EFFECT OF MELATONIN TREATMENT ON ANTIOXIDANTS IN THE ASCORBATE-GLUTATHIONE CYCLE AND COLOR CHANGES IN AVOCADO FRUIT DURING RIPENING

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ABSTRACT

Melatonin acts as a growth regulator with various functions in plants, particularly significant in modulating the ripening and senescence of several fruits. Currently, there is no research on the effects of melatonin on avocado fruit. This study aimed to assess the impact of melatonin on the Ascorbate-Glutathione cycle activity and the colour alterations in avocado peel and flesh during ripening. Avocado 034 was immersed in melatonin (0.1 mM and 0.5 mM) for 2 hours and then stored at 22 °C ± 1 and 75 - 80% humidity. The findings indicated that 0.5 mM melatonin treatment elevated the levels and limited the reduction of ascorbic acid and glutathione while enhancing the activities of antioxidant enzymes ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase in the ascorbate-glutathione cycle. Moreover, the melatonin treatment of avocados resulted in a significant increase in L* and a decrease in a* and b* compared to the control. This suggests that the melatonin treatment could potentially slow down the physiological maturation process, thereby maintaining the freshness and extending the shelf life of avocados. The alterations in color of avocado peel and flesh following exogenous melatonin treatment were suppressed and may be related to the activation of the ascorbate-glutathione cycle.

ẢNH HƯỞNG CỦA XỬ LÝ MELATONIN ĐẾN CÁC CHẤT CHỐNG OXY HÓA TRONG CHU TRÌNH ASCORBATE-GLUTATHIONE VÀ SỰ THAY ĐỔI MÀU SẮC QUẢ BƠ TRONG QUÁ TRÌNH CHÍN

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Melatonin
Chất chống oxy hóa
Chu trình ascorbate-glutathione
Thay đổi màu sắc
Quả bơ

TÓM TẮT

Melatonin hoạt động như một chất điều hòa sinh trưởng với nhiều chức năng đa dạng ở thực vật, đặc biệt quan trọng trong việc điều tiết quá trình chín và lão hóa của nhiều loại quả. Tuy nhiên, hiện chưa có nghiên cứu nào đề cập đến ảnh hưởng của melatonin trên quả bơ. Mục đích của nghiên cứu này là đánh giá ảnh hưởng của melatonin đến sự hoạt động của chu trình ascorbate-glutathione và sự thay đổi màu sắc vỏ quả và thịt quả bơ trong quá trình chín. Quả bơ 034 được ngâm trong melatonin (0,1 mM và 0,5 mM) trong 2h, bảo quản ở 22 ± 1°C và độ ẩm 75 - 80%. Kết quả cho thấy việc xử lý melatonin 0,5 mM đã làm tăng hàm lượng và hạn chế sự sụt giảm axit ascorbic và glutathione, tăng sự hoạt động của enzyme chống oxy hóa ascorbate peroxidase, dehydroascorbate reductase và glutathione reductase trong chu trình ascorbate-glutathione. Hơn nữa, quả bơ được xử lý melatonin thể hiện L* cao hơn và a*, b* thấp hơn đáng kể so với đối chứng, chứng tỏ việc xử lý melatonin có thể làm chậm quá trình chín sinh lý, từ đó góp phần duy trì độ tươi và kéo dài thời gian bảo quản của quả bơ. Sự thay đổi màu sắc của vỏ quả và thịt quả bơ 034 sau xử lý melatonin bị ức chế có thể liên quan đến sự kích hoạt của chu trình ascorbate-glutathione.

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1. Introduction

Avocado (*Persea americana* Mill.) is one of the most nutritious fruits. Avocado fruit is high in energy, fats, proteins, vitamins, fiber, antioxidants, and minerals. Avocado contains various bioactive substances that are good for your health, including vitamin E, ascorbic acid (AsA), carotenoids, and soluble phenolic compounds [1]. Avocado ripens quickly due to strong respiration, resulting in the fruit ripening immediately in a short period of time, with a shelf life of only 5-7 days at room temperature [2]. Furthermore, avocado contains many nutrients and mostly unsaturated fats, which cause quick damage and rot, reducing its quality and commercial value [3].

Fruit ripening is a continuous oxidation process that generates reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl (OH). The ROS content of fruit typically increases progressively during the ripening process, often peaking in the early to mid-ripening stage, and then begins to decline. In addition, the maturation process of fruit is expedited by the corresponding increase in respiration, which is accompanied by an increase in ROS. However, the ROS content reaches a dynamic equilibrium, neutralized by the production and removal of ROS. Conversely, avocados exhibit explosive respiration, which results in the production of a substantial quantity of ROS, their excessive accumulation, and cytotoxicity. If not effectively eliminated, these compounds will peroxide lipids, oxidize proteins, damage membranes, and diminish the nutritional value of the fruit [4].

Plants have evolved effective antioxidant defence mechanisms to reduce ROS accumulation and alleviate oxidative damage. The ascorbate-glutathione (AsA-GSH) cycle is one of the powerful antioxidant systems that is critical for maintaining ROS balance and membrane integrity. This cycle includes antioxidants like AsA and glutathione (GSH), along with key enzymes such as ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR). The AsA-GSH cycle, which is regulated by oxidative and reductive circumstances via changes in AsA/dehydroasocobate (DHA) and GSH/glutathione disulphide (GSSG) levels, is linked to plant senescence. The AsA-GSH cycle, regulated by the oxidative and reductive conditions through an alteration of AsA/dehydroasocobate (DHA) and GSH/glutathione disulfide (GSSG) levels, is related to senescence in plants. Previous research has demonstrated that reduced activity of the AsA-GSH cycle correlate with banana browning in banana [5], peace [6], longan [7], and pear [8]. Consequently, the redox balance must be maintained in order to impede or reduce the aging process of fruits.

Melatonin (N-acetyl-5-methoxytryptamine, MT) is regarded as an endogenous plant growth regulator, which is of great interest because it enhances stress resistance, increases crop yield, and decelerates fruit senescence. Recent studies have showed that MT activates the antioxidant system, enhances the activity of the AsA-GSH cycle, contributes to powerful free radical scavenging, protects cell membranes, and limits the softening and browning of fruit flesh in jujube [9], tomato [10], mango [11], and orange [12]. The studies indicate that MT treatment may effectively preserve postharvest fruit quality. The function of exogenous MT in the avocado ripening process remains mainly unexplored. This study aimed to assess the antioxidant content and enzymatic activity in the AsA-GSH pathway, as well as the alterations in avocado fruit color during ripening and storage.

2. Materials and Methods

2.1. Materials

Avocado sample 034 was harvested from a commercial orchard in Gao commune, Pleiku city, Gia Lai province (13^{o}53'50.9"N, 107^{o}56'51.1"E). Avocado 034 is a local avocado cultivar in Vietnam, grown mostly in the Central Highlands areas, with a high economic value due to its consistent yield and superior quality. This avocado variety has a high flesh ratio, delicious flavor, suitable for consumer taste [13]. The sample was chosen for its homogeneity in shape, size, and

weight, as well as its lack of damage and reaching harvest maturity. After harvesting, the avocado was packed in cartons and transported to the laboratory within 6 hours.

2.2. Sample treatment and storage

After harvest, avocados were rinsed with running water, air-dried at ambient temperature, and randomly divided into three groups, each consisting of 25 fruits, corresponding to three treatments. Two groups were immersed in MT solutions with concentrations of 0.1 mM and 0.5 mM for a duration of 2 hours. The avocados treated with MT were conducted under low light conditions to prevent MT degradation [14]. The remaining fruits were immersed in distilled water as a control. After treatment, the fruits were air-dried and stored at 22 ± 1 ^{o}C, with humidity of 75-80%. Avocado fruit samples were collected at random to assess antioxidant-related parameters in the AsA-GSH cycle, as well as to determine the color of the avocado peel and flesh from treatment (0 days) to the end of the storage procedure, when the avocado was fully ripe (firmness from 2.5 to 5 N). Flesh tissues were sampled from the middle part of a fruit (3 fruits of each replicate in the same formula) every 2 days during the storage period of 12 days. Each analysis was repeated three times.

2.3. Determination of antioxidant content and antioxidant enzyme activity in the AsA-GSH cycle

2.3.1. Determination of antioxidant content in the AsA-GSH cycle

The AsA content was determined by the AOAC method [15]. 5 g of sample was ground in 1% HCl, and the resulting liquid was transferred to a 25 ml volumetric flask, and 1% HCl was added to the mark of the flask. The flask was kept in the dark for 10 min, then the solution was filtered to obtain the clear liquid. 10 ml of the filtrate was placed in a 50 ml conical flask andtitrated with DPIP solution until a persistent pink color appears. AsA content was expressed as mg/100 g FW. Measurements were performed on three fruits for each replicate.

GSH content was determined based on the method of Brehe and Burch [16]. 2 g of avocado pulp was ground in 4 ml of 5% TCA with 5 mM EDTA, then centrifuged at 10,000 rpm for 10 minutes at 4°C to extract the supernatant. The reaction mixture included 1 ml of 0.2 M phosphate buffer (pH 7.7), 0.5 ml of 6.33 mM DTNB, and 1 ml of crude extract. The reaction was incubated at 30 °C for 10 minutes, and the absorbance was measured at 412 nm. The GSH content was calculated using the GSH standard curve, represented as µmol/g FW, and measured on 3 fruits for each replicate.

2.3.2. Determination of antioxidant enzyme activities related to the AsA-GSH cycle

Avocado pulp (0.25 g) was triturated with 1.5 ml of 50 mM K-phosphate buffer, pH 7.5 containing 1 mM AsA, 0.1 mM EDTA, and 2% PVP, then centrifuged at 12,000 rpm for 10 minutes at 4 °C. The resulting clear supernatant served as the enzyme source for the measurement of APX, DHAR, and GR enzyme activities.

APX enzyme activity was determined by the method of Nakano and Asada [17], with modifications. The reaction mixture comprised 2.4 ml of 50 mM K-phosphate buffer at pH 7.5, 0.2 ml of 2 mM H_2O_2, and 0.4 ml of enzyme extract. The APX activity was quantified by measuring the reduction in absorbance at 290 nm, expressed as U/min/g FW.

DHAR enzyme activity was measured using the Nakano and Asada method [17]. The reaction mixture (1 ml) consisted of 50 mM phosphate buffer, pH 7.0; 0.1 mM DHA; 2.5 mM GSH; 0.1 mM EDTA and 100 µl extract. Enzyme activity was determined by measuring the change in absorbance at 265 nm and expressed as U/min/g FW.

GR enzyme activity was determined according to Smith [18]. The reaction mixture contained 2.7 ml of 50 mM K-phosphate buffer, pH 7.5 containing 0.1 mM EDTA, 0.1 ml of 5 mM GSSG and 0.2 ml of extract; then 40 µl of 4 mM NADPH was added. The GR activity was determined based on the change in absorbance at 340 nm in 1 min, expressed as U/min/g FW.

2.3.3. Determine the color of the avocado skin and flesh

The color of the fruit skin and flesh surface was measured with a digital colorimeter (CR-400, Konica Minolta, Japan) using CIELAB coordinates. The results were expressed as L*, a*, and b* values, where L* represents the lightness or darkness of the color, a* represents the color range from green (-60) to red (+60), and b* represents the color range from yellow (-60) to blue (+60). Each measurement was taken at three sites along the equatorial region of each fruit, and the average value of the three spots was calculated [19].

2.3.4. Statistical analyses

All data were analyzed using SAS 8.0 statistical software and presented as mean ± standard deviation (SD). Experimental results were evaluated using ANOVA analysis and a 5% LSD test to determine statistical differences between treatments.

3. Results and Discussion

3.1. Effect of melatonin treatment on AsA and GSH contents in the AsA-GSH cycle

AsA and GSH are non-enzymatic antioxidants found in the AsA-GSH cycle. AsA contributes to the scavenging of superoxide and hydroxyl radicals, as well as the regeneration of tocopherol [20]. AsA is specifically derived from DHA through the action of GSH and serves as a substrate for APX to facilitate the decrease of H_2O_2 and the regeneration of DHA [21]. Meanwhile, GSH converts DHA to AsA, and then GSH is oxidized to GSSG by the enzyme DHAR.

The AsA concentration of MT-treated avocado samples gradually increased, peaking at 7.22 mg/100 g FW (0.1 mM treatment) and 7.55 mg/100 g FW (0.5 mM treatment) at 8 days after harvest, compared to the control of 7.47 mg/100 g FW after 6 days. After that time, the AsA content decreased gradually until the end of storage (Figure 1A). MT treatment inhibited the reduction in AsA content during the ripening and storage. The 0.5 mM MT-treated sample had higher AsA content than the 0.1 mM MT sample ($P < 0.05$).

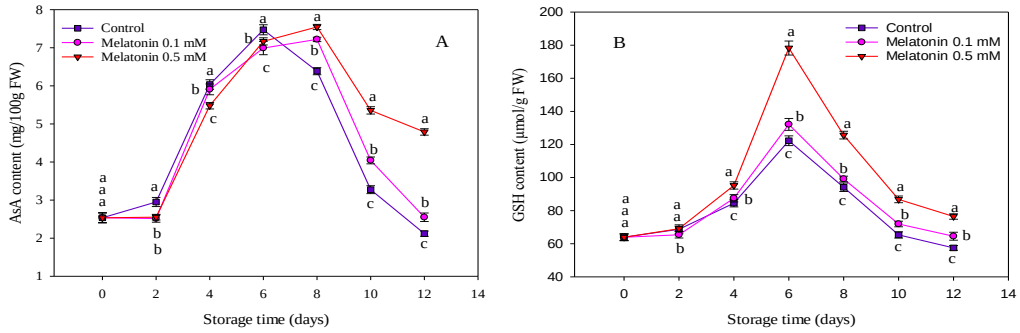

Figure 1. *Changes in AsA (A) and GSH (B) contents of avocado during storage at 22 ± 1 °C and 75–80% RH. Differences are indicated by different letters according to the LSD test (p < 0.05)*

The GSH content in both the control and MT treated avocado samples exhibited a consistent pattern, progressively increasing from day 0 to day 6 and increasing sharply at 6 days, reaching a maximum of 122.28 µmol/g FW (control), 132.16 µmol/g FW (0.1 mM treatment), and 178.3 µmol/g FW (0.5 mM treatment), then gradually decreasing until the end of the ripening process (Figure 1B). The MT treatment elevated GSH levels at all time points, with the 0.5 mM MT treated sample exhibiting a GSH content 1.46 times greater than the control at 6 days post-harvest ($P < 0.05$).

The results of the study on 'Newhall' oranges were identical. The fruit samples treated with 0.2 mM MT exhibited an average 17.45% higher AsA content and an average 37.52% higher GSH content than the control group stored at room temperature (8-12 ^oC) [12]. However, MT-treated strawberries had AsA content lower than the control by approximately 11.32% [22]. The

observed differences may result from the dependence on the harvest maturity of the fruit and MT treatment conditions such as concentration and time.

3.2. Effect of melatonin on APX, DHAR, and GR activities

APX aids plant cells in eliminating H_2O_2 in specific organelles where this molecule is formed. In the AsA-GSH cycle, APX uses AsA as a substrate to catalyze the reduction of H_2O_2 to H_2O while also generating DHA. Unlike APX, DHAR is an enzyme that regenerates AsA directly from DHA when it is oxidized in plant tissues. GR catalyzes the regeneration of GSH directly from GSSG [23], [24].

APX activity in the control and 0.1 mM MT treatment exhibited comparable fluctuations, rising during the initial stages of ripening, peaking on day 6, and thereafter declining, with no statistically significant differences seen at any time point throughout the study. In contrast, the 0.5 mM MT treated sample exhibited a higher APX activity than the control for the majority of the study periods, with a maximum of 5.45 U/min/g FW on day 8, which was 21.65% higher than the control (Figure 2A). During the early stages of ripening, DHAR activity also increased gradually, reaching a maximum on day 6, then decreased. MT treatment promoted this increase and inhibited the decline, which was 25.19% higher than the control at 6 days after harvest (Figure 2B).

The result is similar to a previous study on sweet cherries, where APX and DHAR activities increased significantly in the early stages of storage and peaked on days 14 and 21 after harvest, respectively, and then the activities gradually decreased until the end of storage. The MT treatment significantly reduced the decline in APX and DHAR activities, with an average decrease of 9.37% from days 14 to 56 and 35.46% from days 14 to 63 compared to the control [25]. Additionally, the coordinated activity of APX and DHAR in the AsA-GSH cycle, which is influenced by MT, contributes to the preservation of a high AsA/DHA (or AsA/MDHA) ratio. This eliminates H_2O_2 and toxic ROS, thereby extending the ripening time of peaches [21].

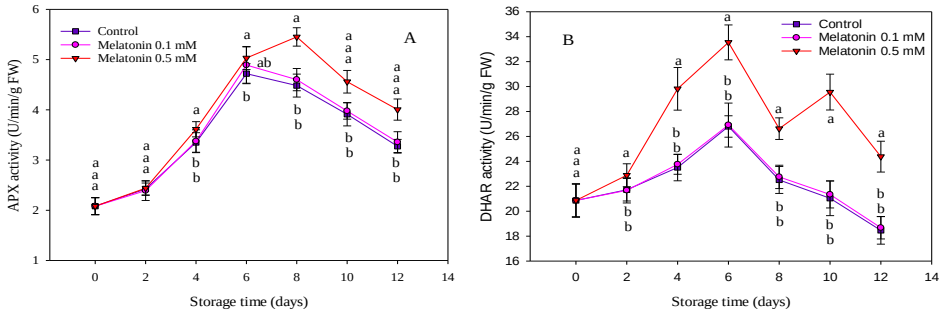

Figure 2. *Changes in the activities of APX (A) and DHAR (B) in avocado during storage at 22 ± 1 °C and 75–80% RH. Differences are indicated by different letters according to the LSD test (p < 0.05)*

GR enzyme activity declined progressively during storage, unlike APX and DHAR. In control and 0.1 mM MT-treated samples, activity declined by 37.21% and 33.52% at 6 days. However, 0.5 mM MT treatment significantly slowed this decline, with only a 21.4% reduction at 6 days (Figure 3). The decrease in GR activity of avocado during storage was consistent with a research on MT treatment on jujube fruit, which showed a 78% decrease after 5 days of storage [26]. The study on 'Newhall' oranges also indicated that MT treatment resulted in an average increase of 29.54% in GR activity compared to the control [12].

The AsA-GSH cycle is a vital ROS-scavenging pathway in plant cells, playing a key role in maintaining postharvest fruit quality by enhancing antioxidant defenses [12]. H_2O_2 is detoxified directly by CAT or indirectly via the AsA-GSH cycle enzymes, using AsA and GSH as electron donors. The levels of AsA and GSH are critical indicators for assessing the resistance of postharvest fruits to oxidative stress [27]. APX oxidizes AsA to MDHA or DHA while eliminating excess H_2O_2. GR regenerates GSH from GSSG, maintaining a high GSH:GSSG ratio

crucial for cellular redox balance and oxidative stress resistance. DHAR further supports redox homeostasis by reducing DHA back to AsA. Elevated levels of AsA, GSH, and related enzymes are essential for sustaining reducing potential and normal cellular functions [25].

In this investigation, exogenous MT treatment enhanced AsA and GSH levels while also increasing the enzyme activities of APX, DHAR, and GR, ensuring the proper functioning of the AsA-GSH cycle, preserving the balance of reducing agents, and contributing to the reduction of ROS content. The increased activity of the AsA-GSH cycle was accompanied by a considerable decrease in the formation of $O_2^{\cdot-}$, H_2O_2, and membrane lipid peroxidation in avocado during ripening, thereby slowing the aging process in the fruit [28].

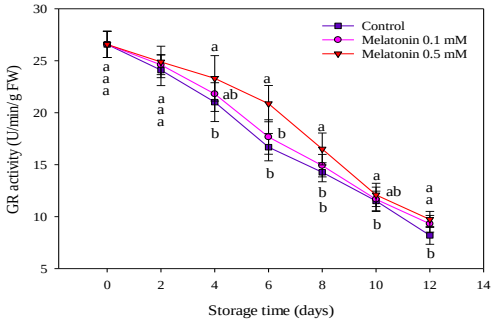

Figure 3. *Changes in the activity of GR in avocado fruit during storage at 22 ± 1 °C and 75–80% RH. Differences are indicated by different letters according to the LSD test (p < 0.05)*

3.3. Effects of MT on avocado peel and flesh color during ripening and storage

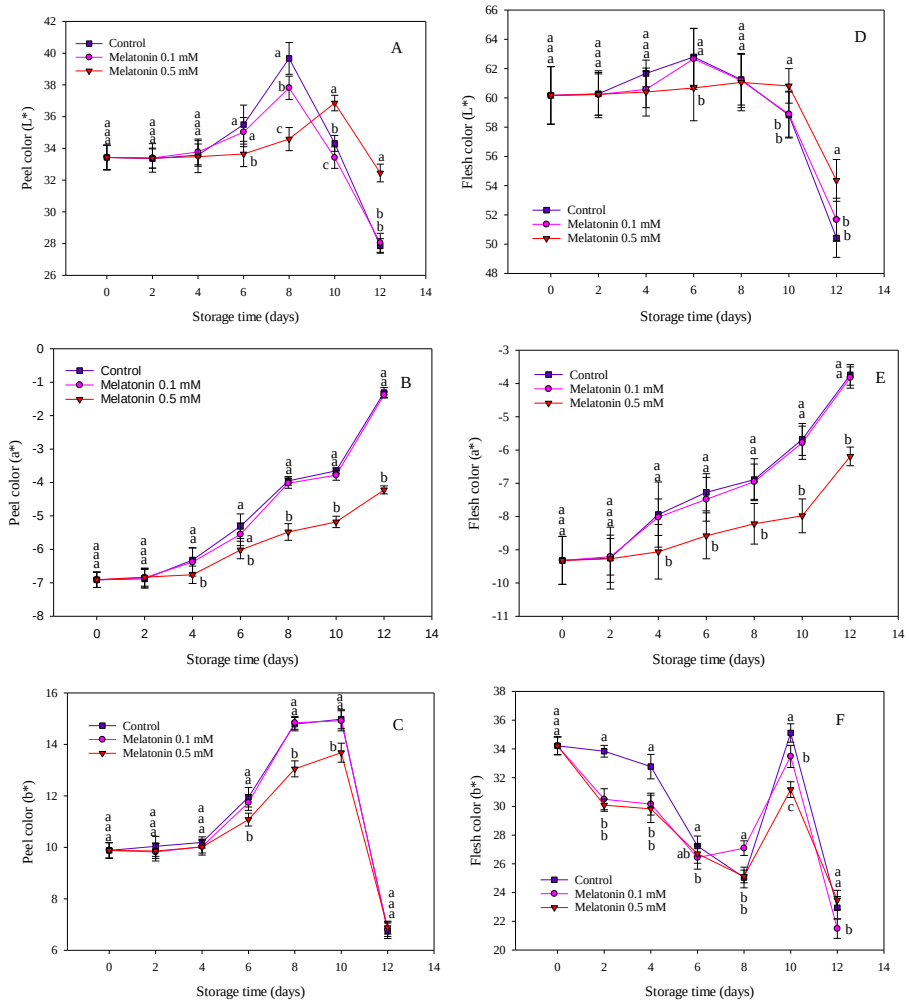

Figure 4. *Changes in peel (A, B, C) and flesh (D, E, F) color of avocado treated with MT during storage at 22 ± 1 °C and 75–80% RH. Differences are indicated by different letters according to the LSD test (p < 0.05)*

Color is a significant sensory element that affects consumer acceptance. During the ripening process, the color of avocados changes from light green to dark green and ultimately to dark yellow. The L* index indicates brightness, a* shows the transition from green to red, and b* represents the change from blue to yellow, all utilized for color assessment.

In the control fruit, the fruit peel brightness (L*) remained almost unchanged for the first four days, and then increased slightly and decreased sharply on the tenth day due to chlorophyll decomposition and dark pigment formation, while the a* value increased from -6.91 to -1.31 and the b* value increased from 9.88 to 14.98 before decreasing to 6.76 on the twelfth day, indicating that the fruit changed from green to dark yellow. For MT-treated fruit, the 0.5 mM treatment was more effective than the 0.1 mM treatment; L* remained high on the 12th day, while a* only reached -4.22, suggesting that the fruit skin remained green (Figure 4A, 4B, 4C).

The fruit flesh also changed similarly to the fruit peel. In the control and 0.1 mM treatments, L* decreased sharply, and dark spots appeared on the 12th day. Meanwhile, the fruit flesh treated with 0.5 mM MT retained better brightness (L* = 60.82 on day 10, compared to the control, which decreased sharply after day 6), and the a* and b* values decreased, demonstrating that MT slowed down the aging and ripening process of avocado fruit after harvest (Figure 4D, 4E, 4F).

Our findings are consistent with Liu et al. [22], showing that strawberry fruits treated with 10 µM MT had higher L* values than controls after 9 and 12 days at 4 °C. Similarly, Zhang et al. [29] reported that MT-treated litchi maintained more stable L* and a* values, indicating delayed senescence. Another study also found that MT slowed color change and chlorophyll loss in mango during storage [30]. In our study, MT enhanced antioxidant enzyme activity in the AsA-GSH cycle, helping preserve tissue integrity and reduce browning caused by ROS [4].

4. Conclusion

Treatment with MT at 0.5 mM for 2 hours in avocado 034 raised the levels of the antioxidants AsA and GSH, and boosted the activity of the enzymes APX, DHAR, and GR, which are part of the AsA-GSH cycle. This may contribute to the elimination of ROS and the reduction of oxidative membrane damage in avocado fruit. In addition, MT reduced the progression of avocado peel and flesh color during the ripening process at 22 ± 1 °C and 75-80% RH. The study contributes to the understanding of the role of exogenous MT in postharvest storage of avocado.

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