio Fukuoka Co., Ltd., Japan. Green yuzu and Nagato-yuzukichi fruits were stored at 20℃ in a covered container (30 L) under a stream of humidified air (200 mL・min-1). A device (Panasonic, Japan) that generated EAWP was fitted to the downward direction on the lid

of the container, and the EAWP formed was applied continuously to yuzu fruit and hourly to Nagato-yuzukichi fruit, respectively, as treated by an effective EAWP level to each fruit. Fruit were removed at scheduled intervals during storage and the flavedo tissues were used for the analyses.

2.2 ESR analyses of active oxygen species in electrostatic atomized water particles

For analysis of the active oxygen species included in EAWP, a small Petri dish contained 100 µl 100 mM CYPMPO and 100 µl distilled water put at a distance of 5 cm from the device and held for 30 min in a covered container (30 L). Active oxygen species, superoxide anions and hydroxyl radicals, included in EAWP were measured using an EPR spectrometer (E500, Bruker, Germany). Measurement was carried out at room temperature under the following experimental conditions: microwave frequency, 9.8 GHz; microwave power, 10 mW; field modulation amplitude, 4 gauss; averaged scans, 100. CYPMPO (Radical Research, Japan), which shows selectivity for both superoxide anions and hydroxyl radicals, was used as a spin trapping reagent. All the experiments were conducted at room temperature.

2.3 Electrostatic atomized water particles on the surface of a fruit peel

Flavedo segments (3 x 20 mm) were prepared from lime fruit peel and held for 1 hour in a desiccator. Ten µl aliquot of 500 mM

126 CYPMPO in 0.1% Triton X-100 was dropped to the surface of a
127 flavedo segment and then held for 20 min to be perfused.
128 Afterward, the flavedo segments were exposed to EAWP for 1
129 hour, and the active oxygen species on the flavedo tissue was
130 determined directly using ESR.
131
132 *2.4 Formation of hydrogen peroxide from electrostatic atomized*
133 *water particles*
134 For the assay of hydrogen peroxide formed from EAWP, 6
135 Petri dishes containing 30 ml distilled water were put down into
136 the container and held at 20℃ for 24 hours in a covered container
137 (30 L) under a stream of humidified air (200 ml・min^{-1}).
138 Hydrogen peroxide was measured using a Pack Test (WAK-H_2O_2,
139 Kyoritsu Chemical-Check Lab., Corp.) based on the peroxidase
140 reaction with 4-aminoantipyrine.
141
142 *2.5 Surface color and chlorophyll assay*
143 The surface color of the fruit was determined by measuring
144 the hue angle with a color difference meter (Nihon Denshoku NF
145 777). The Chl content was measured using *N*,
146 *N*-dimethylformamide (Moran, 1982).
147 *2.6 Total peroxide and potassium ion leakage assays*
148 Total peroxide was measured according to the method of
149 Sagisaka (1976). Flavedo tissue (1.0 g) was homogenized in 10
150 ml of cold 5% trichloroacetic acid with a mortar and pestle on ice.

The homogenate was filtered through Miracloth (Calbiochem, USA) and the filtrate centrifuged at 15,000×*g* for 10 min at 4℃. The reaction mixture contained 2 ml supernatant solution, 0.5 ml 50% trichloroacetic acid, 0.5 ml 10 mM ferrous ammonia sulfate and 0.25 ml 2.5M potassium thiocyanate. With the addition of potassium thiocyanate to the mixture, the color was developed and measured spectrophotometrically at 480 nm. Using hydrogen peroxide, the standard curve was prepared.

For the assay of K+ ion leakage, 2 g of flavedo segments were incubated at 25℃ for 2 hours in a conical flask containing 50 ml deionized water after rinsing the flavedo segments. The samples were then incubated at 80℃ for one day to measure the total K+ ions. The K+ ion was analyzed using an atomic absorption spectrophotometer (Hitachi Z8200, Japan). The K+ ion leakage showed a ratio of leakage from flavedo segments during 2-hour incubation for a total K+ ion level of the flavedo segments.

3. Results and discussion

3.1 Active oxygen species in electrostatic atomized water particles and their effects on the fruit surface

Figure 1A shows ESR spectrum of active oxygen species within EAWP. In the ESR spectrum of CYPMPO spin adducts, the split in some signal such as lowest or highest field signal was observed. It is thought that both superoxide anion and hydroxyl radicals could be definitely present in the EAWP since the split

of each signal was not found in the spectrum of CYPMPO-hydroxyl radical or CYPMPO-superoxide adducts (Mukohda et al., 2010). The ESR spectrum of these radicals was also detected from the surface of the flavedo tissue, as is apparent in Fig.1B, indicating that the superoxide anion radical and hydroxyl radical in the EAWP reached the fruit and were present in unchanged form on the surface of the fruit peel. Figure 2 shows the changes in hydrogen peroxide formed from EAWP during the treatment. Both levels of hydrogen peroxide with continuous treatment and hourly treatment increased to almost the same extent for the first 24 hours. However, the level with continuous treatment was a little higher than that with hourly treatment. Moreover, the hydrogen peroxide level in each Petri dish placed in the container showed almost no difference. The changes in the level of total peroxide, which is mainly hydrogen peroxide, showed the same tendency as those in the hydrogen peroxide level (data not shown). It is suggested that hydrogen peroxide could be formed from a hydroxyl radical included in the EAWP with a radical-radical reaction, as is well known (Czapski, 1984), and that the EAWP is evenly treated inside the container.

These findings show that hydrogen peroxide could be formed from the hydroxyl radical of EAWP in the fruit peel after treatment. In addition, the superoxide anion might also react with formed hydrogen peroxide, and afterward, hydroxyl radical is produced again in the cell, known as the Harber-Weiss reaction

(Harber & Weiss, 1934; Kehrer, 2000).

3.2 Inhibitory effect of electrostatic atomized water particles on degreenig during storage

Figure 3 shows the changes in surface color (hue angle) of green yuzu and Nagato-yuzukichi treated with or without EAWP during storage at 20°C. Hue angle values of green yuzu fruit with EAWP treatment decreased slightly during storage, whereas those of the control decreased gradually for first 7 days and then showed a sharp decrease during storage. Changes in hue angle values of Nagato-yuzukichi fruit with EAWP treatment were also suppressed as compared with those in the control, though a significant differences were not almost observed between EAWP treatment and the control. Unlike green yuzu fruit, the hue angle values of Nagato-yuzukichi fruit with EAWP treatment decreased after 14 days of storage at 20℃, which could indicate that the EAWP treatment is more effective in green yuzu fruit than that in Nagato-yuzukichi fruit. In the Chl assay, Chl *a* content in the control of green yuzu fruit dropped to 17% on day 24 of the storage, while that in EAWP-treated fruit dropped slightly to 86% as compared with that in fresh fruit flavedo (Table 1). The changes in Chl *b* content with or without EAWP treatment were consistent with those in Chl *a* with or without the treatment. In Nagato-yuzukichi fruit, the EAWP treatment also efficiently inhibited the decline in Chl content during storage.

As is apparent in Fig.2, it was implied that hydrogen peroxide could be formed from EAWP treated to the flavedo tissue. Unexpectedly, the total peroxide content in EAWP-treated green yuzu and Nagato-yuzukichi flavedos showed almost no change during storage (Fig.4).

Figure 5 shows the changes in K+ ion leakage level during storage. In the EAWP-treated flavedo tissues of green yuzu fruit, the increase in K+ ion leakage level was significantly suppressed as compared with that in the control concomitantly to the suppression of degreening. In the EAWP-treated Nagato-yuzukichi fruit, the increment in K+ ion leakage level also showed an inhibitory trend, but no significant differences were observed.

EAWP treatment was effective for the suppression of degreening during storage in green yuzu and Nagato-yuzukichi fruits. It is thought that this beneficial effect could be due to the action of hydrogen peroxide formed from the hydroxyl radical included in EAWP (Czapski, 1984). Hydrogen peroxide seems to be relatively stable in comparison with the other oxygen radical species and to act in the cell as a signal transducer (Neil, Desikan, & Hancock, 2002). Morita, Kaminaka, Masumura, and Tanaka (1999) reported that hydrogen peroxide could relate to oxidative stress signaling, resulting in the induction of cytosolic ascorbate peroxidase in suspension cultures of a germinating rice embryo. Orozco-Cárdenas, Narváez-Vásquez, and Ryan (2001)

also noted that hydrogen peroxide acted as a second messenger for the activation of wound response genes in tomato mesophyll cells.

Spraying a treatment of hydrogen peroxide on soybean plants suppressed the occurrence of drought stress, as reported by Ishibashi et al. (2011). The suppression was in order to increase oliogosaccharide levels such as myo-inositol and galactinol, which could be involved in the drought tolerance, by induction of the gene expression of key enzymes relating to oligosaccharide biosynthesis with hydrogen peroxide treatment. In sweet pepper fruit, hydrogen peroxide treatment was reported to be involved in enhancement of the ascorbic acid level with activation of the AsA-GSH cycle (Endo & Imahori, 2012). Imahori, Kanetsune, Ueda, and Chachin (2000) also proved that the increase of the hydrogen peroxide level with maturation could induce the enhancement of antioxidant enzyme activities such as ascorbate peroxidase and superoxide dismutase. Moreover, in postharvest broccoli florets, EAWP treatment effectively retarded the reduction of the ascorbic acid level during storage by up-regulating the biosynthesis and the regeneration genes of ascorbic acid (Ma et al., 2012). A possible explanation from these findings is that hydrogen peroxide formed from EAWP could affect the metabolic reaction by the action of a signaling transducer and that hydrogen peroxide could induce the gene expression and the action of the antioxidant enzymes, resulting

276 in the suppression of degreening in stored green citrus fruit.
277 Postharvest stress treatments such as heat and UV treatments
278 are known to have some beneficial effects for the control of
279 ripening and senescence and the delay of the occurrence of
280 chilling injury (Costa, Vicente, Civello, Chaves, & Martínez,
281 2006; Lurie, 1998; Sivakumar & Fallik, 2013; Yamauchi, 2013).
282 We reported that both heat and UV-B treatments retarded Chl
283 degradation in stored broccoli florets and that hydrogen peroxide
284 levels with those treatments were higher than those in the control
285 (Aiamla-or, Kaewsuksaeng, Shigyo, & Yamauchi, 2010;
286 Funamoto, Yamauchi, Shigenaga, & Shigyo, 2002; Shigenaga,
287 Yamauchi, Funamoto, & Shigyo, 2005; Takino, Yamauchi,
288 Aiamla-or, & Shigyo, 2009). Furthermore, the degreening of lime
289 fruit treated with UV-B was suppressed during storage at 25℃
290 concomitantly with the increase in hydrogen peroxide level,
291 resulting in activation of the AsA-GSH cycle (Kaewsuksaeng,
292 Urano, Aiamla-or, Shigyo, & Yamauchi, 2011; Urano,
293 Kaewsuksaeng, Shigyo, & Yamauchi, 2011). In this paper, the
294 level of total peroxide, which seems to be mainly hydrogen
295 peroxide, hardly changes during storage in both EAWP-treated
296 fruits, and the changes in K+ ion leakage of both EAWP-treated
297 fruits were suppressed during storage as compared with those in
298 the control. These results indicate that hydrogen peroxide
299 formed by the EAWP treatment as well as the stress treatments
300 might be involved in delaying the progress of senescence and

retarding Chl degradation by activating the antioxidant system.

In conclusion, the hydrogen peroxide formed from EAWP, which contains both hydroxyl and superoxide anion radicals, could act as a signal transducer in the cell and control Chl degradation of green yuzu and Nagato-yuzukichi fruits during storage. In this way, postharvest EAWP treatment seems to be effective to suppress degreening in stored green sour citrus fruit.

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426 Figure Legends

427 Fig. 1. ESR spectra of active oxygen species. Figure 1A shows
428 ESR spectrum of active oxygen species included in
429 electrostatic atomized water particles (EAWP). Figure 1B
430 shows ESR spectrum of active oxygen species on the surface
431 of fruit peel with the treatment of EAWP.

432 Fig. 2. Hydrogen peroxide accumulation with the treatment of
433 electrostatic atomized water particles. Six Petri dishes (A to
434 F), in which was contained 30 ml distilled water, were put
435 down in the container and held at 20℃ for 24 hours in a
436 covered container (30 L) under a stream of humidified air
437 (200 ml・min^{-1}). Each value represents the mean of duplicate
438 analyses. (a): Continuous treatment, (b): Treatment at hourly
439 intervals.

440 Fig. 3. Changes in the hue angle value of the surface color of
441 green yuzu (A) and Nagato-yuzukichi (B) fruit with or
442 without the treatment of electrostatic atomized water
443 particles. Vertical bars represent the average with SE (n=3).
444 Significant differences are shown between EAWP treatment
445 and the control (**P<0.01; *P<0.05; NS by *t*-test).

446 Fig. 4. Total peroxide contents of green yuzu (A) and
447 Nagato-yuzukichi (B) fruit with or without the treatment of
448 electrostatic atomized water particles. Vertical bars
449 represent the average with SE (n=3). Significant differences
450 are shown between EAWP treatment and the control

(**P<0.01; *P<0.05; NS by *t*-test).

Fig. 5. Potassium ion leakages of green yuzu (A) and Nagato-yuzukichi (B) fruit with or without the treatment of electrostatic atomized water particles. Vertical bars represent the average with SE (n=3). Significant differences are shown between EAWP treatment and the control (**P<0.01; *P<0.05; NS by *t*-test).

476 Table 1. Chlorophyll contents of green yuzu and
477 Nagato-yuzukichi fruit with or without the treatment of
478 electrostatic atomized water particles.

	Chl[1] *a*			Chl *b*		
	EAWB[2]	Control	*t*-test[3]	EAWB	Control	*t*-test
Green yuzu						
Day 0	339±15.0[4]			91.9±2.20		
Day 7	271±30.7	206±15.1	NS	64.4±6.81	42.7±3.36	NS
Day 24	280±60.6	46.2±23.5	*	80.0±20.2	11.0±5.95	*
Nagato-yuzukichi						
Day 0	315±15.9			97.6±4.71		
Day 7	263±7.50	221±1.73	*	83.6±2.15	70.5±0.31	*
Day 14	172±5.28	73.7±3.53	**	45.4±1.24	15.7±1.23	**

[1] Chlorophyll (μg・g^{-1}flavedo), [2]Electrostatic atomized water particles, [3] Significant differences are shown between EAWB treatment and the control (**P<0.01; *P<0.05; NS by *t*-test), [4]Average value±SE (n=3)

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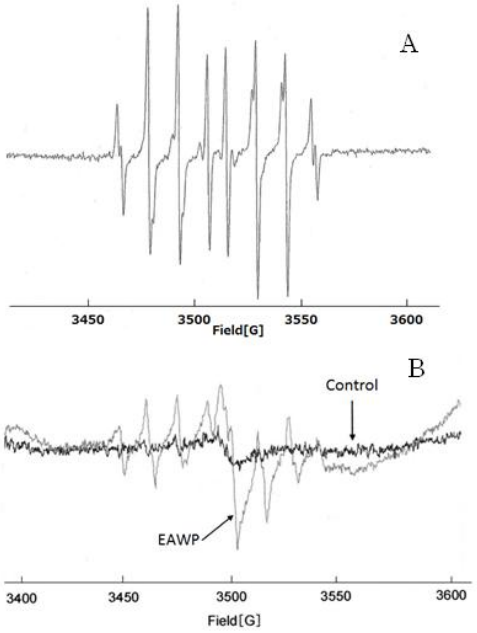

Fig. 1

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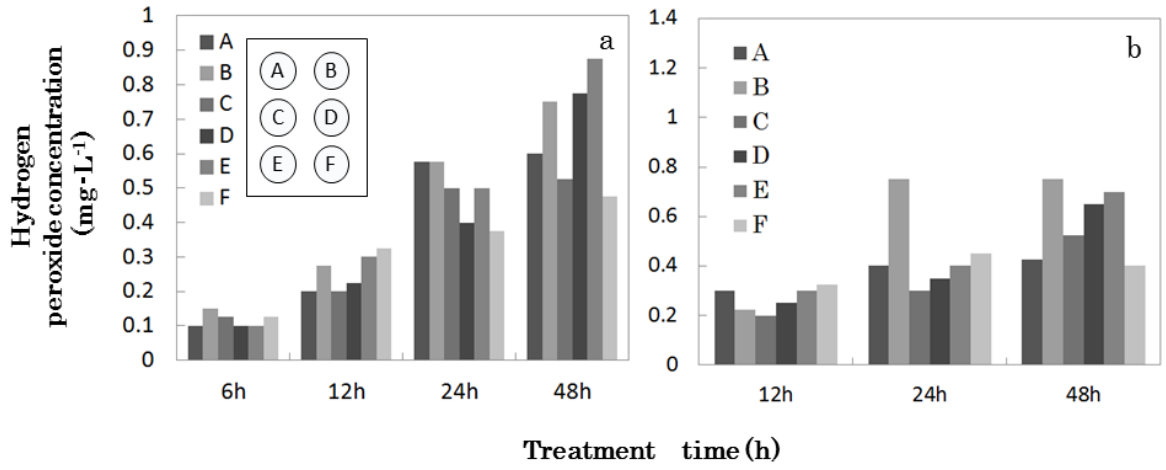

Fig. 2

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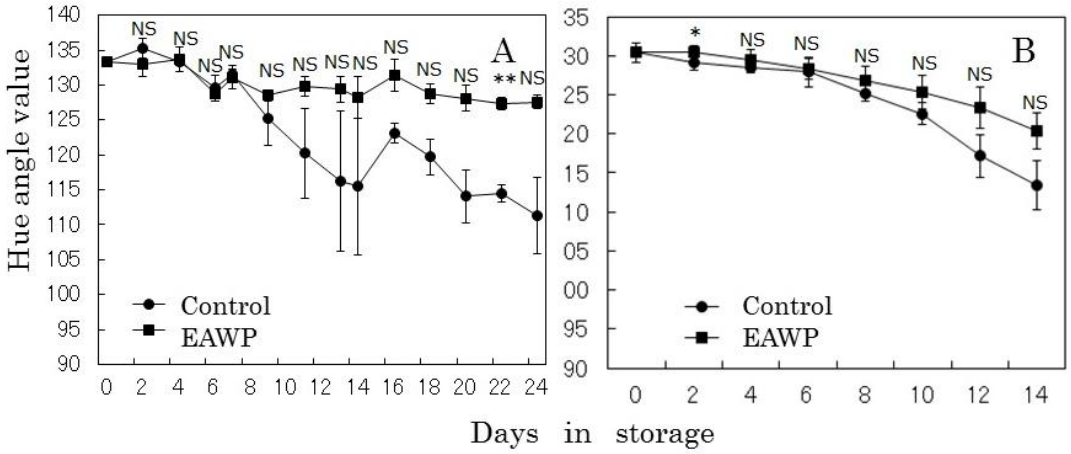

Fig. 3

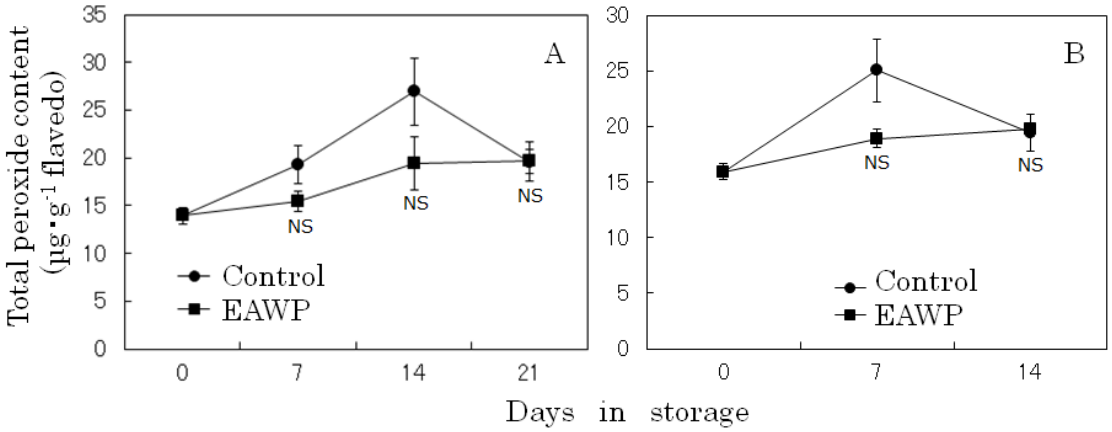

Fig. 4

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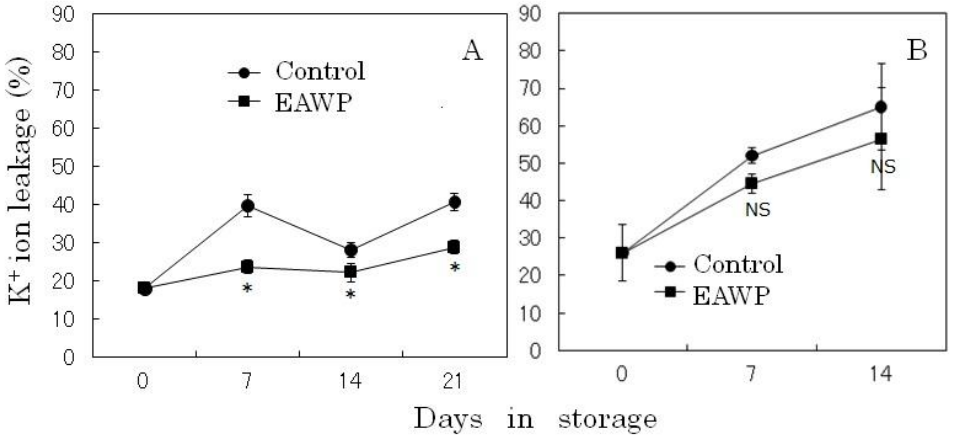
A
B
Control
EAWP
K+ ion leakage (%)
Days in storage
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Fig. 5

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