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# Hydrogen-rich water delays postharvest ripening and senescence of kiwifruit

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ABSTRACT

The effect of hydrogen-rich water (HRW) on prolonging the shelf life of kiwifruit and possible underlying mechanisms were assessed. Our results revealed that HRW (30%, 80%, and 100%) displayed different effects in inhibiting the rot of kiwifruit. Among these treatments, 80% HRW had the most significant effect by decreasing the rot incidence and preserving the firmness of kiwifruit. This conclusion was supported by the fact that 80% HRW treatment could effectively alleviate pectin solubilization and reduce the activities of cell wall-degrading enzymes. On the other hand, HRW treatment was able to reduce the respiration intensity, increase the activity of superoxide dismutase, decrease lipid peroxidation level, and maintain the radical (DPPH$^{\cdot}$, $O_2^{\cdot-}$, and $^{\cdot}OH$)-scavenging activity of kiwifruit. Moreover, the inner membrane of mitochondria exhibited higher integrity. Thus, our results demonstrate that HRW treatment could delay fruit ripening and senescence during storage by regulating the antioxidant defence.



## 1. Introduction

Kiwifruit is an economically important subtropical fruit crop. There are presently more than 70 species of kiwifruit in the genus *Actinidia*, which are widespread in Asia. It is also grown commercially in other countries or continents, such as the United States, Canada, Chile, New Zealand, and parts of Europe (Garcia, Stevenson, Atkinson, Winz, & Quek, 2013). In China, kiwifruit is one of the most common fruits, widely grown in the northwest region. It contains a wealth of phytonutrients, including ascorbic acid, phenolics, flavonoids, vitamin E, carotenoids and minerals, and serves as the best source of lutein and *myo*-inositol among daily consumed fruits (Zhang, Li, Liu, Song, & Liu, 2012). Thus, consumption of kiwifruit can be beneficial for specific health conditions. For example, regular kiwifruit consumption could reduce DNA fragility (Rush et al., 2006) and exert beneficial effects on the antioxidative status and the risk factors for cardiovascular disease (Chang & Liu, 2009).

Despite its wide popularity, the kiwifruit has a short shelf life (approximately 3–4 days) because of ripening and rapid deterioration (Jhalegar, Sharma, Pal, Arora, & Dahuja, 2011), which affects its economic performance. The decay of kiwifruit is closely related to structural changes in the cell wall. Associated processes, e.g., pectin solubilization and depolymerization (Fischer, Wegryzn, Hallett, & Redgwell, 1996), primarily cause kiwifruit softening by hydrolyzing the cell wall. The key enzymes involved in this process are cellulase, pectinmethylesterases (PME), and polygalacturonase (PG) (Ramana-Rao, Gol, & Shah, 2011).

To extend the storage life and maintain the quality of kiwifruit, various methods have been developed, among which cold storage is the most common. However, the kiwifruit is susceptible to relatively low temperature (e.g., 0, −0.8 °C), resulting in the development of physiological disorders, along with rapid softening after cold storage (Mworia et al., 2012). Although 1-methylcyclopropene can reduce ethylene production and prevent flesh softening of kiwifruit more effectively at 20 °C than at 0 °C (Jhalegar et al., 2011), it has no significant effect on the firmness of kiwifruit during the mid-to-late period of storage (Kim, Hewett, & Lallu, 2001). Therefore, alternative postharvest handling strategy is needed for delaying kiwifruit softening and thus extending its shelf life.

In recent years, accumulating evidence has implicated gases of small molecules such as nitric oxide (NO) and hydrogen sulphide ($H_2S$) in various developmental processes, including adventitious rooting (Xuan et al., 2012), alleviation of cadmium toxicity (Li, Wang, & Shen, 2012), seed germination (Wang et al., 2012), and preservation of fruits and vegetables (Hu et al., 2012; Lai, Wang, Li, Qin, & Tian, 2011). Despite certain delaying effects of low-dose NO and $H_2S$ on the senescence of plants, high doses of NO and $H_2S$, especially the latter, can be poisonous to fruits and vegetables (Perna et al., 2011). Owing to stringent safety requirements, the application of NO and $H_2S$ for preservation of fruits and vegetables

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is still largely limited. A previous study has demonstrated hydrogen gas ($H_2$) evolution and uptake by illuminated leaves (Sanadze, 1961), while other *in vitro* studies have demonstrated $H_2$ evolution by isolated chloroplasts and postulated the existence of hydrogenase in specific higher plants (Esquivel, Amaro, Pinto, Fevereiro, & Malcata, 2011). Recently, $H_2$ is proven to have the ability of alleviating various abiotic stresses, including high salinity (Xie, Mao, Lai, Zhang, & Shen, 2012; Xu et al., 2013), and low temperature (Jin, Zhu, Cui et al., 2013) by regulating the antioxidant defence system. It is well known that the senescence of fruits and vegetables is generally accompanied by excessive production of reactive oxygen species (Singh & Singh, 2013). Thus, we speculated that $H_2$ might also be involved in the regulation of postharvest physiological and biochemical behaviour of kiwifruit during storage. Most important, $H_2$ is non-toxic and does not react with most compounds, including oxygen gas ($O_2$) at room temperature (Ohsawa et al., 2007).

In this study, to confirm above deduction and provide new evidence for the function of $H_2$, we investigated the effects of hydrogen-rich water (HRW) on the parameters of physical quality and antioxidant capacity of kiwifruit during storage. Our results support the idea that $H_2$ delays the ripening process, reduces lipid peroxidation and maintains the free radical ($DPPH^{\cdot}$, $O_2^{\cdot-}$, and $\cdot OH$)-scavenging activity in kiwifruit. These results suggest that HRW treatment may be a useful technique for maintaining kiwifruit quality and extending its postharvest life.

## 2. Materials and methods

### 2.1. Kiwifruit

Kiwifruit (*Actinidia chinesis* cv. Huayou) is selectively bred from the cv. 'Zhonghua' and 'Meiwei' seedlings at an orchard in Yangling District (Shaanxi Province, China). The fruit ripen from the end of September to the middle of October. Fruit were harvested at commercial maturity (Brix $\geqslant$ 6.5) and obtained from the Zhongcai Market in Nanjing (Jiangsu Province, China) on October 15. The fruits of uniform size with no physical injuries or infections were chosen for subsequent treatments.

### 2.2. Treatment with hydrogen-rich water (HRW)

Purified hydrogen gas ($H_2$) (99.99%, v/v) was generated from a $H_2$ generator (AYH-300, Beijing Keshi Xingye Technology Co., Ltd., China) and bubbled into 10 L of distilled water at a rate of 200 ml/min. The bubbling was continued for 3 h to allow the saturation of water with $H_2$ (Jin, Zhu, Cui et al., 2013). Subsequently, an aliquot of the saturated hydrogen-rich water (HRW; 100% concentration) was immediately diluted to the indicated concentrations for the further experiments. In the preliminary test, we chose a series of HRW concentrations and found that the concentrations of 30–80% HRW obviously maintained a better quality of kiwifruit and reduced the rot incidence during storage, in comparison with distilled water treatment, whereas 100% HRW treatment accelerated the rot incidence (Supplementary Fig. 1). Therefore, HRW at 30%, 80%, and 100% was used in the following experiments.

Afterwards, the fruits were immersed in plastic boxes with 10 L of distilled water (reagent control, $CK_1$), 30% HRW, 80% HRW, or 100% HRW for 5 min, followed by air-drying at 20 °C for 1 h. Then, the fruits were placed in 21-L Lock & Lock boxes (two holes with a diameter of 1 cm in the diagonal position, which kept the gas composition in the box similar to that in ambient air and prevented an escape of water), then stored at 20 ± 0.2 °C in an MIR-254 culture incubator (Sanyo, Japan) for 16 days and 90–95% relative humidity (RH). Fruit without distilled water and HRW treatment were also used as the blank control ($CK_0$). In view of the fact that the time of HRW treatment was only about 5 min, the effect of hypoxia occurring in headspace (about 10 L) of the plastic box [for example, upon 80% HRW treatment, 0.78% hydrogen and 16.13% oxygen were detected by gas chromatography (GC 7890, Agilent) in the headspace of plastic boxes, in comparison with about 0% hydrogen and 21% oxygen in the outside air] on fruit was almost negligible. During storage, flesh samples were taken from the fruit at 4 d intervals for physicochemical assays. Each treatment was done in triplicate, with 50 fruits in each replicate group.

### 2.3. Determination of $H_2$ content

For analyzing the $H_2$ content, we took 10 ml of freshly prepared HRW into a vial (20 ml). Afterwards, the vials were immediately capped and kept for 2 h, then 0.5 ml of sample was withdrawn from the headspace with a microsyringe and measured by gas chromatography (GC).

The chromatographic system (GC 7890, Agilent) was composed of a gas chromatograph equipped with a thermal conductivity detector (TCD) and a column containing the Molecular Sieve 5 Å stationary phase (MSA). The column was held isothermally at 70 °C. The injection and detector temperatures were adjusted to 200 and 220 °C, respectively. Nitrogen was used as carrier gas. In our experimental conditions, the $H_2$ concentration in freshly prepared HRW (100% concentration) analysed by GC was about 0.66 mM.

### 2.4. Rot incidence evaluation

The external appearance of each fruit and the presence of macroscopic fungal growth were visually evaluated. Fruits with visible decay were defined as 'rot', and no visible changes in the tissues were defined as 'good'. At each sampling day, the number of rotted kiwifruits was recorded. Rot incidence for the treatment unit was calculated as follows:

$$\text{Rot incidence} = \left(\frac{\text{the number of rotted fruit}}{\text{the total number of investigated fruit}}\right) \times 100.$$

### 2.5. Electronic tongue measurements

The original data were acquired through the αASTREE electronic tongue system (Alpha M. O. S. Co., Toulouse, France), which includes 7 different liquid cross-selective sensors (ZZ, BA, BB, CA, GA, HA, and JB). Each sensor is made from silicon transistors with a specific organic membrane, which interacts with ionic, neutral, and chemical compounds present in the liquid sample in a specific manner. Any interaction at the membrane interface is detected by the sensor and converted into an electronic signal to be analysed. The principle of the method is to detect the potentiometric difference between each individually coated sensor and the Ag/AgCl reference electrode. Therefore, an integral signal of each sample comprises a vector with 7 individual sensor determinations.

All the samples were detected at the room temperature of 25 °C. Before data acquisition from the electronic tongue, a sequence of sample preprocessing was implemented: an 8 ml kiwifruit juice sample was diluted to 80 ml with 72 ml distilled water. Then, the 80 ml dilution of each sample was analysed by the electronic tongue. The measurement time was set at 120 s for each sample, and then the sensors were rinsed for 10 s with distilled water before detecting the next sample. Five samples were tested at one measurement sequence. According to the experience of pre-experiment and the requirement of system stability, each sample was measured 7 times.

### 2.6. Determination of the ratio of SSC/TA

Ten kiwifruits per treatment were used to determine soluble solids contents (SSC), titratable acidity (TA) and measured twice in each fruit, using a refractometer WYT-4 (Shanghai Cany Precision Instrument Co., Ltd, China). A previously described method was used to measure TA, and the results were expressed as percentage of malic acid (Zhu, Sun, & Zhou, 2010).

### 2.7. Flesh firmness assay

Flesh firmness was assayed by the penetration test that measured the necessary strength required to insert (into the flesh-fruit) a metal point of a dynamometer to an established distance. The test was performed on two opposite faces of the equatorial zone by using a digital penetrometer installed on a driving column equipped with a 1.0 cm probe (Model 53205, Made in Italy). Flesh firmness of fruits was expressed as $kg/cm^2$.

### 2.8. Determination of water-soluble pectin (WSP) and protopectin contents

Alcohol insoluble solids were extracted from the fruits with 95% alcohol and then air-dried. Water-soluble pectin and protopectin contents were extracted from 2 g of dry alcohol insoluble solids with 200 ml of 0.05 M NaOH and 200 ml of boiling water, respectively. After cooling, the extracts were centrifuged at 6000×*g* for 20 min. Each extraction and centrifugation step was repeated four times, and the supernatants derived from the same sample were pooled. The WSP and protopectin contents were determined according to the method of Li and Zhang (2006).

### 2.9. Assay of enzyme activity

Extraction and enzyme activity assay of cellulase was performed, following the method of Ramana-Rao et al. (2011) with slight modifications. The reaction mixture contained 2.0 ml of carboxymethyl cellulose (1%; w/w) and 1.0 ml of sodium acetate buffer (100 mM, pH 5.0) at 37 °C. The reaction was initiated by adding 1.0 ml of crude enzyme, and 0.5 ml aliquots were taken at 2, 4, 6, and 12 h. The cellulase activity was assayed by measuring the content of reducing groups released from carboxymethyl cellulose. One unit of enzyme activity was defined as the amount of enzyme required to form 1 μmol of reducing groups per hour, per gram of the original fresh sample.

Extractions of pectinmethylesterase (PME) and polygalacturonase (PG) were similar to cellulase. The determination of PME and PG activities followed the methods of Pathak and Sanwal (1998).

Superoxide dismutases (SOD) activity was determined by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Huang et al. (2006) with minor modifications. Fruit tissues (5.0 g) were ground with 10 ml of 50 mM sodium phosphate buffer (pH 7.8). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 14 mM methionine, 3 μM EDTA, 1 μM NBT, 60 μM riboflavin and 0.1 ml of crude enzyme extract. One unit of SOD activity is defined as the amount of enzyme that caused a 50% inhibition of NBT.

### 2.10. Respiration intensity assay

The respiration intensity of fruits was assayed by detecting the production of carbon dioxide ($CO_2$). Ten fruits of each replicate were sealed in a 6.14-L gas-tight container for 2 h at 20 ± 0.2 °C prior to gas sampling. The production of $CO_2$ was monitored, using an Agilent 7820 model gas chromatograph equipped with a Poropak column (1/8 inch, 8 foot). The oven and detector temperatures were 40, and 300 °C, respectively. Nitrogen was used as the carrier gas.

### 2.11. Lipid peroxidation assay

Fruit tissues (5.0 g) were homogenized with 15 ml of 5% (w/v) trichloroacetic acid (TCA). After centrifugation, 3 ml of 0.5% thiobarbituric acid in 15% TCA were added to 5 ml of supernatant. Lipid peroxidation was assayed by estimating the concentration of thiobarbituric acid-reactive substances (TBARS) in the supernatant according to Han et al. (2008) and Jin, Zhu, Xie, and Shen (2013) with slight modifications. The TBARS concentration was calculated using the following equation: $TBARS\ (nmol/g) = [6.45 \times (A_{532} - A_{600})] - [0.56 \times A_{450}]$.

### 2.12. Measurement of free radical-scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·).-scavenging activity was measured according to Fu et al. (2011) with some modifications. Briefly, 10 μl of methanol extract were mixed with 3 ml of 0.1 mM DPPH-methanol solution. After the solution was incubated for 30 min at 25 °C in the dark, the decrease of the absorbance at 517 nm was measured. Control contained methanol instead of the methanol extract, while blanks contained methanol instead of DPPH· solution. The scavenging of DPPH· radicals by the samples was calculated according to the following equation: DPPH· scavenging activity (%) = [1 − (absorbance of sample − absorbance of blank)/absorbance of control] × 100.

The superoxide anion ($O_2^{\cdot-}$)-scavenging activity was measured according to the method of Siddhuraju, Mohan, and Becker (2002) with slight modifications. Aliquots (0.4 ml) of the extracts at 0 μg/ml (control), 50, 100, 250, and 500 μg/ml were mixed with 3 ml of the reaction solution (containing 1.3 μM riboflavin, 13 mM methionine, 63 μM NBT, 100 μM EDTA, and 0.05 M, pH 7.8, sodium phosphate buffer) and then incubated for 15 min under 4000 lux illumination at 24 °C. The absorbance values of the reaction mixtures were measured at 560 nm ($A_{560}$) spectrophotometrically. The relative ($O_2^{\cdot-}$)-scavenging activity (%) was computed as [(1 − $A_{560}$ of the sample)/$A_{560}$ of the control] × 100.

The hydroxyl radical (**.**OH)-scavenging activity was measured according to Isabel-Egea, Sanchez-Bel, Martinez-Madrid, Flores, and Romojaro (2007). The results are expressed as a percentage inhibition of the deoxyribose attack, where 100% attack is defined as the absorbance of deoxyribose without the addition of the samples.

### 2.13. Morphological examination by transmission-electron-microscopy (TEM)

Small pieces (1 $mm^3$) of fruit flesh tissues were fixed in 2% (w/v) *p*-formaldehyde and 2.5% (v/v) glutaraldehyde in a 0.1 M Na phosphate buffer solution (pH 7.2) at 4 °C for 5 h. Then, the samples were washed three times in the same Na phosphate buffer for 10 min at 4 °C, post-fixed with 2% osmium tetroxide for 2 h at 4 °C, and rinsed again in the Na phosphate buffer (pH 7.2). The tissues were dehydrated in a graded series of ethanol solutions (50–100%, v/v), embedded in LR White resin, and polymerized at 60 °C for 48 h. For TEM examination, ultra-thin (60–90 nm thick) sections were cut and mounted onto copper grids, counter-stained with uranyl acetate and lead citrate for 30 and 10 min, respectively, and viewed under an EM 301 electron microscope (Phillips, Drachten, The Netherlands) at 80 kV.

### 2.14. Statistical analyses

Data are presented as means ± SE (standard error) from three independent experimental replications. Statistical analysis and principal component analysis (PCA) were performed using SPSS 18.0 software. Differences among treatments were analysed by one-way ANOVA, taking $P < 0.05$ level as significant according to Duncan's multiple range test.

## 3. Results

### 3.1. Effect of HRW on the rot of kiwifruit

As shown in Table 1, in comparison with the treatment of distilled water ($CK_1$), 30% HRW treatment decreased the rot incidence in kiwifruit; especially on days 8 and 12, significant differences were observed, whereas the 80% HRW treatment significantly reduced the rot incidence during the whole storage. In contrast, 100% HRW treatment accelerated the rot symptom. Notably, when compared with the $CK_0$, the $CK_1$ treatment increased the rot incidence; this may be due to the fact that the process of pretreatment resulted in some injuries to fruit tissue. However, in this case, 80% HRW treatment could reduce the rot incidence of kiwifruit, especially on day 4, suggesting that $H_2$ has a beneficial role in the alleviation of wounding.

### 3.2. Electronic tongue measurements and SSC/TA ratio

Data obtained from the electronic tongue were used to monitor changes in the kiwifruit samples during storage due to the ripening process. The results of principal components analysis (PCA) performed with data obtained from the electronic tongue are shown in Fig. 1. With this PCA model, we are able to reduce the dimensionality of the data, while it also helps to visualize the different categories present.

As shown in Fig. 1A, after 4 days of storage, the PCA plot shows the discrimination of different kiwifruit samples. PC1 vs. PC2, together explaining 94.59% of the variance, shows those samples close to each other in the PCA score plots that share similar characteristics, according to which the kiwifruit samples were divided into two independent clusters along the axis-X (PC1): cluster I, kiwifruit samples of freshly harvested, 80% HRW treatment; cluster II, kiwifruit samples of $CK_0$, $CK_1$, 30% HRW treatment, and 100% HRW treatment. The kiwifruit samples of cluster I were fresher than those of cluster II.

As seen from Fig. 1B, after 8 days of storage, PC1 explained 75.31% of the variance, and PC2 explained 16.8% of the variance. These two principle components accounted for 92.11% of the data variance. All the samples were separated into clusters along the axis-X (PC1):cluster I, sample of freshly harvested; cluster II, samples of 80% HRW treatment; cluster III, sample of $CK_1$, 30% HRW treatment, and 100% HRW treatment; cluster IV, sample of $CK_0$. The freshness of the samples was decreased along the axis-X (PC1) from left to right. Together with the results of Fig. 3A and B, it was found that 80% HRW treatment could maintain the freshness of kiwifruit, namely better sensory characteristic.

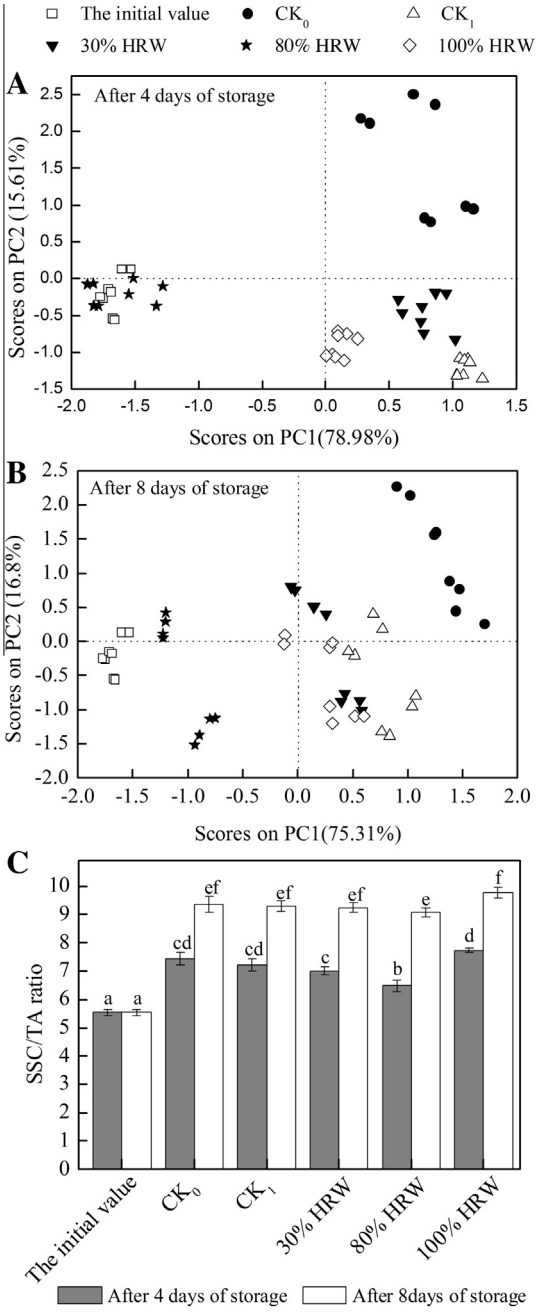


**Fig. 1.** Changes of sensory characteristic of kiwifruit on days 4 (A) and 8 (B), and SSC/TA (C) ratio after pretreatments with different concentrations of hydrogen-rich water (HRW) for 5 min. Bars above column represent standard deviations. Values followed by a different letter were significantly different according to Duncan's multiple range test at $P < 0.05$.

**Table 1**
Changes in the rot incidence (%) of kiwifruit during storage after pretreatments with different concentrations of HRW.

| Treatment | Storage time (d) | | | |
|---|---|---|---|---|
| | 4 | 8 | 12 | 16 |
| $CK_0$ | $4.68 \pm 0.58^{b}$ | $7.60 \pm 0.58^{b,c,d}$ | $19.30 \pm 1.01^{f}$ | $32.75 \pm 1.17^{h,i}$ |
| $CK_1$ | $5.85 \pm 0.44^{b,c}$ | $14.62 \pm 0.94^{e}$ | $30.99 \pm 2.52^{h}$ | $35.09 \pm 0.72^{i,j}$ |
| 30% HRW | $4.68 \pm 0.72^{b}$ | $8.19 \pm 0.52^{c,d}$ | $19.30 \pm 0.95^{f}$ | $32.75 \pm 1.10^{h,i}$ |
| 80% HRW | $0.00 \pm 0.00^{a}$ | $4.68 \pm 0.58^{b}$ | $17.70 \pm 0.15^{f}$ | $27.49 \pm 0.68^{g}$ |
| 100% HRW | $9.36 \pm 0.72^{d}$ | $17.54 \pm 0.61^{f}$ | $36.26 \pm 1.89^{j}$ | $42.69 \pm 2.09^{k}$ |

[a-k] Means with the same letters are not significantly different at $P < 0.05$ using Duncan's multiple range test.

Simultaneously, the changes of SSC/TA were measured, as shown in Fig. 1C. As compared with the initial value of SSC/TA, the SSC/TA ratio in all fruit increased after 4 days of storage. Nevertheless, the ratio of 80% HRW treatment was significantly lower than that in other treatments, showing that the ripening process of this treatment was relatively slow. We also noticed that the 80% HRW treatment brought about a slight decrease in SSC/TA ratio after 8 days of storage. These results were in accordance with the sensory characteristics (Fig. 1A and B), showing that short-term

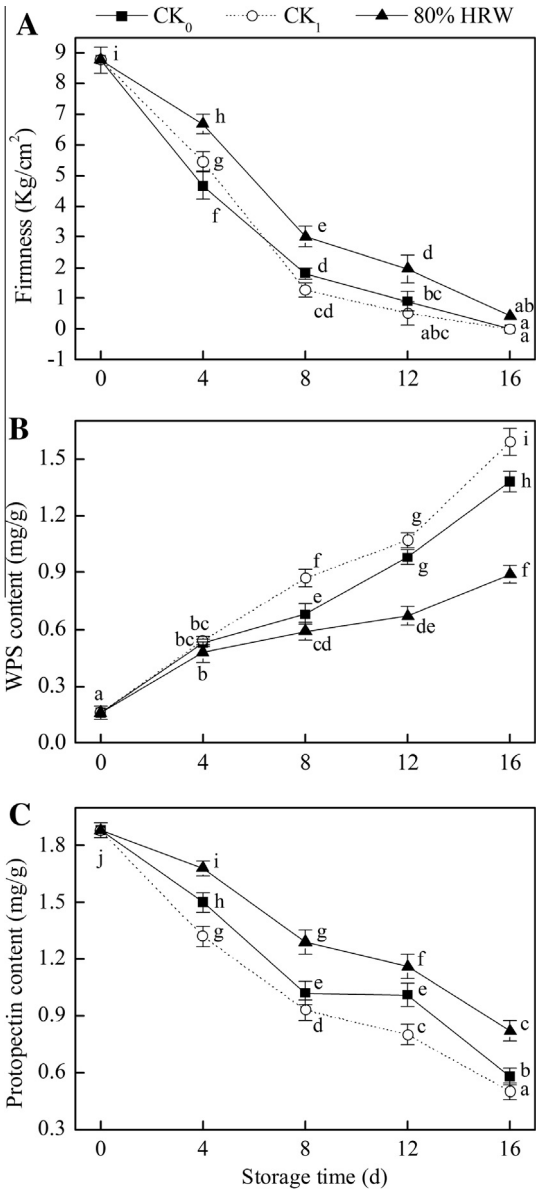


**Fig. 2.** Effects of 80% HRW pretreatment on firmness (A), water-soluble pectin (WSP, B) and protopectin (C) contents of kiwifruit during storage. The vertical bars represent standard deviations. Values followed by a different letter were significantly different according to Duncan's multiple range test at $P < 0.05$.

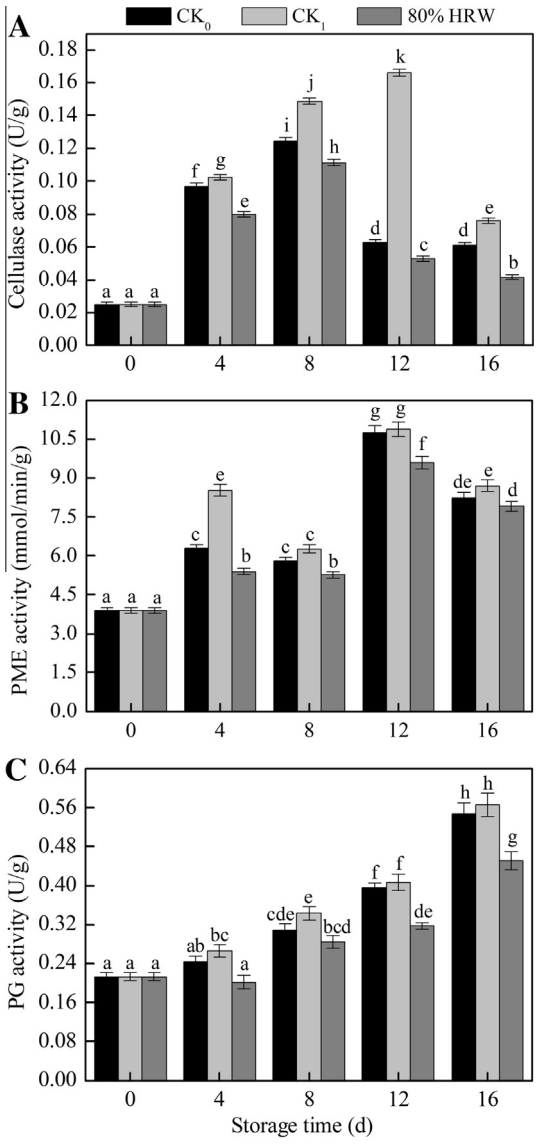


**Fig. 3.** Effects of HRW pretreatment on cellulase (A), pectinmethylesterases (PME, B) and polygalacturonase (PG, C) activities of kiwifruit during storage. The vertical bars represent standard deviations. Values followed by a different letter were significantly different according to Duncan's multiple range test at $P < 0.05$.

pretreatment of 80% HRW affected the change of quality of kiwifruit to some extent. Based on the above results, 80% HRW treatment was chosen, in further analysis, for exploring the mechanism of $H_2$ in delaying senescence of kiwifruit.

### 3.3. HRW delays the decrease of firmness by alleviating pectin solubilization in kiwifruit

Loss of texture is one of the main factors limiting quality and the postharvest shelf-life of fruits and vegetables. Changes in firmness between controls and treated fruits during 16 d of storage are shown in Fig. 2A. All fruits, treated or not, showed a fast decrease in firmness during the first 8 days of storage. After 8 days of storage, the firmness values of $CK_0$, $CK_1$ and HRW treatments decreased by 79.3%, 85.5% and 65.7% in comparison with the initial values, suggesting that 80% HRW treatment could slow the softening rate of kiwifruit. With the prolongation of storage time, the decline of firmness in all fruit were slowed, and the HRW-treated fruits were also significantly firmer ($P < 0.05$) than were $CK_0$ and $CK_1$, with the only exception of 16 d of storage, because all fruit developed textural breakdown in this period. Overall, the results demonstrated that 80% HRW treatment is beneficial for firmness retention of kiwifruit.

Differences in firmness of kiwifruit during storage may be related to pectin solubilization and depolymerization (Ramana-Rao et al., 2011). Therefore, the water-soluble pectin (WSP) and protopectin contents were measured. As shown in Fig. 2B, the WSP contents of all fruit increased over time, while the corresponding protopectin contents constantly decreasing during storage (Fig. 2C). Among different treatments, the WSP content of 80% HRW-treated fruit was significantly lower than those in the controls between day 8 and day 16 of storage. By contrast, the corresponding protopectin contents were significantly higher in the 80% HRW treatment than those in the controls during days 4–16. These results clearly demonstrated that 80% HRW treatment could effectively alleviate the degradation of insoluble protopectin to the

more soluble pectin, thereby preserving the firmness of kiwifruit during storage (Fig. 2A).

### 3.4. HRW inhibits cell wall degrading enzyme activities in kiwifruit

The solubilization of cell wall pectin is primarily mediated by the activities of fruit-softening enzymes, such as cellulase, PG, and PME (Pathak & Sanwal, 1998). Thus, we further assayed the activities of relevant enzymes in kiwifruit during storage. Results showed that the cellulase activity of $CK_0$ and 80% HRW treatment increased significantly during days 0–8 and obviously decreased during days 8–12, followed by a steady period (Fig. 3A). Differently, cellulase activity of $CK_1$ fruit increased obviously during days 0–12 and decreased thereafter. Among different treatments, the 80% HRW treatment had significantly lower ($P < 0.05$) cellulase activity than had the corresponding controls ($CK_0$ and $CK_1$) during all the storage periods, suggesting that 80% HRW posed an inhibitory effect on the cellulase activity of fruit tissues.

The results of PME enzyme activity are shown in Fig. 3B. All fruit reached peak values on days 4 and 12. During days 4–12, the PME activities of kiwifruit were significantly lower ($P < 0.05$) in the 80% HRW treatment than in the controls (except for $CK_0$ on day 16), indicating that 80% HRW suppressed the PME activities of kiwifruit during storage.

Additionally, the PG activity of fruit continuously increased during storage. However, when treated with HRW, the activities of PG were significantly lower ($P < 0.05$) than controls (except for $CK_0$ on day 4 and day 8; Fig. 3C).

### 3.5. HRW reduces the respiration intensity and the lipid peroxidation level, and activates the activity of superoxide dismutase (SOD) in kiwifruit

Generally, respiration is a good indicator of the metabolic rates of harvested fruits and vegetables. Decreasing of respiratory intensity of fruit can be an effective means of regulating general metabolism and extending postharvest storage life of these commodities (Kim et al., 2001). The effects of $H_2$ on the respiratory intensity in the kiwifruits were examined thereafter. As shown in Fig. 4A, the initial respiration intensity was about 4.22 mg/kg/h, and the respiration intensity reached the peak value of 53.9 mg/kg/h (12.8-fold of initial value) after 4 days of storage in $CK_1$. Meanwhile, the respiration intensity of fruits of $CK_0$ and HRW treatments could reach corresponding maximum values on day 8, but 80% HRW exhibited low levels of the respiration intensity throughout the storage, in comparison with $CK_0$ and $CK_1$. For example, it was observed that the peak for the HRW treatment fruit was 26.7% lower than that in $CK_0$ ($P < 0.05$).

To assess whether the beneficial effects of $H_2$ were related to oxidative stress, TBARS, an indicator of oxidative damage, was measured. As shown in Fig. 4B, the senescence caused significant increase in the TBARS contents during storage. However, the TBARS levels in $CK_1$ were 36.4%, 18.9%, 13.1% and 11.0% higher ($P < 0.05$) than those in HRW treatment on the 4th, 8th, 12th, and 16th day of storage, respectively. On the other hand, when compared with $CK_0$, TBARS content in HRW-treated fruit was also significantly lower ($P < 0.05$), except on day 8, suggesting that the application of exogenous $H_2$ protected kiwifruit against oxidative damage.

To confirm the above deduction, the SOD activity, which acts as the pioneer for defence against free radicals, was analysed (Fig. 4C). As expected, 80% HRW-treated fruit had significantly higher SOD activities than had the controls after 4 days of storage. Since the increasing of SOD activity could enhance the ability of kiwifruit tissues to dismute $O_2^{\cdot-}$, a decreased lipid peroxidation accumulation during storage was observed (Fig. 4B).

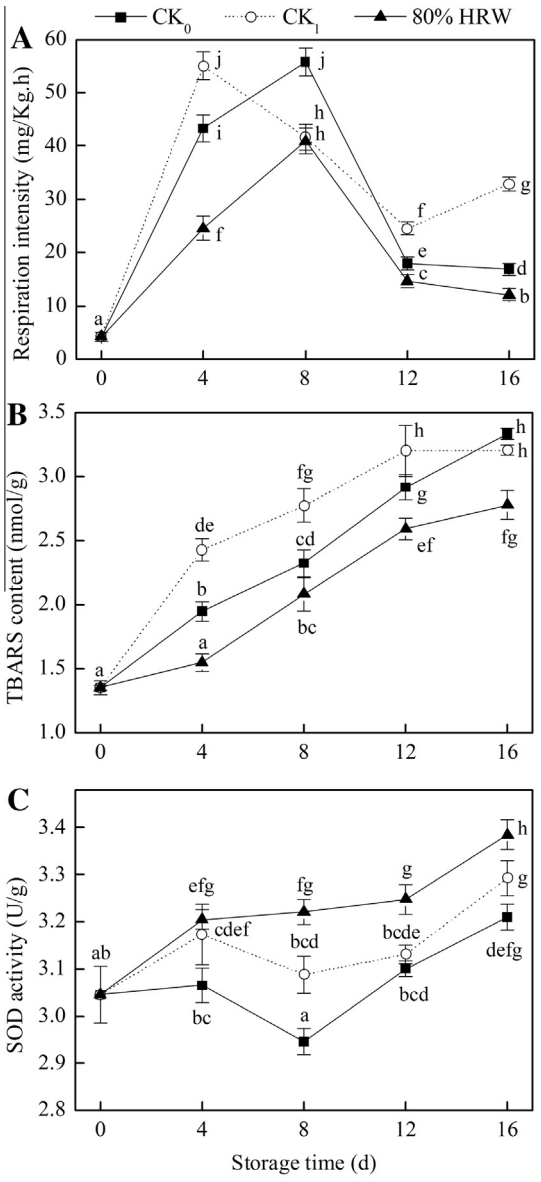


**Fig. 4.** Effects of HRW pretreatment on respiration intensity (A), TBARS content (B) and superoxide dismutase activity (SOD, C) of kiwifruit during storage. The vertical bars represent standard deviations. Values followed by a different letter were significantly different according to Duncan's multiple range test at $P < 0.05$.

### 3.6. HRW excites the radical-scavenging activities of 2,2-diphenyl-1-picrylhydrazyl (DPPH·), superoxide anions ($O_2^{\cdot-}$) and hydroxyl radical (·OH) in kiwifruit

Fruit internal quality and pace of senescence during storage are correlated with antioxidants potential. In addition to the antioxidant enzyme system, plants possess a network of low molecular mass antioxidants, including ascorbate, glutathione and phenolic compounds (Singh & Singh, 2013). Thus, such non-enzymatic antioxidant activity was determined according to the scavenging activities of DPPH·, $O_2^{\cdot-}$ and ·OH. As shown in Fig. 5A, the scavenging activity against DPPH· in HRW treatment exhibited an increase during the first 4 days and then decreased during further storage. Most importantly, the DPPH·-scavenging activity of HRW-treated fruit was significantly higher ($P < 0.05$) than those in the controls throughout the storage.

Meanwhile, the $O_2^{\cdot-}$-scavenging activity of kiwifruit was initially 70% (day 0), which remained relatively stable within 8 days of

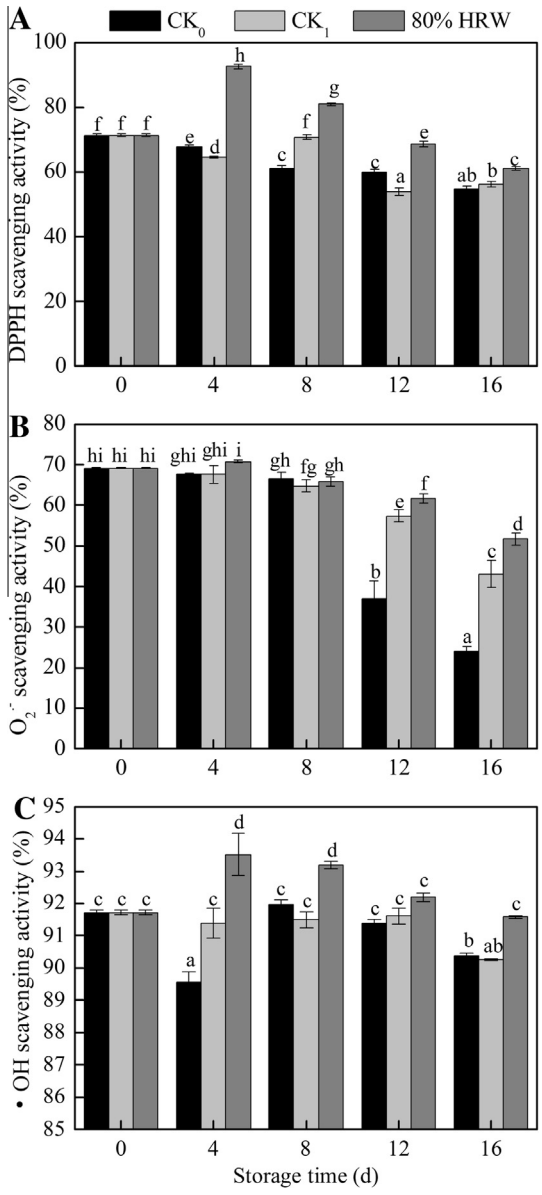


**Fig. 5.** Effects of HRW pretreatment on scavenging activities of 2,2-diphenyl-1-picrylhydrazyl (DPPH., A), superoxide anions ($O_2^{\cdot-}$, B), hydroxyl radical (.OH, C) of kiwifruit during storage. The vertical bars represent standard deviations. Values followed by a different letter were significantly different according to Duncan's multiple range test at $P < 0.05$.

storage in all treatments and obviously decreased thereafter (Fig. 5B). Comparatively, 80% HRW treatment could significantly retard the decline of $O_2^{\cdot-}$-scavenging activity of kiwifruit after 8 d of storage as compared to the control samples.

Similar to the DPPH·-scavenging activity, on day 4, the .OH-scavenging activity in kiwifruit was also increased by 80% HRW (Fig. 5C), while, on the same day, the corresponding values of the controls remained stable ($CK_1$) or decreased 2.4% ($CK_0$) as compared to the initial value (91.7%). Afterwards, the .OH-scavenging activity of all fruit decreased over time. As expected, we also noticed that 80% HRW treatment deferred the decreases on days 8 and 16.

### 3.7. Effect of HRW on the morphology of cellular structures in kiwifruit

To confirm the regulatory roles of $H_2$ to the senescence at a cellular level, we further observed the cellular structures by transmission-electron-microscopy (TEM). The TEM images showed that there exist a large number of mitochondria in the cells of kiwifruit, and the inner membrane of these mitochondria are intact on day 0 (Fig. 6). However, after 12 days of storage, the number of mitochondria decreased in the cells, and the inner membrane of mitochondria was degraded to some extent. In contrast, the inner membrane of mitochondria in the HRW treatment exhibited higher integrity, which may be associated with the reduction of oxidative stress in the HRW treatment (Fig. 4B). In addition, an examination of the structure of the cell wall in kiwifruit showed that 80% HRW treatment evidently delayed the degradation of cell wall in comparison with the controls (particularly with regard to $CK_1$), which was consistent with the result of firmness assay (Fig. 2A).

## 4. Discussion

Unlike many other fruits, the ripening or softening of kiwifruits does not occur on the tree, but takes place several days after harvest. During ripening, fruit often show physiological responses when exposed to exogenous elicitors (Tian, Wan, Qin, & Xu, 2006). For example, 1-MCP can maintain the firmness of kiwifruit (Jhalegar et al., 2011), and nitric oxide (NO) is involved in ripening and senescence of kiwifruit (Lai et al., 2011). Recently, some studies found that $H_2$ acts as a novel and cytoprotective regulator in the improvement of Arabidopsis (Xie et al., 2012) and rice (Xu et al., 2013) salt tolerance. Additionally, $H_2$ could enhance the tolerance of plants to multiple environmental stresses, including drought and cold stresses (Jin, Zhu, Cui et al., 2013). In this study, we observed that pretreatment of kiwifruit with 80% HRW could effectively reduce the rot incidence (Table 1). Meanwhile, this treatment maintained a higher firmness in kiwifruit (Fig. 2A), indicating that $H_2$ is beneficial for delaying fruit ripening. After 12 days of storage, the firmness of HRW treatment was significantly higher than those in the controls on day 8, which suggested that pretreatment with $H_2$ could prolong the shelf life of kiwifruit for about 4 days.

We also noticed that the treatment of 100% HRW aggravated the rot incidence of kiwifruit (Table 1). It was further observed that the kiwifruit could product $H_2$ (Supplementary Fig. 2). For example, in comparison with the control values, 80% and 100% (in particular) HRW treatment caused increase of $H_2$ content after 4 days of storage. A recent study demonstrated that a higher concentration of HRW (100%) really aggravated the opposite effects on the primary root growth of Arabidopsis seedlings caused by salinity (Xie et al., 2012). Therefore, we speculate that the higher level of $H_2$ provided by 100% HRW might exceed the physiological requirement. Also, the delay of ripening and senescence of kiwifruits might be dependent on $H_2$ homoeostases. This deduction was supported by the observation that $H_2$ was found to alter the effect of individual plant hormones (Zeng, Zhang, & Sun, 2013). Therefore, we further suggest that the high content of $H_2$ might influence plant senescence-related hormone signalling. Certainly, this should be investigated in the near future.

Fruit softening often occurs during ripening, particularly in the climacteric fruit, because of degradation of cell wall components, a consequence of the coordinated action of cell wall-modifying enzymes, including cellulase, pectin methylesterase (PME) and polygalacturanase (PG) (Fischer et al., 1996; Pathak & Sanwal, 1998). For example, cellulase can degrade cellulose and the β-1,4-glucan backbone of xyloglucan, a hemicellulosic polysaccharides that is prominently found in the cell walls of dicotyledons (Ramana-Rao et al., 2011). Lohani, Trivedi, and Nath (2004) showed that the softening changes in banana fruit are reflections of an overall response of cellulase activity levels. In this study, although HRW treatment reduced the cellulase activity, the result of

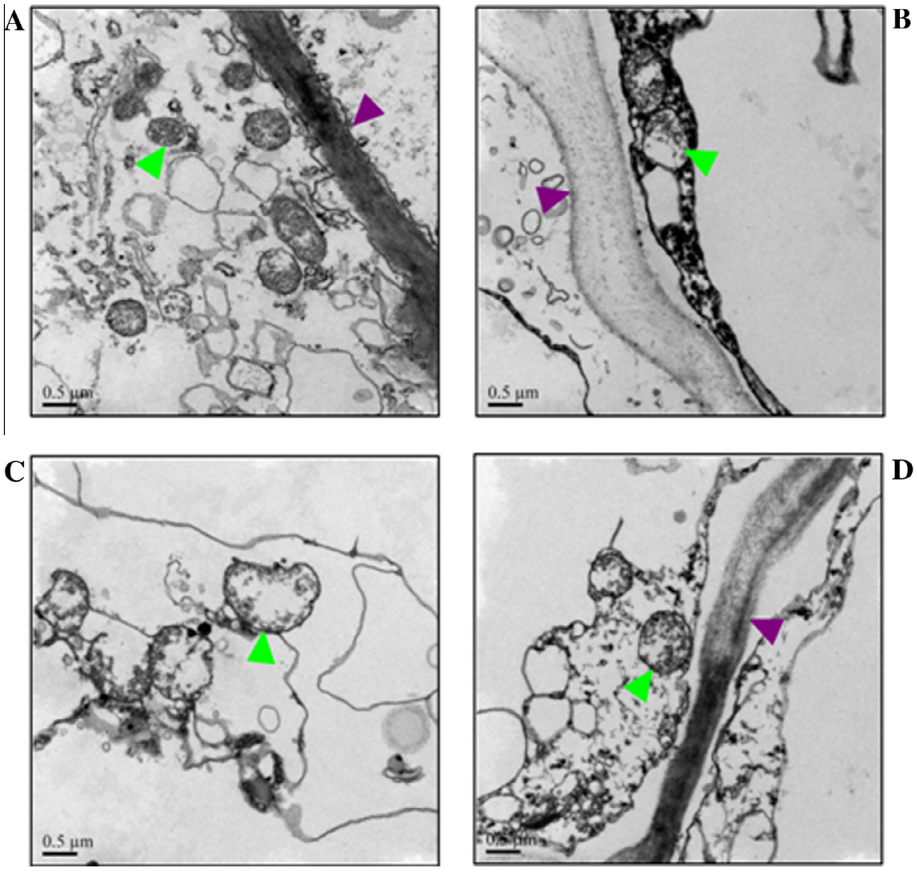


**Fig. 6.** Transmission-electron-microscopy images of kiwifruit flesh on day 0 (A) and on day 12 of storage in $CK_0$ (B), $CK_1$ (C) and 80% HRW treatments (D). The mitochondria (▲) and cell wall (▲) in kiwifruit flesh are marked in the Figure.

correlation analysis (data not shown) illustrated that cellulase was not a key enzyme regulating the cell wall degradation of kiwifruit during storage. Previously, Bonghi, Pagni, Vidrih, Ramina, and Tonutti (1997) also suggested that PG is involved in pectin depolymerization in the late phase of kiwifruit softening. In addition, PME activity is usually linked to chemical changes in cell wall-middle lamella structure during ripening of kiwifruit tissue (Mahboube, Mohammad, Mohsen, & Hamidreza, 2010). In this study, correlation analysis also showed that the PG activity has a significant negative correlation with firmness in all treatments (data not shown). Similarly, the PME activity also has a negative correlation with firmness in all treatments. However, the correlation coefficient was significant only in the 80% HRW treatment ($r = 0.808$; $P < 0.05$), suggesting that PME plays a key role in regulating the softening of kiwifruit. Therefore, HRW treatment suppressed the activities of PME and PG in kiwifruit (Fig. 3B and C), resulting in a delay of cell wall polyuronide solubilization (Fig. 2B), and thus affecting pectin metabolism and fruit softening (Fig. 2A).

The production of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, is considered as an important event in fruits during storage (Singh & Singh, 2013). The accumulation of ROS resulting from an altered balance between ROS production and scavenging capacities can reduce the storage quality and marketability of fruits and vegetables (Hodges, Lester, Munro, & Toivonen, 2004). Previous studies showed that $H_2$ exerted a protective role in many disorders related to oxidative stress in animals (Ohsawa et al., 2007). More recently, Cui, Gao, Fang, Lin, and Shen (2013) also proved that HRW treatment exhibited obvious decreases of Cd-induced ROS overproduction and alleviation of oxidative stress by 3,3′-diaminobenzidine tetrahydrochloride staining. Our results further illustrated that, besides inhibiting the rot incidence of kiwifruits during storage, 80% HRW treatment exhibited a remarkable alleviation of oxidative damage in kiwifruit during storage, which was evaluated by TBARS content (Fig. 4B). This protective effect of $H_2$ might be ascribed to the ability of $H_2$ to activate the activity of SOD (Fig. 4C). The results were consistent with those obtained in animals, showing that low doses of $H_2$ enhance SOD and CAT activities, as well as increasing levels of the well-known antioxidant glutathione (GSH), thereby increasing endogenous antioxidant defences against ROS (Hong, Chen, & Zhang, 2010). Similar results were also observed by Jin, Zhu, Cui et al. (2013) and Xu et al. (2013), demonstrating that $H_2$ treatment could induce SOD activity and reduce lipid peroxidation in alfalfa leaves under paraquat stress, and rice seedlings under salt stress. In addition, Ohsawa et al. (2007) found that $H_2$ could selectively reduce **.**OH content in animals. In the present investigation, HRW treatment caused the increase of scavenging activity of ·OH on day 4 (Fig. 5C). Moreover, the treatment induced the increase of DPPH·-scavenging activity on same day. With the prolonging of storage time, the radical-scavenging activity declined (Duan et al., 2011). Nevertheless, the HRW treatment delayed the decrease of scavenging activity of $O_2^{\cdot-}$ during storage (Fig. 5). These results clearly revealed that $H_2$ could increase the radical-scavenging activity and maintain it at basal physiological levels during storage of kiwifruit. Accordingly, we postulated that $H_2$ may act as a ROS-scavenger or inducer of antioxidant systems.

In general, the increased free radical levels can damage mitochondrial DNA (mtDNA) in organisms, and the damage to mtDNA results in decay of mitochondria, decreased production of ATP, and the activation of the senescence (Dorszewska, 2013). $H_2$ has favourable distribution characteristics: it can penetrate biomembranes and diffuse into the cytosol, mitochondria and nucleus.

Consequently, we further investigated the effect of HRW pretreatment on the structure of mitochondria in the cells of kiwifruit. Related experiments illustrated that 80% HRW was able to maintain a higher integrity of the membrane in mitochondria, and delay the degradation of mitochondria (Fig. 6). The alteration of the membrane system, the primary response of senescence, disrupts the composition and functionality of mitochondria, and then alters the protein complexes within the membranes (Miki & Funato, 2012). In addition, this negatively affects the functionality of such complexes and stimulates an abnormal ROS production, causing an oxidative stress that can be considered as the secondary response of senescence (Picard et al., 2011). In this case, HRW treatment also delayed the process of senescence in kiwifruit, which was consistent with the result in Fig. 2A. These results expand our understanding of the role of $H_2$. Notably, $H_2$ has no risk of flammability or explosion at a concentration of less than 4.7% in air. We propose that $H_2$, one of the most well-known molecules, could be widely used in preservation as a safe and effective antioxidant with minimal side effects.

## 5. Conclusion

This study showed that pretreatment with 80% HRW could effectively reduce the rot incidence and inhibited the respiration intensity of postharvest fruits. 80% HRW treatment could delay decrease in fruit firmness by alleviating pectin solubilization and suppressing the enzyme activities of cellulase, PG, and especially PME. Additionally, the HRW treatment reduced lipid peroxidation by enhancing the SOD activity, as well as the free radical ($DPPH^{\cdot}$, $O_2^{\cdot-}$, and $\cdot OH$) scavenging activity. Reduction of oxidative damage could be one of the main mechanisms by which the HRW treatment delays senescence and inhibits respiration of kiwifruit. Together, these results clearly indicate that HRW treatment has a potential role in the preservation of kiwifruit. However, the detailed mechanisms by which $H_2$ executes function in fruit defence responses should be investigated in the future.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.01.067.

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