

INFLUENCE OF A PREHARVEST MELATONIN APPLICATION ON POSTHARVEST CHILLING INJURY IN BASIL (*OCIMUM BASILICUM* L.)

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ABSTRACT

Postharvest chilling injury is a physiological disorder detrimental to produce quality and shelf-life. Basil (*Ocimum basilicum* L.) is susceptible to postharvest chilling injury at temperatures below 12°C, manifesting as leaf lesions and discoloration. Melatonin, a growth regulator, has reduced postharvest chilling injury severity in produce, but its effect on basil is unknown. Here, we evaluated the impact of an exogenous preharvest melatonin application at 400 µM on basil leaves stored at 3.5°C for 12 days. Visual parameters, including objective color descriptors, chilling injury index, and damaged surface area, as well as biochemical (total soluble solids, malondialdehyde, and total polyphenol content) and physiological (electrolyte leakage and fresh weight loss) markers for cold stress, were assessed. Melatonin-treated leaves showed reduced symptoms (15–22%) relative to the untreated control after cold storage. The biochemical and physiological parameters displayed subtle changes between treatments after storage. However, melatonin induced alterations before storage (70–90%), suggesting it acted as a stressor.

Key words: melatonin, chilling injury, postharvest quality

INTRODUCTION

Postharvest chilling injury (PCI) is a physiological disorder with profound consequences on the physiology and quality of susceptible produce (Albornoz et al. 2022). PCI initiates at low temperatures, which, depending on the species, developmental stage, and genotype, can fluctuate between 0 and 15°C (Raison & Lyons 1986). Early alterations associated with PCI include modifications in membrane fluidity, overproduction and accumulation of reactive oxygen species (ROS), increased rates of respiration and ethylene production, and activation of a signal transduction cascade that causes massive changes at the cellular and tissue levels (Aghdam et al. 2015). These events translate into detectable symptoms that accelerate deterioration and spoilage, ultimately contributing to postharvest loss and waste (Albornoz et al. 2022).

Basil (*Ocimum basilicum* L.) is a culinary herb typically used for its aromatic leaves, both in fresh and processed format. Basil is a crop of tropical origin, and it is susceptible to PCI when stored at

temperatures below 12°C. Symptoms manifest as leaf and stem discoloration, development of dark lesions, and loss of glossiness and aroma (Cantwell & Reid 2002).

Methods for reducing PCI incidence tested at a laboratory scale in basil include storage in controlled atmospheres (Amodio et al. 2004), 1-MCP treatments (Berry et al. 2010) and preharvest applications of abscisic acid (Satpute et al. 2019), among others.

Melatonin is a signaling molecule widely distributed in plant and animal kingdoms. It is relevant in plant physiology and in the response to biotic and abiotic stress (Aghdam et al. 2022). For instance, exogenous melatonin has alleviated PCI symptoms in economically relevant species like banana (Wang et al. 2021), cucumber (Liu et al. 2022), and tomato (Aghdam et al. 2022). However, in basil, the impact of external melatonin applications on PCI development remains unknown.

This study aimed to assess the effect of preharvest melatonin application on the development of PCI symptoms in basil leaves during cold storage using quality and physiological markers.

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MATERIALS AND METHODS

Plant material and growth conditions

Basil (*Ocimum basilicum* L. 'Genovese') seeds (Hortus Sementi, Italy) were germinated in Petri dishes in dark conditions until cotyledons were expanded (~ 7 days) and then transferred to seedling trays. When the first set of true leaves developed, the plants were transplanted into 11 × 11 × 15.5 cm (L × W × H) polyethylene pots containing commercial substrate (potting mix, Anasac, Chile) and grown in an indoor cultivation system, which included light-emitting diodes (LEDs) (ECO LED-1000, ProGarden, China) with a photosynthetic photon flux density (PPFD) of 150–170 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and a light spectrum composed of 68% 660 nm, 12% 460 nm, 14% white light, 4% 730 nm, and 2% 410 nm in 18 h photoperiod. The air temperature was maintained between 18–24°C, and the plants were managed under standard practices.

Melatonin preparation and applications

Melatonin (Sigma-Aldrich, Missouri, USA) was dissolved in 10 mL of food-grade absolute ethanol to produce a 1 mM stock solution and then diluted in water to generate a 400 μM working solution. This concentration was chosen based on previous studies in leafy vegetables, such as cabbage leaves and sprouts, which used 1 to 500 μM of melatonin (Aghdam et al. 2022).

Two hundred four-week-old plants with 4–6 nodes and a height of 22 cm were divided into two groups. The first group was sprayed with 400 μM melatonin (MT400), and the second group with water (control, MT0) containing the same volume of ethanol used to prepare the melatonin solution. Foliar applications were carried out until runoff. Plants were immediately placed into a dark chamber and kept there for 24 hours. The experiment was conducted once in November–December 2022.

Harvest and experimental setup

Leaves were collected with sharp scissors from each group of plants (MT0 and MT400), avoiding the two upper and lower leaves, leaving a 1 cm petiole attached, and combined. Twenty leaves were placed in a 16 × 9 × 6.5 cm (L × W × H) polyethylene clamshell with nine 1-mm perforations on the top to prevent the formation of a modified atmosphere. The bottom of the clamshell was covered with a wet paper towel wrapped in a plastic bag to maintain high relative humidity and prevent

excessive water loss (Larsen et al. 2022a). Samples were stored at $3.5 \pm 1^\circ\text{C}$ for 12 days. Physiological and qualitative parameters were measured on day zero (D0) and after 12 days of cold storage (D12). Six replicates were used for each treatment and storage time, each consisting of 20 leaves packed in one clamshell.

Chilling injury index (CII)

Each leaf in the clamshell box was evaluated after 12 days of cold storage for PCI symptoms, including leaf lesions, discoloration, browning, or necrosis. Symptoms were assessed with a 0 to 5 hedonic scale, where 0 – no damage; 1 – few isolated and small lesions (<2 cm); 2 – numerous small lesions; 3 – larger lesions (>2 cm) that are isolated and cover <50% of the surface; 4 – lesions covering 50–75% of the surface, and 5 – > 75% of the surface damaged by severe lesions (Satpute et al. 2019).

Leaf area affected by PCI

The percentage of leaf surface affected by PCI was quantified by the Compu Eye, Leaf and Symptom Area software after 12 days of cold storage. Leaves in a clamshell were organized into groups of 10, and a picture was taken. Pictures were processed with a detection unit of 0.4 mm^2 (range 0.1–1 mm^2) and a sensitivity of 5 (range 0–30). The damaged surface area (SA) was expressed in percent. A value of 100% indicates a leaf is wholly affected, while 0% indicates the absence of damage (Bakr 2005).

Objective color

A portable colorimeter model CS-10 (CHN Spec, China) was used to assess surface color through a D65/10° illuminant/observer system and the $L^* a^* b^*$ scale. Three readings per leaf were taken, avoiding the midrib, and averaged. The Hue angle was calculated as $\tan^{-1}(b^*/a^*)$, and Chroma as $(a^2 + b^2)^{1/2}$.

Fresh weight loss

The weight of basil leaves was measured at 0 and 12 days of storage, and weight loss was expressed as a percentage.

Total soluble solids

Leaf total soluble solids (TSS) was quantified by a portable digital refractometer HI 96801 (Hanna instruments, Woonsocket, RI, USA) at room temperature. The leaves (2.5 g) were ground with a mortar and pestle, placed in a piece of cheesecloth, squeezed, and a drop of juice was added to the prism of the refractometer. Results were expressed in °Brix.

Total polyphenol content

The determination of total polyphenol (TP) content was based on the Folin–Ciocalteu method (Singleton & Rossi 1965). Phenolic compound extraction was carried out by homogenizing 1.5 g frozen leaves in 15 mL of methanol/water/formic acid (25 : 24 : 1) solution, followed by sonication (Branson 5800, Emerson Electric, Missouri, USA) for 1 h and storage at 4°C for 24 h. The next day, samples were sonicated for 1 h and centrifuged (L535-R, Cence, Hunan, China) at 3,500 rpm for 5 min. Twenty-five microliter of supernatant was aliquoted into a microplate and mixed with 200 µL of distilled water and 25 µL of Folin–Ciocalteu reagent (0.5 N). The microplate was placed into a hybrid multimode microplate reader (Synergy H1, BioTek, Winooski, VT, USA), shaken for 30 s, and incubated in the dark for 5 min at 25°C. Twenty-five microliter of 10% (w/v) sodium carbonate were added, followed by shaking for 30 s and dark incubation at 25°C for 1 h. A calibration curve between 0.05 and 1.0 g·L⁻¹ of gallic acid was prepared as a standard. Sample absorbance was recorded at 765 nm. A reagent blank was prepared by adding water instead of the sample. Three technical replicates were used. Polyphenol content was expressed in mg equivalent of gallic acid per 100 g of fresh weight (FW).

Malondialdehyde content

One gram of frozen leaves was homogenized in 15 mL of 0.1% (w/v) trichloroacetic acid (TCA), followed by centrifugation (L535-R, Cence, Hunan, China) at 4,000 rpm for 20 min at 15°C. The supernatant was aliquoted into 2-mL tubes and centrifuged (TG1650-WS, Bioridge, Shanghai, China) at 11,000 rpm for 10 min. Five hundred microliter of supernatant was added to 500 µL of a mixture of 20% (w/v) TCA and 0.5% (w/v) thiobarbituric acid (TBA), placed in a water bath at 92°C for 20 min and incubated on ice for 1 h. Samples were centrifuged at 11,000 rpm for 10 min, and a 200 µL aliquot was placed into a microplate. Absorbance was recorded at 532, 600, and 400 nm with a hybrid multimode microplate reader (Synergy H1, BioTek, Winooski, VT, USA). Two technical replicates were used. The malondialdehyde (MDA) content was expressed as nmol per mg FW (Heath & Packer 1968; Hodges et al. 1999).

Electrolyte leakage

One 8-mm leaf disk was obtained from each leaf using a cork borer and avoiding the midrib. Six disks were immersed into a 50-mL tube containing 25 mL of distilled water and kept at 3.5 ± 1°C (cold-storage temperature) for 30 min. The initial conductivity (ECI) was measured with a benchtop electric conductivity meter (COND/TDS Meter 3700, Goldpoint, Shanghai, China). Samples were stored at -20°C for 72 h, thawed at room temperature, and lightly shaken. The total conductivity (ECT) was recorded (Cozzolino et al. 2016). A total of six leaves per clamshell were used. Electrolyte leakage was calculated as follows:

$$\text{Electrolyte leakage (\%)} = \left(\frac{\text{ECI}}{\text{ECT}} \right) \times 100.$$

Statistical analysis

The experiment had a completely randomized design. Statistical analyzes were performed in SAS OnDemand for Academics, Microsoft Excel, and MetaboAnalyst 5.0. An unpaired *t*-test was used to detect differences between treatments. The Kruskal–Wallis test was used to identify significant differences between treatments for weight loss, CII, and electrolyte leakage. Data plotted at https://jeehyoung-kim-19.shinyapps.io/Split_Violin_plot/

RESULTS AND DISCUSSION

The effect of exogenous melatonin applications on PCI symptoms has been studied in numerous horticultural products (Cao et al. 2018; Janatizadeh et al. 2019; Wang et al. 2021; Liu et al. 2022), but to date, there have been no reports on basil. The response of melatonin-treated at 400 µM (MT400) and untreated (control, MT0) basil leaves that were stored at PCI-inducing conditions, i.e., 3.5°C for up to 12 days, was assessed.

Exogenous melatonin influence on the development of PCI symptoms

The external appearance of basil leaves based on objective color descriptors and the development of PCI symptoms was monitored after cold storage and expressed as CII and SA percentages (Fig. 1).

Treatment with melatonin reduced PCI severity in basil leaves. Control leaves treated with water developed more obvious symptoms, including browning, discoloration, and necrotic lesions (Fig. 1A–B). CII, which rates PCI based on a subjective scale, was 21.8% higher in untreated than in melatonin-treated

samples (Fig. 1C). SA, which assesses PCI severity digitally, was 15.1% greater in the control group without melatonin (Fig. 1D).

These results are consistent with the delayed peel browning achieved by 200 μM melatonin application in banana stored at 7°C for 9 days (Wang et al. 2021) and the lower development of surface lesions and depressions in cucumber treated with 100 μM melatonin and exposed at 2°C for up to 12 days (Liu et al. 2022).

Regarding objective color, MT400 responded similarly to CII and SA and resulted in leaves with

higher lightness (L) values after cold storage, indicative of less blackening. Treated leaves also maintained a greener color than the control by exhibiting more negative greenness (a^*) levels. The strength of color given by the chroma value was higher in treated leaves as well (Fig. 1E). Likewise, lemon basil (*Ocimum × citriodorum*) and holy basil (*Ocimum sanctum*) leaves sprayed preharvest with 5mM salicylic acid, a growth regulator involved in biotic and abiotic stress tolerance, and stored at 7°C for 4 or 8 days displayed more greenness ($-a^*$) and higher chroma levels than the untreated control (Supapvanich et al. 2015; Suamuang et al. 2016).

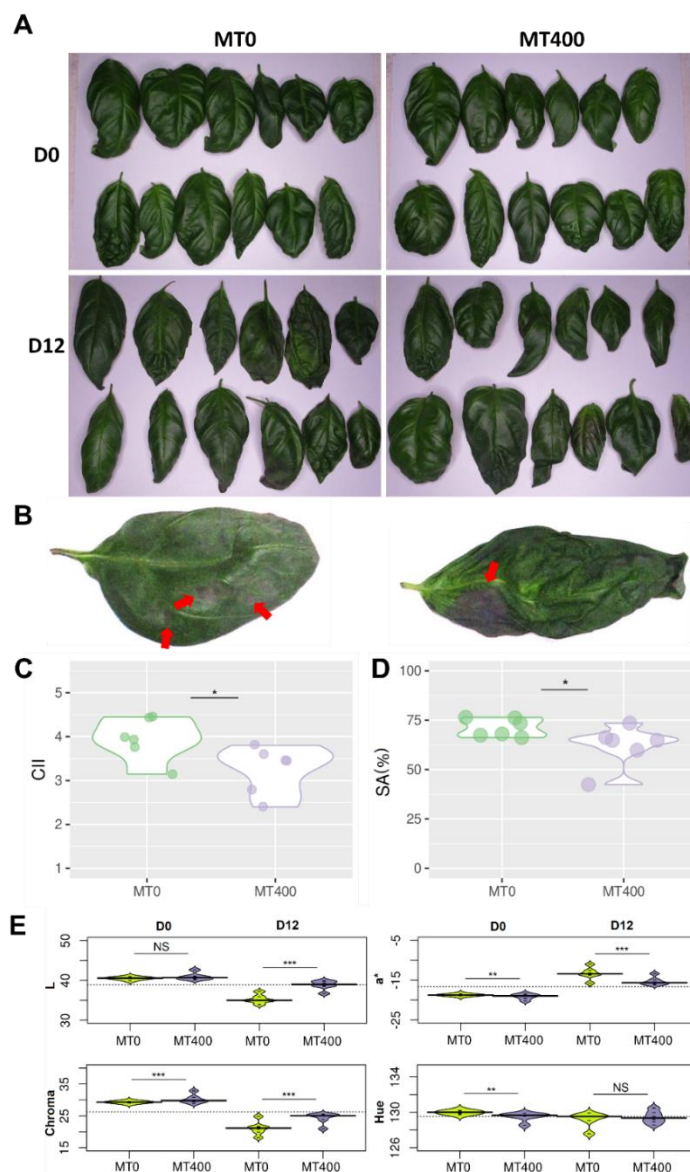


Figure 1. External changes of control (MT0) and melatonin-treated (MT400) basil leaves during cold storage, visual progress of PCI symptoms (A), close view of leaf PCI (B), symptoms shown with red arrows, discoloration and darkening (left), darkening and water loss (right), CII (scale 1–5) (C), symptom area (%) (D), violin/jitter plots of data distribution, *($p < 0.05$) by Kruskal–Wallis test, objective color descriptors (E), violin plots of data distribution, **($p < 0.01$), ***($p < 0.001$) by unpaired t -test; NS – nonsignificant

In our experiment, most color parameters of MT400 samples before cold exposure were significantly different from the control (MT0). This suggests that the preharvest melatonin application carried out 24 hours before cold storage induced physiological changes in the leaves. In the field of abiotic stress, melatonin has a wide array of functions that include acting as an antioxidant by neutralizing ROS, modifying gene expression, activating signaling networks, and modulating, among others, primary and secondary metabolic pathways (Shi et al. 2016), which in this case could be partly responsible for developing a protective response to postharvest cold stress.

Melatonin-induced physiological and compositional alterations associated with stress responses

Physiological parameters, including the percentage of FW loss, TSS ($^{\circ}$ Brix), electrolyte leakage rate, MDA, and TP content, were monitored before and after cold storage.

Loss of FW after 12 days at 3.5 $^{\circ}$ C did not fluctuate significantly between treatments, with the MT400 and MT0 samples reporting values of 13.7% \pm 1.7 and 11.2% \pm 1.4, respectively (mean \pm SE).

Electrolyte leakage, a marker of physiological status in response to stress (Satpute et al. 2019), increased 107–108% after cold storage in both treatments ($p < 0.01$), aligned to the development of PCI symptoms and consistent with previous studies (Supapvanich et al. 2015; Suamuang et al. 2016). Under the current experimental conditions, there were no changes that could be directly attributed to melatonin; however, longer storage could reveal differences between treatments (Table 1).

TSS content can be correlated with the abundance of sugars in basil leaves (Larsen et al. 2022b). TSS did not change between treatments before and after cold storage, but it increased ($p < 0.01$) in MT400 samples after 12 days at 3.5 $^{\circ}$ C from 3.98 to 4.87 $^{\circ}$ Brix compared to MT0 leaves. This was not observed in MT0 leaves (Table 2). Melatonin could elicit the accumulation of osmoprotectants, like sucrose, glucose, and fructose, due to starch breakdown associated with postharvest cold stress (Larsen et al. 2022b).

As for MDA (Fig 2A), an indicator of lipid peroxidation, there was no difference between MT0 and MT400 at D12. Similarly, the TP content remained unchanged at D12 in the control and melatonin applications (Fig. 2B). TP content indirectly informs on the antioxidant activity since oxidative stress in postharvest induces the synthesis of antioxidants such as phenolic compounds (Pedreschi & Lurie 2015). At D0, MT400 revealed higher MDA (70%) and polyphenol (90%) levels than MT0 but decreased after 12 days by 48 and 56% in MDA and polyphenol content, respectively. This finding suggests that melatonin induced the synthesis of compounds associated with oxidative damage before exposure to cold stress, returning to a basal state after storage. Likewise, lemon basil treated with salicylic acid preharvest reported increased TP levels 24 h after treatment but before cold storage compared to the untreated control (Supapvanich et al. 2015). These findings also align with reports of basil under tissue culture conditions, where melatonin has been proposed as a stressor that elicits the synthesis of bioactive compounds by activating signal transduction cascades (Duran et al. 2019; Nazir et al. 2020).

Divergence of melatonin-induced responses before and after cold storage

To identify patterns in response to the application of preharvest melatonin before cold storage, a partial least-squares discriminant analysis (PLS-DA) was conducted, integrating the visual, physiological, and compositional parameters (Fig. 3).

The first and second principal components could explain 78.6 and 17.1% of the data, respectively. Remarkably, the MT400 data separated from MT0 in opposite quadrants at D0 concerning both treatments after 12 days of cold storage. At D12, the melatonin and control treatments overlapped.

This finding highlights the potential role of melatonin as a stressor under nonstressful temperature conditions at D0. Although biochemical changes induced by melatonin after storage were minor, they could have elicited rearrangements that reduced the severity of PCI symptoms qualitatively and quantitatively.

Table 1. Electrolyte leakage (%) of control (MT0) and melatonin-treated (MT400) basil leaves during cold storage

	D0	D12
MT0	16.2 ± 2.5Ab	33.7 ± 6.7Aa
MT400	16.1 ± 0.8Ab	33.4 ± 9.7Aa

Values are the mean ± standard error (SE) of 6 clamshells and were calculated after 12 days of storage at 3.5 ± 1°C; different capital letters indicate significant difference ($p < 0.05$) between treatments, and different lower-case letters indicate significant difference between days of storage by Tukey's test

Table 2. Total soluble solids (°Brix) of control (MT0) and melatonin-treated (MT400) basil leaves during cold storage

	D0	D12
MT0	4.08 ± 0.17Aa	4.42 ± 0.20Aa
MT400	3.98 ± 0.07Ab	4.87 ± 0.21Aa

Note: see Table 1

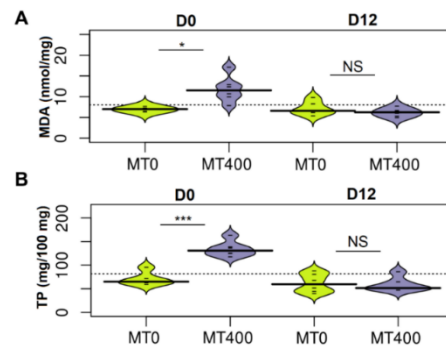


Figure 2. Compositional changes of control (MT0) and melatonin-treated (MT400) basil leaves during cold storage, violin plots of data distribution, leaves were stored for 0 (D0) and 12 (D12) days at 3.5 ± 1°C, MDA content (A), TP content (B), expressed as mg gallic acid per 100 mg sample, *($p < 0.05$); ***($p < 0.001$) by unpaired t -test, NS – nonsignificant

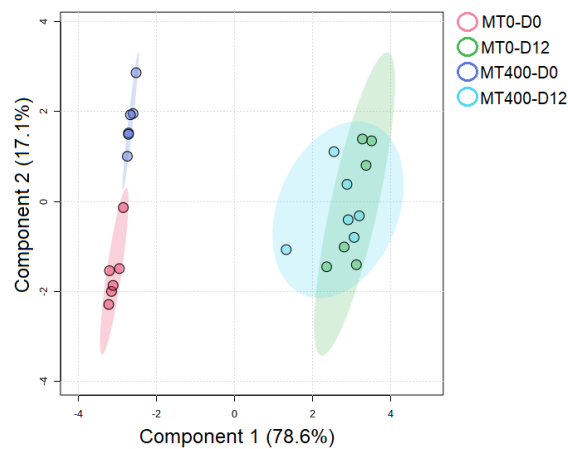


Figure 3. Partial least-squares discriminant analysis of visual and physiological parameters of control (MT0) and melatonin-treated (MT400) basil leaves during cold storage, scores plot, leaves were stored for 0 (D0) and 12 (D12) days at 3.5 ± 1°C, data were normalized (square root transformation) and displayed at 95% confidence region, each color represents a treatment, the first two components account for 95.7% of the variance

CONCLUSIONS

PCI affects the produce quality and shelf life of basil leaves. Our study aimed to characterize the responses of basil leaves treated with 400 µM of melatonin preharvest and stored at PCI-inducing conditions, i.e., 3.5 °C for 12 days. Objective and

subjective visual parameters revealed that melatonin reduced PCI symptoms. As for the biochemical and physiological markers, there were no detectable differences between melatonin-treated and untreated leaves after 12 days of cold storage. However, melatonin-induced alterations before storage could be linked to a stress response. This is the first

report exploring the impact of melatonin on PCI in basil. Future research on the biochemical and molecular basis of melatonin-induced changes will be needed to improve our understanding of the role of this molecule in chilling injury and design long-term solutions to this complex disorder.

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