Afyon Kocatepe Üniversitesi Fen ve Mühendislik Bilimleri Dergisi

Afyon Kocatepe University Journal of Science and Engineering

AKÜ FEMÜBİD **22** (2022) 025401 (315-323) AKU J. Sci. Eng. **22** (2022) 025401 (315-323)

DOI: 10.35414/akufemubid.1003264

Araştırma Makalesi / Research Article

The Effects of Hydrogen Peroxide Application on Physio-chemical Properties of Strawberry (*Fragaria ananassa*, var. Festival) Fruits in Postharvest Storage Period

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Geliş Tarihi: 01.10.2021 Kabul Tarihi: 10.03.2022

Keywords
Postharvest; H_2O_2; Strawberry; Pectate lyase; Pectin; Polygalacturonase

Abstract

Disassembly of cell wall polysaccharides play an important role in postharvest fruit texture softening. Reactive oxygen species (ROS) are involved in fruit ripening. Here, the role of hydrogen peroxide (H_2O_2), acting as a ROS, on physical, biochemical and molecular properties and polysaccharide content of strawberry fruits during storage at +4 °C was investigated. Strawberry fruits harvested at commercial ripening stage were immersed in H_2O_2solutions (0, 100 and 500 mM) for 30 minutes, then stored at +4 °C for 8 days. The results showed that 100 mM H_2O_2treatment significantly increased fruit firmness, decreased water soluble pectin and expression of cell wall related genes, *polygalacturonase* (*PG*) and *pectate lyase* (*PL*). These results suggested that overall morphological and biochemical quality of strawberry could be effectively maintained by 100 mM H_2O_2treatment in postharvest storage conditions.

Hidrojen Peroksit Uygulamasının Hasat Sonrası Depolama Döneminde Çilek (*Fragaria ananassa*, var. Festival) Meyvelerinin Fizyo-kimyasal Özellikleri Üzerine Etkileri

Anahtar kelimeler
Hasat Sonrası; H_2O_2; Çilek; Pektat liyaz; Pektin; Poligalakturonaz

Öz

Hücre duvarı polisakkaritlerinin parçalanması, hasat sonrası meyve dokusunun yumuşamasında önemli bir rol oynamaktadır. Olgunlaşma ile birlikte Reaktif oksijen türlerinin (ROS) akümülasyonu farklı çalışmalarda gösterilmiştir. Bu çalışmada bir ROS görevi gören hidrojen peroksitin (H_2O_2) +4 °C'de depolanması sırasında çilek meyvelerinin fiziksel, biyokimyasal ve moleküler özellikleri ile polisakkarit içeriği üzerindeki rolü araştırılmıştır. Ticari olgunluk aşamasında hasat edilen çilekler farklı konsantrasyonlardaki H_2O_2 solüsyonlarında (0, 100 ve 500 mM) 30 dakika bekletildikten sonra +4 °C'de 8 gün saklanmıştır. Elde edilen veriler, 100 mM H_2O_2 uygulamasının meyve sertliğini önemli ölçüde artırdığını, suda çözünür pektini ve hücre duvarı ile ilişkili *poligalakturonaz* (*PG*) ve *pektat liyaz* (*PL*) enzimlerini kodlayan genlerin ekspresyonunu azalttığını göstermiştir. Bu sonuçlar, hasat sonrası depolama koşullarında çileğin genel morfolojik ve biyokimyasal kalitesinin 100 mM H_2O_2 uygulaması ile etkili bir şekilde korunabileceğini göstermiştir.

1. Introduction

Strawberry (*Fragaria × ananassa*), which is commercially produced, is a hybrid that resulting from the cross of two wild octaploid species (*Fragaria verginiana x Fragaria chiloensis*) with a chromosome number (2n = 8X = 56). Strawberry is a fruit with a very short shelf life, and its production and consumption are increasing rapidly due to its characteristic aroma and nutritive components (8.8 million tons of production FAO, 2020). Turkey, on the other hand, is the largest strawberry producer in the world after the USA, with an annual production of 669 195 tons (TUIK 2021).

Fruit softening in strawberries, similar to all fleshy fruits, is due to excessive dissolution from the middle lamella of parenchyma cells, increasing the intercellular spaces and breaking their connections (Santiago-Doménech *et al*. 2008). When examined at the cell wall level, a moderate pectin dissolution and degradation and a slight decrease in the molecular weight of hemicellulosic polymers are the general characteristics of strawberry softening (Figueroa *et al*. 2010). When the cellulose structure in the cell wall is analysed, this polymer is found in very small amounts compared to pectin, and almost not affected during the softening period (Koh *et al*. 1997; Figueroa *et al*. 2008). Therefore, most of the studies carried out to reveal the softening mechanism of strawberry and to control the softening have focused on pectinolytic enzymes and different chains and regions targeted by these enzymes. The reason for this is that pectin degradation plays a fundamental role in fruit softening. For example, it was shown in the characterization study of the rhamnogalacturonan lyase (RG-lyase) in strawberry that the *FchRGL1* gene activity increased considerably during the ripening period (Méndez-Yañez 2020). Xue et al. (2020) identified 54 PME genes in *F. vesca* 'Hawaii 4' strawberry, characterized *FvPME38* and *FvPME39* genes by over-expression and RNAi silencing, and proved that these genes have important functions such as fruit softening and pectin dissolution in ripening fruits. Silencing the *FaβGal4* gene, a candidate of β-galactosidase, resulted in 30% firmer transgenic strawberry fruits (Paniagua *et al*. 2016). However, in general, these transgenic studies were very limited in increasing fruit tissue firmness in strawberry. However, transgenic strawberry fruits obtained by silencing the *polygalacturonase* (*PG*) (Quesada *et al.* 2009) and *pectate lyase* (*PL*) (Jiménez-Bermúdez *et al*. 2002) resulted with much firmer texture.

Reactive oxygen species (ROS) are composed of several free radical-containing molecules that are regularly found in plants and are harmless at normal levels (Foyer *et al*. 2018). The level of ROS increases during the ripening period of strawberry and this is associated with the transition of molecules from chloroplasts to chromoplasts (Song *et al*. 2020). However, during the ripening/softening period or under stress conditions, the level of ROS in fresh fruits can exceed a certain threshold, causing irreversible DNA damage and cell death, leading to senescence and reduced shelf life of fresh fruits (Chen *et al*. 2019; Lin *et al*. 2020). ROS containing mainly hydrogen peroxide (H_2O_2) have been shown to participate in cell differentiation, senescence, PCD (programmed cell death) and cell wall formation in plants (Ribeiro *et al*. 2017). H_2O_2, a compound considered safe and environmentally friendly (Felizini *et al*. 2016), has been implicated as a key signalling molecule in various biochemical pathways associated with the a/biotic stress response. H_2O_2 regulates the plant development process and stress responses by interacting with hormones (Liu *et al*. 2020). In addition, H_2O_2 treatment has been proposed as an alternative to chemical treatments to improve the quality of fresh fruits in postharvest conditions (AL-Saikhan and Shalaby 2019). In this study, it was aimed to examine the changes in cell wall polysaccharides during the postharvest softening process in strawberry. For this purpose, the main quality parameters, pectin fractionation, and gene expression of pectin-degrading key enzymes were characterized in strawberry fruits treated with different H_2O_2 concentrations.

2. Materials and Methods

2.1. Plant Material

Strawberry fruits (*Fragaria ananassa*, var. Festival) used in the experiments were taken during the commercial harvest period from a producer in Aziziye Village of Burdur Province, Turkey.

Harvested strawberry fruits were brought to Burdur Mehmet Akif Ersoy University, Plant Molecular Genomics Research Laboratory to carry out all analysis. The 100 mM and 500 mM H_2O_2 solutions used in the experiments was prepared by diluting it in distilled water completed to 2 Liters. Distilled water was used for the control group. Fifty fruits were used for the group and each concentration group. For each concentration group, fruits were immersed in solutions for 30 minutes, then dried

and stored at 4°C for 8 days, then brought to room temperature (RT) and three 3 days. Samples were taken every 2 or 3 days to observe the physiological, morphological, and molecular changes of the fruits.

2.2. Evaluation of fruit quality

Colour of the strawberries was evaluated using a calorimeter (PCE-CSM 1) by randomly taking 10 fruits from each concentration group and control. The measured L*, a*, b* values were calculated as colour index data (CI) (Nangare *et al.* 2016). The firmness of fruits was measured from the two sides of pericarp with a penetrometer (PCE-PTR 200, 6 mm probe) and the results were recorded in Newton (N). The firmness of five fruits of all treatments and control fruits were measured at intervals of three days. In order to determine the weight loss of the fruits (fruit weight loss, FWL) during the storage, 10 strawberry samples were taken from the two concentration groups and the control fruits. In this process, the morphological changes of the fruits were also photographed.

2.3. Extraction and fractionation of cell components

For the extraction of pectin, alcohol-insoluble solids (AIS) were prepared following the procedure (Lunn *et al.* 2013). Pectin in different forms found in the cell wall was extracted by sequential chemical extraction of the cell wall material. In order to extract pectin in water-soluble form, 7.5 mg of AIS was mixed with water (WSP, Water Soluble Pectin) at room temperature for four hours and centrifuged at 10000 g for 20 minutes. After the liquid phase was taken, the residue was mixed with 50 mM CDTA for four hours at RT to remove the ionic bound pectin and centrifuged at 10000 g for 20 minutes. After the liquid phase was removed, the last remaining residue was mixed with 50 mM Na_2CO_3 + 20 mM $NaBH_4$ for overnight at 4 °C to remove pectin covalently bound to the cell wall, and the liquid phase was taken and centrifuged again. 300 µL of each sample was taken into a test tube and incubated in a hot water bath at 70 °C for 40 minutes by adding 300 µL of boric acid and 5 mL of sulfuric acid (H_2SO_4). After incubation, 200 µL of dimethyl-phenol solution was added to the test tubes and left for 5 minutes at RT for colour change. For the quantitative analysis of the samples, uronic acid measurement was read with a spectrophotometer (BioTek, Epoch Microplate) at a wavelength of 405nm and 450 nm. The average of readings from two biological replicates and two technical replicates were used for statistical analyses. Results are expressed as milligrams of GA g^{-1} AIS.

2.4. Total RNA isolation and RT-qPCR analysis

Total RNA was extracted from the fruits, 0, 2, 5, 8 (days) of storage using the PureLink™ Plant RNA isolation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Total RNA concentration was measured using nanodrop (BioTek, Epoch Microplate) and then translated into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The expression level of each gene was calculated by normalizing it to the endogenous reference gene *actin* (Hou et al., 2021). The primer sequences used for RT-qPCR are listed in Table 1. The program used for PCR was set using the following conditions: 95°C for 5 minutes, 35 cycles at 95°C for 5s, 30s at 60°C. Amplifications were performed using the CFX96™ Real Time System (Bio-Rad) and gene expression levels were calculated using the $2^{-\Delta Ct}$ method (Livak and Schmittgen 2001).

Table 1. Primer sequences used for RT-qPCR

Gene	Forward Sequence	Reverse Sequence
PG	ACTTCAACTGCGGAGGCTTT	TAGCACCAAGAGGCGGT
PL	GCCTTGCTCGTTTGCGTATC	TCCTTGGCTCCTCTACTACTTCC
Actin	TGCATATATCAAGCAACTTTACACTGA	ATAGCTGAGATGGATCTTCCTGT

2.5. Statistical Analysis

The statistical analyses were conducted according to completely randomised design with at least five different strawberry fruits for fruit firmness and 10 fruits CI measurements. Three different fruits were used for cell wall analysis. All statistical analyses were performed through XLSTAT (version 2016.02.28451, Addinsoft, France). Duncan test was utilised for the comparison of means ($P \leq 0.05$).

3. Results and Discussion

Strawberry fruits harvested at commercial maturity stage were treated with 100 mM and 500 mM H_2O_2. In order to determine clearly the effects of H_2O_2 on biochemical composition and gene expression level, fruit samples were stored at +4 °C for 8 days, then brought to RT and stored for 3 more days. Phenotypic changes occurring in fruits during this period are shown in Figure 1.

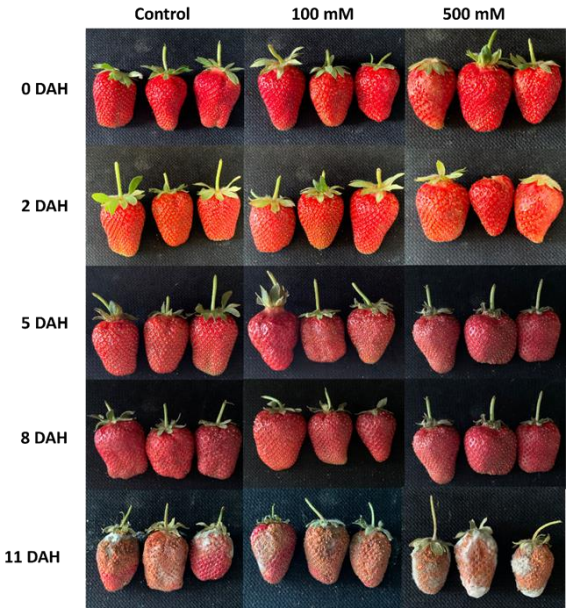

Figure 1. The effects of H_2O_2 treatment during the postharvest storage period of strawberry fruits harvested at commercial ripening stage. H_2O_2 applications at control, 100 mM and 500 mM. DAH: (days after harvesting).

The colour index value (CI) of the fruits were measured on the days when photographs were taken. However, since the fruits at the 11DAH were brought to room temperature, they deteriorated extremely rapidly, and their colour index values could not be measured properly. There was no significant change in the colour index values of the fruits in the first five days of storage. However, at the end of 8 days, the fruits started to change to a darker colour from their bright red colour and a decrease in CI values occurred (Figure 2a).

Regardless of the treatments, a gradual decrease in the firmness of the strawberry fruits was observed during storage (Figure 2b). While this decrease progressed slower at +4 °C, it continued sharply after the fruits were taken to room temperature. Even if there was no difference between the groups in terms of fruit firmness on the last day (8DAH) when the fruits were stored at +4 °C, after the fruits were brought to RT, the firmness of strawberry fruits treated with 100 mM H_2O_2 was statistically significant compared to the control and 500 mM H_2O_2 treated fruits ($p < 0.05$). FWL of strawberry fruits treated with H_2O_2 and control (non-treated with H_2O_2) are shown in Figure 2c. Although high differences were not observed in FWL throughout the entire analysis, fruits treated with 100 mM H_2O_2 had slightly less weight loss.

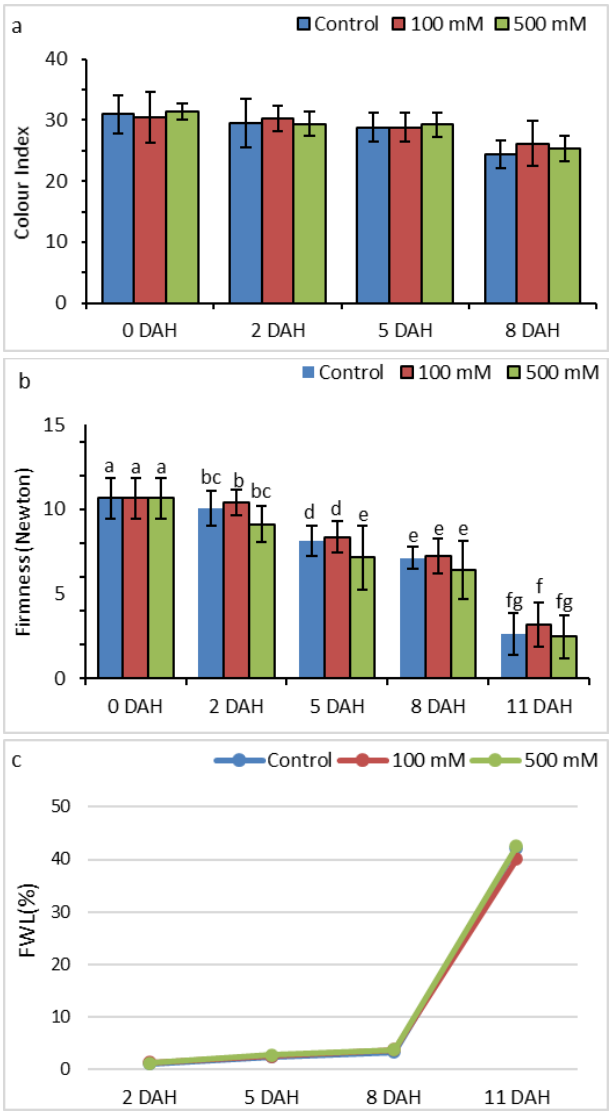

Figure 2. Effect of H_2O_2 treatments on CI (a), firmness (b) and fruit weight loss (c) of strawberry fruits during postharvest storage up to 11 days. Data were calculated over the averages of ten fruits for CI and FWL analyses, and five fruits for firmness values (SD). Different letters above the columns indicate statistically significant $p \leq 0.05$ differences.

3.1. Changes in cell wall composition of fruits during storage

In order to show the differences in fruit firmness at the level of cell wall composition, H_2O_2 treated, and control strawberry samples were powdered, and the material was separated into pectin fractions as described in the method section. Fractionation of cell wall material (CWM) revealed differences in WSP levels at different ripening/softening stages (Figure 3). As expected, the amount of WSP in all groups was approximately 40 mg/UA in AIS^{-1} in the first harvested period, while this amount increased to approximately 70 mg/UA in AIS^{-1} in the 2 DAH stage, especially in control fruits. The amount of WSP remained the same in fruits treated with H_2O_2 (2DAH fruits) in the same period. During the 8-day shelf life of all samples, a significant reduction in WSP amount was observed ($p<0.05$). In the last period of the shelf life (measurable amount of WSP at 8DAH), fruits treated with 100 mM H_2O_2 had the least amount of WSP (Figure 4). A gradual and significant increase in CDTA-soluble pectin content was found over time in H_2O_2-treated and control strawberry fruits. The highest amount of CDTA soluble pectin content in all strawberry groups was observed at 8 DAH period and this showed that CDTA content increased as ripening/softening progressed. When the amount of pectin dissolved in Na_2CO_3 was examined, clearer results emerged as in the WSP pectin content. Strawberry fruits treated with 100 mM H_2O_2 contained significantly higher amounts of Na_2CO_3 soluble pectin than 500 mM H_2O_2 and control fruits. These results showed that the application of 100 mM H_2O_2 slowed down the degradation of pectin, which plays an important role in the softening of strawberry.

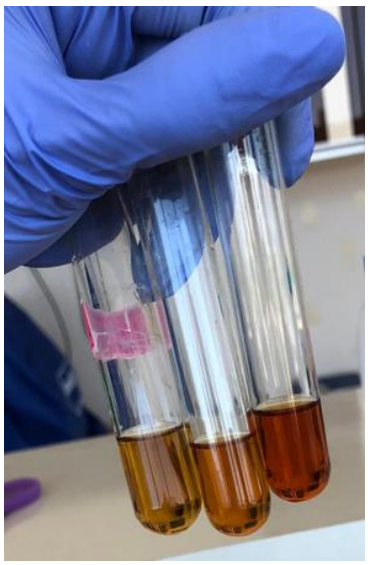

Figure 3. An image of each of the pectin fractions soluble in WSP, CDTA and Na_2CO_3 obtained from the strawberry fruits, respectively.

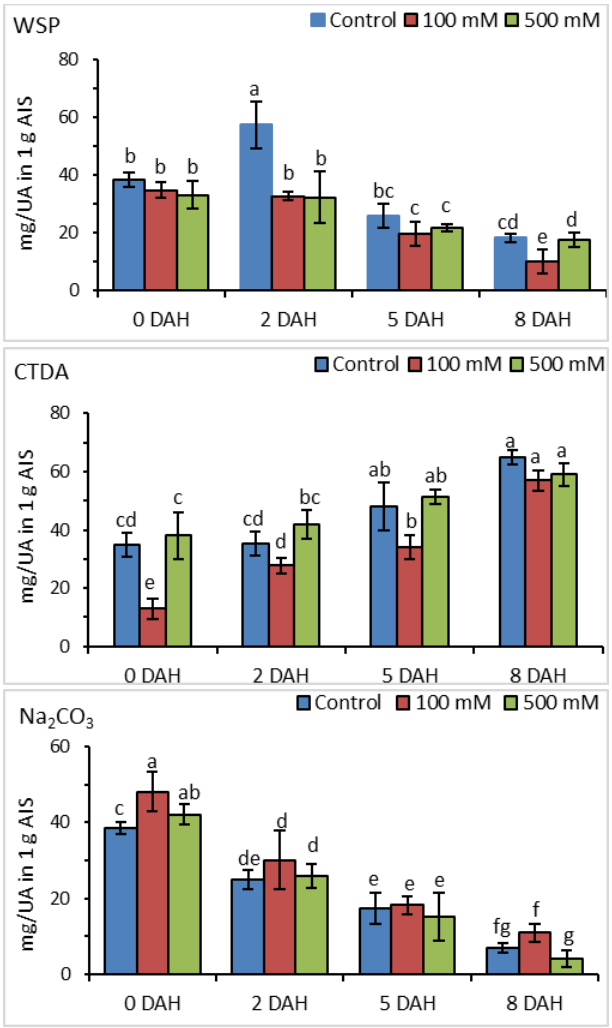

Figure 4. Effect of 100 mM and 500 mM H_2O_2 treatment on WSP, CDTA, and Na_2CO_3 soluble pectin contents. Data were calculated by taking the mean ± standard deviation (SD) of five fruits at each stage and treatment. Different letters indicate statistically significant differences $p\leq0.05$.

3.2. Analysis of expression levels of PG and PL genes by RT-qPCR

PG and *PL* genes were expressed almost at the same level in all treatment groups on the 8th day of storage and did not show a statistical difference (Figure 5). However, there was less *PG* gene expression level in fruits treated with 100 mM H_2O_2 in all analysed days compared to the other two groups. The *PL* gene, on the other hand, was less expressed in strawberry fruits treated with 100 mM H_2O_2 at the 5DAH stored fruits compared to the other two groups. It can be said that the lower expression level of *PG* and *PL* genes in fruits treated with 100 mM H_2O_2, compared to the control and fruits treated with 500 mM H_2O_2 might have caused the firmer fruits treated with 100 mM H_2O_2.

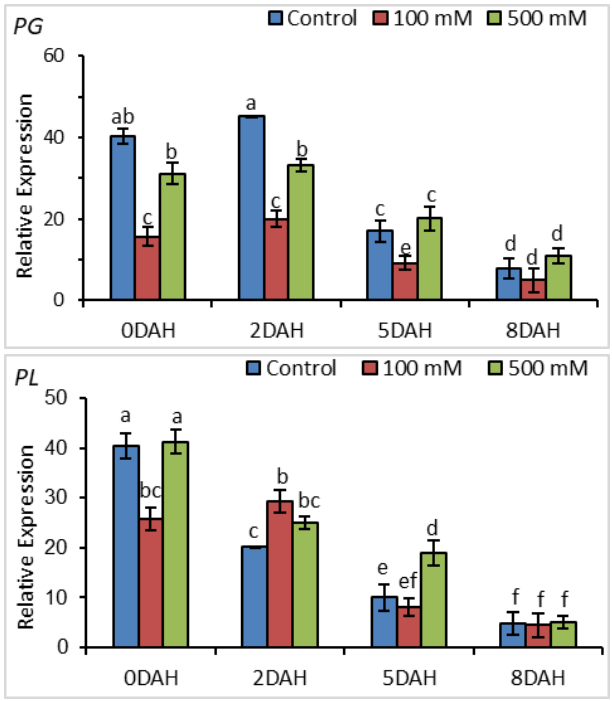

Figure 5. Expression levels of *PG* and *PL* genes during 8 days of storage. The fold-change of genes was normalized to relative expression of the control group. Error bars in each column indicate ±SD of three biological and two technical replicates. Different letters indicate statistically significant differences p≤0.05.

It is well known that H_2O_2 acts as a signalling molecule facilitates various biochemical and physiological processes and can potentially improve tolerance against biotic and abiotic stress conditions Čamagajevac *et al.* 2019). However, if H_2O_2 accumulates in fruits or plant tissues at higher levels than they should be, it can initiate programmed cell death (Marinho *et al.* 2014). In previous studies, it has been shown that H_2O_2 levels increase during the fruit softening period in Kyoho grape (Guo *et al.* 2020), mango (Ren *et al.* 2016), peach (Qin *et al.* 2009) and melon (Lacan and Baccou 1998).

In this study, it was attempted to determine the effects of post-harvest applications of H_2O_2 on morphological, biochemical, and molecular aspects of strawberry fruits. Fruit firmness and FWL are the two important criteria that affect fruit quality and determine shelf-life. Our results indicated a gradual increase in FWL during storage at 4°C in all treated and control fruits, but a sharp increase occurred at RT. However, the FWL was slightly lower in fruits treated with 100 mM H_2O_2 than other two groups (control fruits and 500 mM H_2O_2 treated fruits). The results showed that treatment of higher concentration of H_2O_2 did not reduce FWL in strawberry under postharvest conditions.

During fruit softening period, pectin is accompanied by the disassembly of cell wall components, including cellulose and hemi-cellulose, all of which play important roles in cell-to-cell adhesion and firmness (Brummell and Harpster 2001). The resulting differences in WSP, CDTA and N_2CO_3 fractions may eventually lead to a decrease in cell wall strength and cell separation of the fruit. (Lin *et al.* 2018). An increase in WSP content is closely related with reduced intercellular adhesion, and higher WSP will lead to loss of tissue integrity (Wakabayashi *et al.* 2000). In our results, it was observed that increased WSP content in control and 500 mM H_2O_2 treated fruits resulted in decreased firmness of this fruits. In a study supporting our results, longan fruits treat with 1.96 mmol L^{-1} of H_2O_2 had higher amount of WSP compared to their controls (Lin *et al.* 2020). In our analysis, there was statistically lower amount of WSP and higher amount of N_2CO_3 soluble pectin fraction in fruits treated with 100 mM H_2O_2 compared to other two groups. This result indicates that fruits treated with 100 mM H_2O_2 have much more covalently bound pectin ready to be converted to CDTA and/or WSP in later strawberry softening stages.

Postharvest degradation of fruit tissue is closely associated with changes in mechanical properties of the cell wall (Figueroa *et al.* 2010). Polygalacturonase (PG) and pectate lyase (PL) are the key enzymes which are sequentially involved in cell wall degradation of soluble pectin and depolymerization of de-esterified homo-galacturonan (HG) (Wang *et al.* 2019). The activity of *PG* and *PL* decreased in strawberry fruits treated with 100 mM H_2O_2, especially at 5DAH fruits (Figure 5), resulting in increased firmness (Figure 2b) and decreased WSP content (Figure 4). In contrast, strawberry fruits treated with 500 mM H_2O_2 had higher expression levels of *PG* and *PL*, especially at 5 days stored fruits (Figure 5), a higher WSP (Figure 4) and greater loss of fruits firmness (Figure 2b) and fruit weight (Figure 2c). Consistent with these results, Lin *et al*. (2020) showed that H_2O_2 up-regulates the expression levels of genes that disrupt the longan pulp cell wall. Bayoumi (2008) showed that treatment of 15 mM H_2O_2 reduces the decomposition of white pepper fruits and prolongs their shelf-life. Dipping the carrots in 0.5% H_2O_2 was very effective in controlling decomposition of the samples. However, these results contradict the findings from the study, which have found increased PG activity and softer tissue in berry fruits treated with 300 mM H_2O_2 (Guo *et al.* 2019). These results suggest that application of H_2O_2 at certain concentration might up/down-regulate the activity cell wall modification, thus either preventing or stimulating the softening of strawberry.

4. Conclusion

The present results indicated that different H_2O_2 concentrations have different effects on strawberry quality in postharvest storage conditions. Treatment of fruits with 500 mM H_2O_2 accelerated fruit softening, FWL, increased WSP and increased the expression of cell wall degrading enzymes, suggesting that 500 mM H_2O_2 treatment is not an optimum concentration for increasing strawberry quality parameters in postharvest storage. However, treatment of 100 mM H_2O_2 is the most effective concentration as it significantly affects the physiological properties of fruits, such as extends shelf-life and reduces FWL. Nevertheless, further research is required to better understand the effects of H_2O_2 at different concentrations, especially between 100 mM and 500 mM and treatment durations.

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