

Brief Report

Pre- and Postharvest Melatonin Treatment: A Comparative Study of Lemon Cultivars on Melatonin and Flavanone Content During Cold Storage

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Abstract

Adverse environmental and postharvest conditions challenge the functional quality of lemons, an economically vital citrus crop. Melatonin (MEL) has emerged as an effective regulator of plant stress responses and secondary metabolism. This study evaluated the effects of pre- and postharvest MEL treatments, combined with cold storage, on the fruit quality of two lemon cultivars ('Fino' and 'Verna'). The research focused specifically on endogenous MEL and flavanone dynamics. Three experimental conditions were assessed: (a) preharvest MEL application at 0.1 and 1 mM; (b) preharvest treatment followed by cold storage; and (c) combined pre- and postharvest MEL treatment followed by cold storage. Preharvest treatments increased endogenous MEL at harvest in a dose- and cultivar-dependent manner. Specifically, 1 mM being optimal for 'Fino', while 0.1 mM was more effective for 'Verna'. During cold storage, 'Fino' fruit, characterized by low basal endogenous MEL levels, showed a marked increase in MEL accumulation, suggesting the stimulation of biosynthesis. In contrast, 'Verna' fruit, which had initially high endogenous MEL content, exhibited a pronounced decline, indicating MEL consumption to counteract oxidative stress. Flavanone content increased dose-dependently after preharvest treatment and was preserved during storage in 'Fino' but declined in 'Verna'. These findings demonstrate that the fruit cultivar must be considered a critical factor in MEL-based strategies, as identical treatments may yield markedly different outcomes even within the same species.



Keywords: *Citrus limon* L.; flavanones; Fino; Verna; endogenous; melatonin

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1. Introduction

The lemon (*Citrus limon* L. Osbeck) belongs to the Rutaceae family, which comprises several economically important fruits within the *Citrus* genus [1]. Spain is currently the seventh-largest producer of lemons worldwide, with an annual production of 1,148,870 tonnes. National consumption of lemons is projected to rise by around 4.5% during the 2024–2025 season [2,3]. This growing demand highlights the importance of maintaining and enhancing lemon quality.

Lemon quality is defined by parameters such as juiciness, titratable acidity, freshness and bioactive compound content, particularly vitamin C and phenolic compounds. Flavanones are the predominant class of phenolic compounds in lemons and have been widely associated with health-promoting properties due to their antioxidant and anti-inflammatory activities. These compounds have been shown to protect against metabolic disorders, including cardiovascular diseases. For example, hesperidin, the most abundant citrus flavanone, has been reported to have antihypertensive effects [4,5]. Due to these functional attributes, lemons are widely used in the food, beverage, cosmetic and pharmaceutical industries, which further increases their commercial relevance.

However, increasing climatic variability and adverse environmental conditions have been linked to a decline in fruit quality. This is particularly evident regarding the accumulation of bioactive compounds. These challenges have prompted the development of innovative strategies to preserve or enhance the functional quality of lemons, extend their shelf life and improve their marketability. Among these approaches, pre- and postharvest elicitation techniques using compounds such as melatonin (MEL) have gained considerable attention. These treatments stimulate antioxidant defences and secondary metabolism, leading to increased phenolic accumulation [6,7].

In this context, MEL has emerged as a particularly promising elicitor. Beyond its well-known role in regulating circadian rhythms in animals, MEL exhibits multiple health-promoting properties, including immunomodulatory and anti-apoptotic effects. It also acts as a powerful antioxidant, capable of scavenging reactive oxygen species and inducing endogenous antioxidant enzymes [8]. In plants, MEL plays a key role in stress tolerance and metabolic regulation. Its application has been shown to enhance the activity of enzymes such as catalase and ascorbate peroxidase. This reduces oxidative damage in crops like sweet cherries, where MEL elicitation effectively lowered hydrogen peroxide accumulation [9].

More recently, the effectiveness of combined pre- and postharvest MEL treatments with cold storage has been evaluated in citrus fruits. Studies on 'Fino' lemons and blood oranges demonstrated improvements in functional quality through the increased accumulation of endogenous MEL and polyphenols, such as flavanones and anthocyanins. This is likely to be mediated by the activation of MEL-related biosynthetic genes [10,11]. These MEL-related genes have also been shown to be involved in flavonoid synthesis in other fruit species, such as limes (*Citrus aurantifolia* Swingle) and kiwifruit [12,13]. However, the magnitude and direction of these responses depend strongly on factors such as the dose of MEL and the citrus species or cultivar.

Based on these considerations, the present study aimed to evaluate, for the first time, the effects of pre- and postharvest MEL treatments, also combined with cold storage, on lemon fruit quality. It particularly focused on endogenous MEL and flavanone levels in two cultivars ('Fino' and 'Verna').

2. Materials and Methods

2.1. Plant Material and Experimental Design

The research was carried out in a commercial orchard in Orihuela, Alicante, Spain. The study used twenty-year-old lemon trees grafted onto *Citrus macrophylla* rootstock. Two cultivars, 'Fino' and 'Verna', were evaluated. For each cultivar and treatment, three field replicates were established, with each replicate consisting of three trees (nine trees per treatment). The experimental unit was defined as a sample of 30 fruits (lot), harvested from one field replicate (10 fruits per tree), to ensure biological representativeness.

Preharvest treatments of MEL were applied by foliar spraying of newly prepared aqueous solutions of MEL (Sigma-Aldrich, Madrid, Spain) at concentrations of 0.1 and

1 mM, supplemented with 1 mL L⁻¹ of the surfactant Tween 20. Each tree received 1.5 L of solution per application. Control trees were sprayed with distilled water containing 1 mL L⁻¹ Tween 20. The selected MEL concentrations were based on previous studies reporting optimal stimulation of secondary metabolism in citrus fruit within this range [10]. The application of each treatment was conducted monthly, starting after the natural de-greening stage and continuing until seven days prior to the harvest date [10]. Fruits were harvested at the yellow commercial ripening stage, selecting only those free of visible defects or physiological disorders. A total of 90 fruits were harvested for each treatment and cultivar (10 fruits per tree), separated from the stalk and a small number of leaves, and immediately transported to the laboratory.

To establish the experimental conditions, three homogeneous lots of 30 fruits ($n = 3$ biological replicates) were prepared from each field replicate. (a) Fruits analysed immediately at harvest (Pre-AH); (b) fruits subjected to cold storage at 1 °C and 85–90% relative humidity for 21 days (Pre + CS); and (c) fruits subjected to a postharvest MEL treatment consisting of immersion in a 1 mM MEL solution for 15 min, followed by air-drying at room temperature and subsequent cold storage under the same conditions (Pre + Post + CS). Dose of 1 mM for postharvest treatments was selected based on previous studies that demonstrated to be the most effective dose for improving the quality of lemons [6,7,10]. Control fruits for the postharvest treatment were immersed in distilled water. No visual symptoms of chilling injury, such as pitting or browning, were observed in any treatment during the 21 days of cold storage.

2.2. Juice Extraction and Sample Preparation

Samples were freshly squeezed from each lot of 30 lemons and subsequently stored in a freezer at −20 °C until further analysis, to preserve the chemical composition. This storage temperature was sufficient to maintain the stability of melatonin and flavanones for the duration of the analysis (less than 21 days), as previously reported for bioactive compounds and melatonin in aqueous solutions [14–16]. Each biological replicate ($n = 3$) consisted of the pooled juice from its respective lot.

2.3. Quantification of Melatonin and Flavanone Content

The MEL content of the juice was analysed using the protocol described by Agulló et al. [10]. Briefly, processing involved centrifugation at 10,500 rpm for 5 min (Sigma 1–13, B. Braun Biotech International, Osterode, Germany), filtration through a 0.22 µm polyvinylidene fluoride filter (Millex HV13, Millipore, Bedford, MA, USA) and storage at −20 °C prior to analysis. MEL determination and quantification were then performed using a UHPLC-QqQ-MS/MS (UPCL-1290 Series and a 6460 QqQ-MS/MS; Agilent Technologies, Waldbronn, Germany) with an Acquity BEH C18 column (2.1 mm × 150 mm, 1.7 µm; Waters, Milford, MA, USA). Chromatographic separation was achieved using a binary linear gradient of milli-Q water (mobile phase A) and methanol (mobile phase B), both containing 5% formic acid (v/v). The flow rate was 0.30 mL min⁻¹ with the elution programme (time in min; % B): (0.00; 40), (1.50; 40), (1.51; 90), (3.50; 90) and (3.51; 40). The injection volume was 10 µL, with a flow rate of 0.9 mL min⁻¹ during analysis. Nitrogen was used as the collision gas to facilitate compound fragmentation in the triple quadrupole mass spectrometer. The operating parameters of the instrument were configured as follows: drying gas flow: 8 L min⁻¹; sheath gas flow: 12 L min⁻¹; sheath gas temperature: 350 °C; nebuliser pressure: 30 psi; capillary voltage: 4000 V; and nozzle voltage: 1000 V. Results were expressed as µg L⁻¹ of fresh juice.

The flavanones were quantified using HPLC-DAD by applying a previously reported method [17]. Chromatographic analysis was performed using a Luna C18(2) column

(250 mm × 4.6 mm, 5 µm, 100 Å), coupled with a Security Guard Cartridge (4 mm × 3.0 mm). The Agilent Technologies 1220 Infinity HPLC system was equipped with an autosampler (G1313B) and a diode array detector (model 1260, Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of acidified water (95.0:5.0 *v/v*, Milli-Q water/formic acid; solvent A) and methanol (solvent B). The elution was conducted at a flow rate of 0.9 mL min^{−1} with an injection volume of 10 µL, following this linear gradient (time in min; %B): (0, 15%); (20, 30%); (30, 40%); (35, 60%); (40, 90%); (44, 90%); (45, 15%); and (50, 15%). Instrument control and data acquisition were performed using MassHunter software (version B 04.00). ChemStation for LC 3D Systems software Rev. B.01.03-SR2 (2 0 4) (Agilent Technologies Spain S.L., Madrid, Spain) was used for chromatographic processing. Finally, the flavanones were quantified as hesperidin equivalents at 280 nm. The results were expressed as mg 100 mL^{−1} of fresh juice (FW basis).

To ensure consistency in the analytical process, standard calibration curves for MEL and flavanones were analysed in duplicate at the beginning and end of the chromatographic sequence. Each biological sample was analysed once, since the stability of the system was confirmed by the consistency between the initial and final standard injections.

2.4. Statistical Analysis

Data are presented as the mean ± standard deviation (SD). The statistical unit was the lot (*n* = 3), which represented the biological replicates from the field design. To ensure maximum sensitivity and prevent the high basal metabolite levels of one cultivar from masking variations in the other, independent statistical models were applied for each factor level. For inter-cultivar comparisons, Student's *t*-test was employed to perform pairwise comparisons between 'Fino' and 'Verna' cultivars under same treatment and condition. For intra-cultivar comparisons, one-way analysis of variance (ANOVA) followed by Tukey's multiple range test was used to identify statistical differences between treatments and storage conditions within each cultivar separately. This dual approach ensures that the significance markings for treatment, storage, and cultivar are derived from independent statistical assessments. A significance level of *p* < 0.05 was applied. All statistical analyses were performed using SPSS 25.0 software.

3. Results

The endogenous MEL content of the 'Fino' and 'Verna' lemon cultivars has been evaluated across all treatments and conditions (Figure 1). For the 'Fino' cultivar, preharvest application of 1 mM MEL significantly increased endogenous MEL content four-fold ($1.02 \pm 0.06 \mu\text{g L}^{-1}$) compared to the control. The respective endogenous MEL content at harvest for the control and 0.1 mM treatments was $0.24 \pm 0.10 \mu\text{g L}^{-1}$ and $0.26 \pm 0.06 \mu\text{g L}^{-1}$. Furthermore, after cold storage, the endogenous MEL content of the control fruits ($5.93 \pm 0.40 \mu\text{g L}^{-1}$) and the fruits treated with 0.1 mM ($1.43 \pm 0.27 \mu\text{g L}^{-1}$) significantly increased. However, it was reduced in the fruits treated with 1 mM ($0.48 \pm 0.15 \mu\text{g L}^{-1}$). Finally, postharvest treatment followed by cold storage significantly increased endogenous MEL levels in all treatments, with control fruits showing the highest values ($6.99 \pm 0.35 \mu\text{g L}^{-1}$).

Conversely, the 'Verna' cultivar exhibited significantly higher endogenous MEL values than the 'Fino' cultivar at harvest: $25.42 \pm 3.76 \mu\text{g L}^{-1}$ for control fruits. Additionally, preharvest MEL treatments significantly increased its endogenous concentration. The 0.1 mM treatment nearly tripled this level to reach the highest concentration ($70.79 \pm 2.50 \mu\text{g L}^{-1}$), followed by the 1 mM treatment ($35.47 \pm 1.59 \mu\text{g L}^{-1}$). However, after cold storage, the endogenous MEL content decreased by more than 99% regardless of whether the fruit

were treated only at harvest (Pre + CS) or received a combination of pre- and postharvest treatments (Pre + Post + CS).

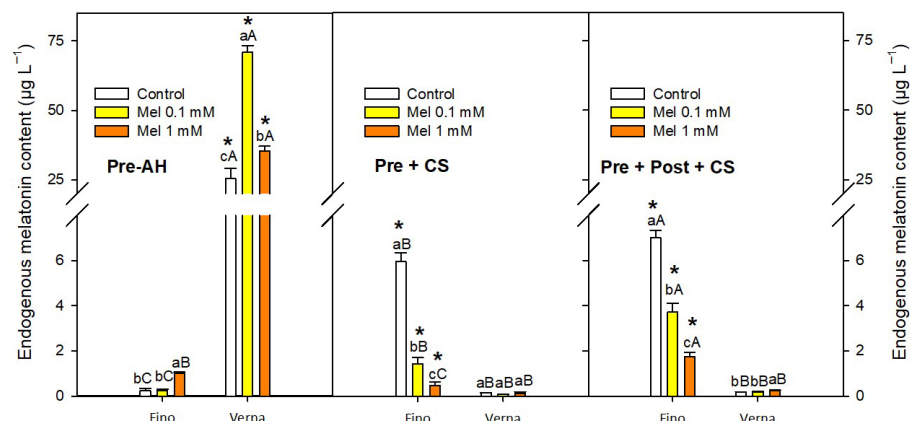


Figure 1. Endogenous melatonin content in control and treated lemons with preharvest melatonin (MEL) treatments at different doses (0.1 and 1 mM) at harvest (Pre-AH), after cold storage (Pre + CS), and after a postharvest application of 1 mM MEL combined with cold storage (Pre + Post + CS) of ‘Fino’ and ‘Verna’ cultivars. Statistical significance ($p < 0.05$) is indicated by three independent marking systems: (i) Asterisks (*) represent pairwise comparisons between ‘Fino’ and ‘Verna’ cultivars for each specific treatment and condition using Student’s t-test; (ii) Lowercase letters denote differences between treatments within the same cultivar and storage stage; and (iii) Uppercase letters indicate differences between experimental stages for a given treatment and cultivar. Intra-cultivar differences (letters) were determined by separate one-way ANOVA followed by Tukey’s multiple range test for each cultivar.

Regarding the flavanone content of lemon juice (Figure 2), its levels were significantly higher for the ‘Fino’ cultivar compared to the ‘Verna’ cultivar. Additionally, pre-harvest treatments with 1 mM of MEL significantly increased flavanone content in a dose-dependent manner in both cultivars. The highest flavanone content was observed at harvest (Pre-AH) for 1 mM MEL treatment.

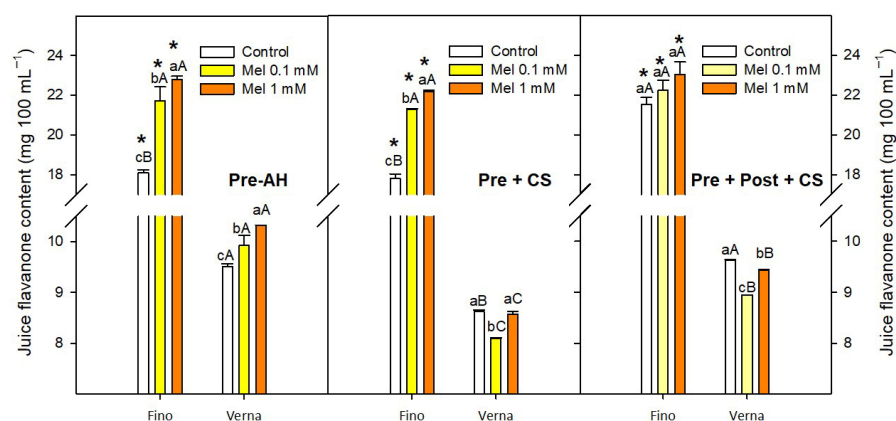


Figure 2. Juice flavanone content (mg 100 mL⁻¹) in control and treated lemons with preharvest melatonin (MEL) treatments at different doses (0.1 and 1 mM) at harvest (Pre-AH), after cold storage (Pre + CS), and after a postharvest application of 1 mM MEL combined with cold storage (Pre + Post + CS) of ‘Fino’ and ‘Verna’ cultivars. Statistical significance ($p < 0.05$) is indicated by three independent marking systems: (i) Asterisks (*) represent pairwise comparisons between ‘Fino’ and ‘Verna’ cultivars for each specific treatment and condition using Student’s t-test; (ii) Lowercase letters denote differences between treatments within the same cultivar and storage stage; and (iii) Uppercase letters indicate differences between experimental stages for a given treatment and cultivar. Intra-cultivar differences (letters) were determined by separate one-way ANOVA followed by Tukey’s multiple range test for each cultivar.

After cold storage (Pre + CS), the flavanone content of the 'Fino' cultivar remained stable, whereas it decreased significantly in the 'Verna' cultivar, regardless of the treatment. Ultimately, the highest flavanone values were observed in the 'Fino' cultivar after postharvest treatment followed by cold storage (Pre + Post + CS), showing a significant increase compared to harvest values. Although no significant differences were observed between treatments, the MEL 1 mM treatment resulted in the highest flavanone levels. In contrast, postharvest treatment only maintained the flavanone content of control fruits in the 'Verna' cultivar. Treated fruits showed a significant decrease similar to that observed after cold storage.

4. Discussion

Melatonin is a naturally occurring molecule in plants that plays a key role in regulating physiological processes. When applied externally, it enhances fruit quality and improves stress tolerance in various citrus fruits, such as lemons and blood oranges [6,11]. While the general role of MEL as a postharvest regulator is documented, our results reveal that this response is far from universal and is governed by complex, cultivar-specific metabolic strategies. This study shows that the response to MEL treatments varies greatly depending on the lemon cultivar, as demonstrated by the contrasting behaviour of the 'Fino' and 'Verna' cultivars.

The application of exogenous MEL increased endogenous MEL levels in both cultivars, likely through absorption and stimulation of MEL biosynthesis pathways. Indeed, MEL has been reported to upregulate genes involved in its own metabolic pathway. These genes include tryptophan decarboxylase, serotonin *N*-acetyltransferase and *N*-acetylserotonin methyltransferase [10,18]. Notably, we identified a dose–response inversion between cultivars. The effective concentration required to maximize endogenous MEL accumulation was cultivar-dependent. Specifically, 1 mM was optimal for 'Fino', while a lower dose of 0.1 mM was better for 'Verna'. This suggests that a high-dose approach may be counterproductive depending on the initial metabolic state of the cultivar.

A key finding of this study is that the initial endogenous MEL content is important in determining the response of fruit to cold storage. We observed an opposite endogenous dynamic between the two varieties. The 'Fino' cultivar, which had low levels of MEL at harvest, showed a pronounced increase in MEL during cold storage, particularly in the control and low-dose treatments. This suggests that cold storage might stimulate MEL biosynthesis as a defensive mechanism when levels are initially low. Although no visual chilling injury was observed, cold storage could induce MEL biosynthesis due to its antioxidant activity and pivotal role in abiotic stress conditions [10,11,19]. Cold stress may enhance the expression of genes involved in MEL biosynthesis and the production of antioxidant enzymes, such as catalase and ascorbate peroxidase, in a similar manner to the response to other abiotic stressors [20,21]. Similarly, MEL levels increased after postharvest treatment followed by cold storage. This increase was related to both external MEL absorption and stimulation of the plant's antioxidant defence system for the 'Fino' cultivar, involving endogenous MEL synthesis [18,22].

By contrast, the 'Verna' cultivar exhibited high levels of endogenous MEL at harvest, which decreased markedly after cold storage across all treatments. This "Consumption vs. Synthesis" dichotomy represents a novel insight. When basal MEL levels are naturally high, as in 'Verna', the fruit appears to prioritize the immediate mobilization and consumption of these reserves to neutralize cold-induced reactive oxygen species, rather than activating new synthesis. Similar behaviour has been reported in other fruit species, such as sweet cherries, where MEL depletion occurs under prolonged cold stress due to its scavenging activity against reactive oxygen species [19]. As previously mentioned, MEL could mitigate

the oxidative stress caused by cold storage by acting against free radicals and reducing oxidative stress [8,21,23]. This oxidative stress results in a greater reduction in endogenous MEL content in the 'Verna' cultivar when stored under stressful conditions [8,23,24]. Furthermore, postharvest MEL application did not restore endogenous levels in 'Verna', likely because MEL continued to be rapidly consumed under stressful conditions [10,11]. These findings suggest that MEL homeostasis during storage is driven by a dynamic balance between biosynthesis and consumption, which is strongly influenced by initial endogenous concentrations and cultivar-specific metabolic responses.

Conversely, citrus fruits are characterised by their high polyphenolic content, particularly flavanones. These compounds have significant pharmaceutical, biological, and biomedical applications [5]. Given the potential role of flavanones and polyphenolic compounds in general in combatting certain diseases, analysing their content is a fundamental quality parameter [25]. The present results confirm that MEL acts as an effective modulator of phenolic metabolism. It significantly enhanced flavanone accumulation in a dose-dependent manner following preharvest application. This effect could be attributed to the enhanced expression of enzymes involved in flavonoid synthesis, such as phenylalanine ammonia-lyase, cinnamate-4-hydroxylase and chalcone synthase [12,13]. In both cultivars, 1 mM MEL induced the highest flavanone levels at harvest, consistent with previous findings in lemons [10].

Regarding cold storage, distinct patterns on flavanone content between cultivars emerged. The 'Fino' cultivar showed no significant changes in flavanone content. It could be related to the low activity of phenolic degradative enzymes such as peroxidase and polyphenol oxidase at low temperatures [26]. In 'Fino', the biosynthesis of MEL coincided with the stability of its phenolic profile. Conversely, the 'Verna' cultivar exhibited reduced flavanone content following storage across all treatments. The simultaneous reduction in both endogenous MEL and flavanones in 'Verna' suggests a higher susceptibility to chilling-induced oxidative depletion in this genotype. Prolonged exposure to MEL, particularly when pre- and postharvest applications are combined, could activate phenolic biosynthetic pathways by upregulating key enzymes such as 4-coumarate:CoA ligase and cinnamate-4-hydroxylase [22]. In this context, the flavanone content of the 'Fino' cultivar increased further following postharvest treatment combined with cold storage, demonstrating an additive effect. In contrast, a stabilising effect was observed in the 'Verna' cultivar, consistent with previous reports on the response of citrus fruits to MEL under cold stress [10,11].

Nevertheless, it should be noted that the present study focused primarily on the metabolic shift in melatonin and flavanones. While no visual symptoms of chilling injury were detected, the lack of traditional physiological markers, such as weight loss, firmness, or enzymatic antioxidant activities, represents a limitation. This should be addressed in future research to provide a more holistic understanding of melatonin-mediated stress tolerance.

Although numerous studies propose the application of MEL as an effective strategy to improve the functional quality of fruit, the present results demonstrate that this approach is not entirely accurate. It depends on the fruit cultivar, treatment dose and storage conditions. Substantial varietal differences in MEL metabolism and response to exogenous application were observed between lemon cultivars depending on their initial endogenous MEL content. This determines whether MEL biosynthesis is stimulated or, conversely, whether endogenous reserves are consumed to mitigate oxidative stress during storage.

5. Conclusions

The two lemon cultivars studied, 'Verna' and 'Fino', exhibited an increase in endogenous MEL levels following MEL treatments. In this study, pre- and postharvest MEL

treatments, applied individually or in combination and followed by cold storage, induced contrasting physiological responses in ‘Fino’ and ‘Verna’ lemons. This highlights the cultivar-specific nature of MEL functionality. Therefore, the fruit cultivar must be considered a critical factor when designing MEL-based pre- and postharvest strategies, since the same treatment can lead to markedly different outcomes, even within the same fruit species. Future research should expand these studies to additional cultivars and fruit species to elucidate the diverse MEL-dependent mechanisms involved. It will enable the development of precise, cultivar-specific postharvest management strategies.

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